NUTRITION IN THE REGULATION OF MUSCLE DEVELOPMENT AND REPAIR

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PUBLISHED IN: Frontiers in Nutrition and Frontiers in Physiology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-699-6
DOI 10.3389/978-2-88974-699-6

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NUTRITION IN THE REGULATION OF MUSCLE DEVELOPMENT AND REPAIR

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Citation: Adegoke, O., Fu, X., Huang, Y., eds. (2022). Nutrition in the Regulation of Muscle Development and Repair. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-699-6

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frontiers in Physiology

EDITORIAL
published: 24 February 2022
doi: 10.3389/fphys.2022.853007

Editorial: Nutrition in the Regulation of Muscle Development and Repair

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Keywords: skeletal muscle, repair, regeneration, amino acids, fatty acids, micronutrients, satellite cells, insulin resistance

Editorial on the Research Topic

Editorial: Nutrition in the Regulation of Muscle Development and Repair

The significance of skeletal muscle on whole body metabolism and wellbeing is underlined by the fact that when muscle mass, quality and/or functions are suboptimal, there are implications for whole body wellbeing. It is unequivocal that specific nutrients can regulate the growth of skeletal muscle, either alone or in combination with resistance exercise. Mechanisms regulating formation of muscle cells, either during development or muscle repair/regeneration postnatally, are also well understood. However, although formation of new post-mitotic muscle cells (myotubes) requires synthesis of new proteins, and that amino acids (AA) are required for protein synthesis, not much is understood about the link between nutrients (micro and macro) and formation of new muscle cells. Related to this is the contribution of amino acid metabolism to muscle and whole-body well-being. From food production and nutrition perspectives, it is critical that foods be not only nutritious but be desirable: of what value is a highly nutritious/healthy food if consumers find it unsavory? This Research Topic is a collection of articles on these issues.

Two reviews in this collection focus on the role of nutrients in regulating muscle satellite cells and muscle regeneration. In one, Blum et al. reviewed evidence and gaps regarding nutrient and metabolic determinants of muscle regeneration. The nonessential amino acids serine and glycine, as well as vitamins A and D, are candidate nutrients. The review also discussed the impact of whole foods (blueberry supplements) and obesity on satellite cell integrity. In the second review, Latham et al. discussed evidence for the functions of vitamin D in skeletal muscle regeneration following injury. Because vitamin D can regulate maintenance of satellite cell (SC) integrity, it is tempting to speculate a role for vitamin D in the preservation of muscle regenerative ability, especially during aging. Studies on the effects of nutrients on muscle anabolism have typically focused on macronutrients. However, macronutrient and energy metabolism depends on micronutrients (especially the B vitamins) status because of the roles of the latter as cofactors. He et al. examined the effects of maternal methyl donor mix (MET, a mix of folic acid, methionine, choline, vitamins B6 and B12) supplementation in pregnant pigs. Piglets from dams that consumed the supplement had increased body weight and myofiber diameter, though with reduced fiber number. These improvements occurred in parallel with increased circulating levels of relevant hormones and signaling proteins.

Two reports documented the effects of betaine (trimethylglycine), a feed supplement that is used in livestock production, on growth, and meat/carcass yield and quality in piglets. Betaine functions primarily as an osmolyte and methyl donor (Stuart and Craig, 2004). In one report, the authors showed that betaine increased piglet feed intake (consistent with increased blood gastrin level) and growth, though feed/gain ratio was not different. There were varied and modest effects on

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Edited and reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *11 January 2022*
Accepted: *28 January 2022*
Published: *24 February 2022*

Citation:
Adegoke OAJ, Huang Y, Fu X and Mora S (2022) Editorial: Nutrition in the Regulation of Muscle Development and Repair. Front. Physiol. 13:853007. doi: 10.3389/fphys.2022.853007

intramuscular lipid (IMF) content, but no effects on muscle ω3 PUFA levels. Given the significance of ω3 fatty acids in human health, identifying livestock management practices that do not only enhance flavors but also promote the incorporation of "good" lipids are desirable. In the 2nd study, betaine supplementation increased lean weight and measures of meat quality. It also altered muscle fiber type. The data are consistent with the supplement having a positive effect on meat production and quality. Because increased blood levels of alanine and aspartic aminotransferases were observed, it will be useful to examine what the effect of the supplement will be on animal health. In a related study, Wang et al. used meta-analysis to review the effect of polyunsaturated fatty acids (PUFA) on meat quality of pigs. Evidence suggests that PUFA, specifical conjugated linoleic acid (CLA, a derivative of linoleic acid, an ω6 fatty acid,) and linseed (a type of flax seed from which α-linolenic acid, an ω3 FA, is derived) can improve IMF (a main measure of meat quality) but not growth indices. Thus, more still needs to be done to identify practices that will improve both meat production and quality.

AAs are critical for the synthesis of proteins and compounds that are vital for muscle functions (e.g., creatine, carnitine, glutathione). Factors that regulate AA availability include transport, disposal into proteins and other compounds, oxidation, and their release from proteolysis. Acute studies showed a positive correlation between the expression of AA transporters and muscle protein synthesis (Drummond et al., 2011; Roberson et al., 2018). Roberson et al. examined whether chronic resistance exercise and leucine/whey protein concentrate would regulate protein synthesis and expression of amino acid transporters. Resistance exercise suppressed protein abundance of ATF4 (a transcription factor that normally induces the expression of AA transporters) but increased the expression of AA transporters LAT1 and PAT1. For LAT1, the effect of exercise was reduced in the two supplement groups. Surprisingly, C2C12 that overexpressed LAT 1 had reduced

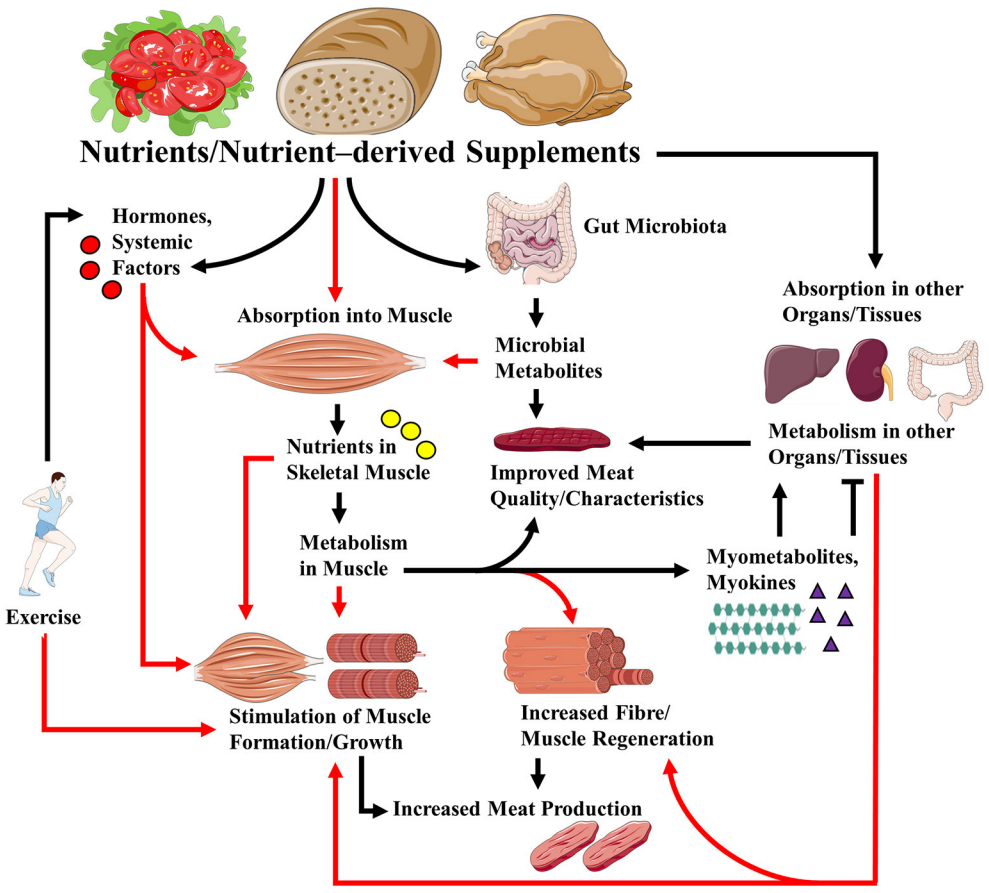

FIGURE 1 | Links between nutrients/ nutrient-derived supplements and skeletal muscle growth/regeneration, health and meat quality. Nutrients and supplements derived from them can regulate muscle growth/regeneration, muscle metabolism, and, in the case of livestock production, meat quality *via* direct effects on muscle, and indirectly through their effects on hormones and systemic factors, their effects on other organs/tissues, and the gut microbiota. Metabolism of the nutrients/supplements in skeletal muscle can lead to the release of myokines and myometabolites that can affect (positively or negatively) metabolism in distal organs and tissues. Note that exercise may modulate the effects of nutrients and supplements *via* its actions on hormones, nutrient absorption, and gut microbiota. For simplicity, all the possible effects of exercise are not indicated. Red arrows indicate absorption into, and/or direct and indirect effects on muscle.

protein synthesis. It is also not clear why the overexpression of the transporter would lead to a reduction in BCKDHa, the enzyme that catalyzes the first irreversible step in branched-chain AA (BCAA) catabolism.

Exercise may modify gut microbiota, leading to the emergence of bacteria genera that favor adaption and improved exercise performance (Scheiman et al., 2019). Because the use of supplements is prevalent amongst athletes (Garthe and Maughan, 2018), Jaago et al. examined the effect of prebiotics mix on exercise-induced changes in microbiota. Prebiotic, but not training, reduced the diversity of some bacteria phyla by >95%. Although this was a study of $n = 1$, and there were no exercise-alone control groups, the data are hypothesis-generating regarding the impact of supplements on exercise and athlete health. It would also be interesting to examine what implications the changes in microbiota have on muscle health/functions. Given the effects of nutraceuticals on health, there is interest in examining their effects on muscle. Black chokeberry (aronia) is rich in phenolic compounds (Tolic et al., 2017; Shikov et al., 2021) and previous studies have documented its effect on health. Yun et al. showed that aronia fruit extracts increased MHC protein levels in C2C12 myotubes and reduced dexamethasone-induced atrophy. Mice treated with the extract had bigger and stronger muscles and better mitochondrial functions. Because animals treated with aronia had higher body weight, it would be interesting to know treatment effects on adipose tissue.

Muscle can regulate metabolism in other tissues through the release of muscle-derived myokines and metabolites (myometabolites) (Rai and Demontis, 2016). Hypoxanthine is a metabolite of purine and of ATP metabolism that is released during muscle contraction and is implicated in muscle fatigue. Yin et al. showed that the negative effects of hypoxanthine on fatigue was linked to reduced muscle glycogen and increased muscle lactate. These observations occurred along with increased expression of uncoupling protein 2 (UCP2) and reduced muscle ATP levels. When UCP2 was deleted in muscle, the effects of hypoxanthine were abated. Given the beneficial effects of exercise, and that increased levels of UCPs are generally perceived as desirable, how does one get the best of both worlds: benefits of exercise while limiting the negative effects of metabolites like hypoxanthine?

Impaired metabolism of BCAAs is linked to chronic diseases like insulin resistance, cardiovascular disease, and some cancers. Mann et al. reviewed the evidence and discussed emerging mechanisms of regulation of BCAA catabolism, including micro RNAs and posttranslational modifications of enzymes involved in BCAA catabolism. The review also discussed the impact of circadian rhythm on BCAA catabolism and the emerging observation that each of the BCAAs has unique metabolic imprints.

These articles shed more light on the roles that nutrients play in regulating muscle growth and regeneration, and how nutritional factors can regulate food (meat) production and quality (**Figure 1**). Some interesting hypotheses were generated, the testing of which will further advance the field.

AUTHOR CONTRIBUTIONS

OAJA: conceptualization. OAJA and SM: writing and original draft preparation. OAJA, YH, XF, and SM: review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a Natural Science and Engineering Discovery Grant (NSERC) to OAJA.

ACKNOWLEDGMENTS

Figure 1 was generated in part using images assembled from Servier Medical Art (https://smart.servier.com/), used under Creative Commons Attribution 3.0 Unported License.

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frontiers in Nutrition

ORIGINAL RESEARCH
published: 30 November 2020
doi: 10.3389/fnut.2020.609022

Maternal Methyl-Donor Micronutrient Supplementation During Pregnancy Promotes Skeletal Muscle Differentiation and Maturity in Newborn and Weaning Pigs

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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: 22 September 2020
Accepted: 27 October 2020
Published: 30 November 2020

Citation:
He Q, Zou T, Chen J, Jian L, He J, Xia Y, Xie F, Wang Z and You J (2020) Maternal Methyl-Donor Micronutrient Supplementation During Pregnancy Promotes Skeletal Muscle Differentiation and Maturity in Newborn and Weaning Pigs. Front. Nutr. 7:609022. doi: 10.3389/fnut.2020.609022

Adequate maternal methyl-donor micronutrient (MET) intake is an important determinant of the organ development and metabolic renovation of offspring. The mechanism involved in skeletal myogenesis and the effect of MET supplementation during pregnancy on the maternal body remain unclear. Thus, this study aimed to investigate the potential effect of methyl donor micronutrients (MET) on skeletal muscle development and metabolism in offspring using pig models. Forty-three Duroc × Erhualian gilts were assigned to two dietary groups during gestation: control diet (CON) and CON diet supplemented with MET (folic acid, methionine, choline, vitamin B6, and vitamin B12). The results showed that maternal MET exposure during pregnancy significantly increased the concentrations of protein, triiodothyronine (T3), and thyroxine (T4) in colostrum and methyl metabolites, including S-adenosylmethionine (SAM), S-adenosyl-L-homocysteine (SAH), 5-methyl-tetrahydrofolate (5-MTHF), and betaine, in the maternal and offspring umbilical vein serum. A similar pattern was demonstrated in the body weight gain and myofiber diameters in offspring. In addition, maternal MET supplementation significantly increased the concentration of offspring serum insulin-like growth factor 1 (IGF-1), T3, and T4; upregulated the mRNA expression of IGF-1 and IGF-1 receptor (IGF-1r) and the phosphorylation level of protein kinases in offspring longissimus dorsi muscle; and upregulated the expression of myogenic genes and fast myosin heavy chain (fast MyHC) in offspring skeletal muscle. Supplementing sows with higher levels of MET during gestation may promote skeletal muscle differentiation and maturity and improve the skeletal muscle mass of the piglets.

Keywords: pregnancy, colostrum, offspring, myogenesis, muscle differentiation

INTRODUCTION

Maternal nutrition during pregnancy plays a role in regulating offspring growth, development, and metabolic health through altering the epigenetic state of the genome (1–3). Previous studies showed that maternal methyl donor micronutrients (MET), such as folic acid, methionine, choline, betaine, etc., are associated with the structure, physiology, and metabolism of the offspring (4–7). MET participates in the synthesis of nucleotides, proteins, and lipids by integrating glucose, amino acid status, and vitamins and feeds into epigenetic mechanisms by methyl group donation or transfer (8). Recently, a study found that maternal MET supplementation has a significant effect on carcass traits and meat quality of pig offspring (9). The mechanism involved in skeletal myogenesis and the effect of MET supplementation during pregnancy on the maternal body remain unclear.

Skeletal muscle is one of the important peripheral tissues affected by maternal diets. It comprises ∼40% of the body mass (10). The net number of skeletal muscle fibers is determined during embryogenesis, and postnatal growth is mainly determined by an increase in the fiber size. Therefore, fetal growth stages are important for skeletal muscle development and could profoundly influence postnatal skeletal muscle growth processes of the offspring (3). Myogenesis is crucial in postnatal muscle growth and regeneration (11), which needs some nutrients; the suppression factor in this process is the usability of maternal nutrients (12). Public health policies recommend periconceptional maternal supplementation of methyl donor, especially in the first trimester of pregnancy. MET is usually provided as supplements during pregnancy to promote cell division during organ growth and the development and metabolic renovation of tissues (13). Methyl donor insufficiency constitutes a risk factor for weaned pups in growth retardation, delayed ossification, and cognitive deficits (14). Previous studies have mainly focused on the impact of methyl donor on neural tube defect, metabolism, and human and mouse offspring diseases (15–17). Recently, some studies have shown that the accumulation of methyl donor in cells is beneficial in improving muscle mass and betaine has a significant effect on skeletal myogenesis, such as proliferation and differentiation of myoblast *in vitro* and *in vivo* (18). In addition, it was reported that maternal MET could regulate growth and proliferation of pig offspring, which was at least partly mediated by insulin-like growth factor 1 (IGF-1) (19). The same effect has been shown in humans (20). However, whether a high level of maternal MET is related to a healthy skeletal muscle mass in offspring remains inconclusive.

Considering the similarities in the anatomical structure and metabolism between pigs and humans, the current study used pig as a translational model for the human infant. Therefore, this study aimed to evaluate whether maternal higher MET supplementation during gestation affects the skeletal muscle developmental characteristics and muscle fiber transform of pig offspring at birth and weaning.

MATERIALS AND METHODS

Ethical Statement

The experiment was conducted following the Chinese guidelines for animal welfare. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Jiangxi Agricultural University (Ethics Approval Code: JXAUA01).

Experimental Design and Animal Material

A total of 43 gilts (Duroc × Erhualian) with initial body weight (BW) of 102.8 ± 6.3 kg and the same genetic background were artificially inseminated with purebred Duroc semen (littermate Duroc boars) and then randomly assigned to receive either a control diet (CON, $n = 21$) or a methyl donor micronutrient diet (MET, $n = 22$). Dietary treatment started at the last insemination and lasted until parturition. Ingredients and composition of pregnant gilts diets are shown in **Table 1**. The nutrient levels met or exceeded the recommendation of the National Research Council (NRC) (2012) (21). The difference between the two diets was that the MET supplementation was replaced by the filler (wheat middlings) in the CON diet. After producing each diet, the main dietary nutritional contents were analyzed by the near-infrared (NIR) analyzer (FOSS, Denmark). There were no differences between the two groups in crude protein, crude ash, crude fat, crude fiber, calcium, and total phosphorus. The MET-supplemented gestation diet contained 4,700 mg kg^{-1} methionine (CJ BIO, Malaysia, purity ≥99%), 16.3 mg kg^{-1} folic acid (Sigma-Aldrich, St. Louis, MO, United States, purity ≥97%), 2,230 mg kg^{-1} choline (NB GROUP, Shandong, China, purity = 60%), 0.15 mg kg^{-1} vitamin B12 (Sigma-Aldrich, purity ≥98%), and 1,180 mg kg^{-1} of vitamin B6 (Jiangxi Tianxin Pharmaceutical, Jiangxi, China, purity ≥98%). Previous reports were used as references to determine the treatment dosages of folic acid (22), vitamin B12 (9, 23), methionine, choline, and vitamin B6 (19, 24). During lactation, sows received a standard lactation diet (**Table 2**). Lactation lasted 24 days. The experiment began with 54 gilts, 27 gilts per treatment. Pregnancy was confirmed by ultrasonic examination 30 days post-mating; 11 gilts were eliminated due to failure of pregnancy.

During gestation, all sows were housed per group with individual feeding. Sows were fed twice per day at 08:00 and 14:00 h, and water was provided *ad libitum*. The feeding amount was based on regular breeding management of pig farm and NRC recommendations (2012). After 110 days of gestation, sows were transferred to farrowing pens. After parturition, all sows were fed on the diet three times per day (i.e., 08:00, 12:00, and 15:00 h). Freshwater was provided *ad libitum*. During the first 5 days of lactation, the amount of feed was progressively increased. Subsequently, lactating sows were fed *ad libitum* until weaning at 24 days.

Sample Collection

After 110 days post-conception, blood samples of sows were collected via the precaval vein at 07:00 h immediately before feeding. During parturition, colostrum samples (30–40 mL) were

TABLE 1 | Ingredients and nutrient composition of diets for the pregnant sows.

Items	CON	MET
Ingredients, %		
Corn	55.00	55.00
Soybean hull	10.00	10.00
Rice bran	10.00	10.00
Expanded soybean	8.00	8.00
Soybean meal	13.00	13.00
Dicalcium phosphate	1.70	1.70
Limestone	0.20	0.20
NaCl	0.30	0.30
Lysine monohydrochloride, 98.5%	0.05	0.05
Choline chloride, 60%	0.17	0.17
Minerals[a]	0.40	0.40
Vitamins[a]	0.05	0.05
Methyl donnor[b]	0	1.11
Filler[c]	1.13	0.02
Total	100.00	100.00
Calculated nutrient composition[b]		
Digestible energy, Mcal kg^{-1}	3.14	3.14
Crude protein, %	15.20	15.20
Total lysine, %	0.86	0.86
Standardized ideal digestible- lysine, %	0.72	0.72
Calcium, %	0.86	0.86
Total phosphorus, %	0.69	0.69
Available phosphorus, %	0.41	0.41
Folic acid, mg kg^{-1}	1.30	16.30
Choline, mg kg^{-1}	1025.00	2230.00
Vitamin B12, μg kg^{-1}	30.00	150.00
Vitamin B6, mg kg^{-1}	3.00	1180.00
Methionine, mg kg^{-1}	2050.00	4700.00
Nutrient composition, %[d]		
Crude protein	15.26	15.22
Crude ash	5.96	5.92
Crude fat	5.63	5.57
Crude fiber	5.23	5.38
Calcium	0.94	0.98
Total phosphorus	0.64	0.64

[a] *Vitamin mixture supplied the following amounts of vitamins/kg of complete diet: 10,000 IU vitamin A; 1,000 IU vitamin D3; 60 IU vitamin E; 2 mg vitamin B1; 4 mg vitamin B2; 3 mg niacin; 15 mg Cu; 110 mg Fe; 100 mg Zn; 20 mg Mn; 0.2 mg I; 0.3 mg Se.*
[b] *Calculated according to the China Feed Database.*
[c] *The filler was wheat middlings (CF = 2.8%, CP = 13.6%, DE = 3.1 Mcal/kg).*
[d] *The nutrient composition is measured value.*

TABLE 2 | Ingredients and nutrient composition of basal diets during lactation.

Items	Content
Ingredients %	
Corn	59.00
Rice bran	7.00
Soybean meal	22.00
Fermented soybean meal	4.00
Soybean oil	2.00
Fish meal	2.00
Dicalcium phosphate	1.40
Limestone	1.20
NaCl	0.30
Lysine monohydrochloride, 98.5%	0.05
Choline chloride, 60%	0.17
Minerals[a]	0.20
Vitamins[a]	0.20
Filler[b]	0.48
Total	100.00
Calculated nutrient composition[c]	
Digestible energy, Mcal kg^{-1}	3.30
Crude protein, %	18.46
Total lysine, %	1.05
Standardized ideal digestible-lysine, %	0.92
Standardized ideal digestible-methionine, %	0.27
Standardized ideal digestible-threonine, %	0.62
Standardized ideal digestible-tryptophan, %	0.20
Calcium, %	0.93
Total phosphorus, %	0.73
Available phosphorus, %	0.43
Nutrient composition, %[d]	
Crude protein	18.86
Crude ash	6.25
Crude fat	4.44
Crude fiber	4.05
Calcium	0.98
Total phosphorus	0.69

[a] *Vitamin mixture supplied the following amounts of vitamins/kg of complete diet: 7,000 IU vitamin A; 1500 IU vitamin D3; 16 IU vitamin E; 1 mg vitamin B1; 2.4 mg vitamin B2; 1 mg vitamin B6; 8 μg vitamin B12; 20 mg niacin; 0.6 mg folic acid; 16 mg Cu; 100 mg Fe; 80 mg Zn; 2 mg Mn; 0.14 mg I; 0.1 mg Se.*
[b] *The filler was wheat middlings (CF = 2.8%, CP = 13.6%, DE = 3.1 Mcal/kg).*
[c] *Calculated according to the China Feed Database.*
[d] *The nutrient composition is measured value.*

collected from sows by hand milking after thoroughly cleaning the udder with water before newborns suckled. Besides, umbilical vein blood was collected from all sows. The colostrum and serum samples were immediately refrigerated at −20°C for further testing. The BW of each piglet was recorded after cleaning the fetal membrane. A total of 12 piglets/treatment (half male and half female) were, respectively, selected from 12 L according to the BW (as close as possible to the average BW in each treatment group). Venous blood samples were collected from piglets that were not fed on colostrum, and then the longissimus dorsi muscles were immediately collected after euthanasia, snap-frozen in liquid nitrogen, and stored at −80°C for further analyses. Longissimus dorsi samples for histological analysis were fixed in 4% paraformaldehyde in a phosphate buffer. Similarly, a total of 8 weaned piglets/treatment (half male and half female) were, respectively, selected from 8 L according to the BW (as close as possible to the average BW in each treatment group). The samples collected from the weaned piglets were similar to those of the newborn piglets.

Serum and Colostrum Hormone Measurement

Serum from sows at day 110 and offspring umbilical cord was analyzed for S-adenosylmethionine (SAM), S-adenosyl-L-homocysteine (SAH), homocysteine (Hcy), 5-methyl-tetrahydrofolate (5-MTHF), and betaine using the enzyme-linked immunosorbent assay (ELISA) kits purchased from MLBIO (Shanghai, China). Sensitivities of the assays were 0.1 μmol/mL, 0.1 μmol/mL, 0.1 nmol/mL, 0.1 ng/mL, and 1.0 ng/mL for SAM, SAH, Hcy, 5-MTHF, and betaine, respectively. Intra- and inter-assay coefficients of variation (CV) were <10 and 15%, respectively. Serum IGF-1, triiodothyronine (T3), and thyroxine (T4) of newborn and weaning piglets were assessed using ELISA kits purchased from Nanjing Jiancheng Biotech (Nanjing, China). Sensitivities of the assays were 0.5 μg/L, 1.5 pmol/L, and 12.5 pmol/L for IGF-1, T3, and T4, respectively. The intra- and inter-assay CV was <10 and 12%, respectively.

Colostrum Composition Analysis

All colostrum samples were analyzed for moisture, total solids (TS), solid non-fat (SNF), lactose protein, and fat content. Colostrum prolactin was assessed with ELISA kits purchased from Nanjing Jiancheng Biotech (Nanjing, China). The sensitivity of the assay was 2 ng/L, and the intra- and inter-assay CV was <10 and 12%, respectively. Colostrum immunoglobulins (Ig), T3, and T4 were determined by immunoturbidimetry and ELISA kits purchased from Sino-UK (Beijing, China). Sensitivities of the assays were 0.1 g/L, 0.1 g/L, 0.1 g/L, 0.05 ng/mL, and 1 ng/ml for immunoglobulin G (IgG), IgA, IgM, T3, and T4, respectively. The intra- and inter-assay CV was <4.5 and 9.5%, respectively, for immunoglobulins and 10 and 12%, respectively, for T3 and T4.

Histological Analysis

Longissimus dorsi muscle samples were embedded in paraffin, sliced at a thickness of 6 μm, and stained with hematoxylin and eosin (H&E). More than ten different microscopic fields of each section were chosen to determine the cross-sectional area of muscle fibers and calculate the muscle fiber density.

Quantitative Real-Time PCR

Total RNA was isolated from the muscle tissue using TRIzol reagent (TransGen Biotech, Beijing, China). The integrity, purity, and concentration of RNA were determined by 1% agarose gel electrophoresis and nucleic acid/protein analyzer. cDNA was synthesized with a commercial RT Master Mix kit (TransGen). qPCR amplification was performed with the BioRad PCR machine with SYBR green master mix (TransGen) following the manufacturer's guidelines. All samples were analyzed in triplicates. The optimal annealing temperature of each primer was determined. The correlation coefficients of all the standard curves were >0.99, and the amplification efficiency values were between 90 and 110% (3.6>slope>3.1). The specificity of amplification was determined by the melting curve analysis at the end of the target gene amplification. The relative mRNA expression of target genes was determined after normalization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference using the $2^{-\Delta\Delta Ct}$ method (25). The primer sequences of the target genes and GAPDH were designed by Primer 5.0 software (Premier Biosoft, Palo Alto, CA, United States) and validated by BLAST sequence alignment in NCBI (**Table 3**).

TABLE 3 | Primer sequences of the target genes and reference genes.

Gene	Primer sequence (5′-3′)	Product (bp)	GenBank ID
IGF1	Forward: TTCAACAAGCCCACAGGGTA	102	XM_005664199.1
	Reverse: CTCCAGCCTCCTCAGATCAC		
IGF-1r	Forward: ATTACCGCAAGGGAGGGAAA	174	NM_214172.1
	Reverse: GAAGGACTTGCTCGTTGGAC		
AKT1	Forward: CTGCCCTTCTACAACCAGGA	66	HQ687753.1
	Reverse: GAAGCGGATCTCCTCCATGA		
AKT2	Forward: GTGCTTCGTGATGGAGTACG	118	HQ687754
	Reverse:C TCCAGAGCCGAGACAATCT		
IGFBP5	Forward: GTGTACCTGCCCAACTGTGA	158	NM_214099.1
	Reverse: AAGCTGTGGCACTGGAAGTC		
Myf5	Forward: AGACGCCTCAAGAAGGTCAA	95	NM_001278775.1
	Reverse: CTGAGGATCTCCACCTTGGG		
Myf6	Forward: CCCTTCAGCTACAGACCCAA	183	NM_001244672.1
	Reverse: GTCCACGATGGAAGAAAGGC		
MyOD1	Forward: GTGCAAACGCAAGACCACTA	125	NM_001002824.1
	Reverse: GATTCGGGTTGCTAGACGTG		
Myog	Forward: AATCTGCACTCCCTCACCTC	73	NM_001012406.1
	Reverse: TTTCATCTGGGAAGGCCACA		
Pax3	Forward: CAGCAGAGCAGCTTGAAGAG	152	XM_021075359.1
	Reverse: CTGCTTCCTCCATCTTGCAC		
Pax7	Forward: TGCCCTCAGTGAGTTCGATT	152	NM_001206359.1
	Reverse: ATCCAGACGGTTCCCTTTGT		
MRF4	Forward: CCCTTCAGCTACAGACCCAA	183	NM_001244672.1
	Reverse: GAGCAGCTGGAAGTAAAGGC		
MSTN	Forward: AGTGATGGCTCCTTGGAAGA	169	AY448008.2
	Reverse: TCCACAGTTGGGCCTTTACT		
TGFβ-1	Forward: GCAGGTACTCCTGGTGAACT	196	AF461808.1
	Reverse: AGGATACCAGTCGGGTAGGT		
MYH7	Forward: TTCAAGCTGGAGCTGGATGA	152	NM_213855
	Reverse: GTGAGGTCGTTGACAGAACG		
MYH2	Forward: TAGGCCCTTTGATGCCAAGA	111	NM_214136
	Reverse: GCTTCCGTCTTCACTGTCAC		
MYH1	Forward: TCAAGGACACCCAGATCCAC	166	NM_001104951
	Reverse: TCCTGTTCTGCGACTTTCCT		
MYH4	Forward: GAATCCCTGGACCAACTGGA	92	NM_001123141
	Reverse: CCTCCCTCTGCAATTTGCTC		
GAPDH	Forward: TGGAAAGGCCATCACCATCT	105	NM_001206359.1
	Reverse: ATGGTCGTGAAGACACCAGT		

IGF1, insulin-like growth factor 1; IGF1r, insulin-like growth factor 1 receptor; AKT, protein kinases; IGFBP5, insulin growth factor-binding protein 5; Myf5, myogenic factor 5; Myf6, myogenic factor 6; MyoD1, myogenic differentiation factor 1; Myog, myogenin; Pax3, paired box gene 3; Pax7, paired box gene 7; MRF4, muscle regulatory factor 4; MSTN, myostatin; TGFβ-1, transforming growth factor-β 1; MYH, myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Immunoblotting Analysis

Protein was extracted from muscle tissues with a total protein extraction kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (TransGen). The total protein was then denatured with a 6× protein loading buffer (TransGen). Western blot analysis for protein kinases (t-AKT; 9272S, Cell Signaling, United States), phosphorylated protein kinases (p-AKT; 9271S, Cell Signaling, United States), myogenic differentiation factor 1 (MyoD1; 18943-1-AP, Proteintech, China), myogenin (Myog) (ab1835, Abcam, United States), slow myosin heavy chain (slow MyHC; ab11083, Abcam, United States), fast MyHC (20140-1-AP, Proteintech, China), and β-actin (66009-1-Ig, Proteintech, China) was carried out as previously described (12). Briefly, protein extracts were separated by 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The membrane was washed with TBS with 0.1% Tween 20 (TBST) and incubated with 5% non-fat dry milk in TBS with 0.1% TBST at room temperature for 60 min, incubated overnight with the selected primary antibodies at 4°C, and washed with TBST followed by incubation with either horseradish peroxidase (HRP)-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit secondary antibodies for 60 min at room temperature. Finally, membranes were visualized using the Image Lab statistical software (Bio-Rad, Laboratories, Hercules, CA, United States). The secondary antibodies were purchased from Proteintech Group (SA00001-1 and SA00001-2). The relative expressions of t-AKT, p-AKT, MyoD1, Myog, slow MyHC, and fast MyHC protein were normalized to β-actin.

Statistical Analyses

Statistical analysis was performed using SPSS 24.0 software (SPSS Inc.). The Shapiro–Wilk test was performed to determine whether measured data sets followed a normal distribution. All data sets were normally distributed and required no transformation prior to analysis. The statistical power was adequate for detecting differences between groups. An independent-sample *t*-test was used to compare the differences between the CON and the MET. The individual sow was considered as the experimental unit for number of born and weaned piglets, and a litter was considered as the experimental unit for litter weight and daily gain weight, whereas each pig was considered as the experimental unit for the remaining data. All results are shown as means ± SD; a value of $P < 0.05$ was considered statistically significant.

RESULTS

Maternal Characteristics

The reproductive performance of sows fed with the MET diet and the growth performance of offspring are shown in **Table 4**. There was no difference in the number of total born, born alive, and weaned piglets ($P > 0.05$). There was no significant difference in the litter weight of birth ($P > 0.05$). However, the litter weight at weaning and average daily gain were greater in piglets from the MET sows compared with those from the CON sows ($P < 0.05$).

TABLE 4 | Effect of maternal methyl-donor micronutrient (MET) supplementation during gestation on growth performance of offspring ($n = 21$ per group).

Items	CON	MET	*P*-value
No. of total born per litter	10.75 ± 2.14	10.92 ± 2.10	0.840
No. of born alive per litter	10.58 ± 2.07	10.69 ± 2.06	0.896
No. of weaned piglets per litter	9.00 ± 1.48	9.36 ± 1.63	0.590
Litter weight at birth, kg	13.10 ± 2.97	13.99 ± 2.05	0.391
Litter weight at weaning, kg	58.09 ± 8.27	69.40 ± 12.96	0.024
Average daily gain during 1–24 days	0.23 ± 0.03	0.26 ± 0.01	0.005

Data are reported as means ± standard deviation. CON, control diet; MET, methyl-donor micronutrient diet.

TABLE 5 | Effect of maternal MET supplementation during gestation on nutrition composition, immunoglobulin, total triiodothyronine (T3), and thyroxine (T4) concentrations in colostrum ($n = 10$ per group).

Items	CON	MET	*P*-value
Nutrition composition, %			
Moisture	73.96 ± 2.77	72.45 ± 4.23	0.377
TS	13.89 ± 2.21	16.96 ± 3.78	0.069
SNF	8.71 ± 0.49	8.74 ± 0.55	0.922
Fat	3.27 ± 0.31	3.36 ± 0.38	0.603
Lactose	3.02 ± 0.33	2.91 ± 0.37	0.493
Protein	5.49 ± 0.80	6.88 ± 1.36	0.018
Colostrum immunoglobulins, ng/L			
IgM	3.18 ± 0.25	3.88 ± 0.59	0.023
IgG	60.75 ± 6.38	67.34 ± 3.68	0.060
IgA	11.11 ± 0.79	11.82 ± 0.82	0.156
Colostrum hormones			
T3, ng/mL	0.12 ± 0.01	0.15 ± 0.02	0.005
T4, ng/mL	4.49 ± 0.94	5.63 ± 0.57	0.007
T3/T4	0.03 ± 0.01	0.03 ± 0.00	0.177

Data are reported as means ± standard deviation. TS, total solids; SNF, solid-non-fat; Ig, immunoglobulin CON, control diet; MET, methyl-donor micronutrient diet.

Colostrum Composition of Sows

Colostrum obtained from the MET-fed sows had a higher protein content than that from the CON-fed sows ($P < 0.05$). Colostrum from the MET-fed sows tended to have a greater TS than that from the CON-fed sows ($P = 0.069$). The IgM level was increased in colostrum from the MET sows compared with that from the CON sows ($P < 0.05$). Colostrum from the MET-fed sows tended to have a greater IgG level than that from the CON-fed sows ($P = 0.060$). T3 and T4 concentrations were significantly higher in the MET group colostrum compared with the CON group colostrum ($P < 0.05$). However, the ratio of T3 to T4 did not differ in the colostrum of the CON and the MET sows ($P > 0.05$; **Table 5**). Moreover, serum prolactin was higher in the supplemented sows than in the control sows ($P < 0.05$). However, there was no significant difference

TABLE 6 | Effect of maternal MET supplementation during gestation on prolactin concentration in sows' serum and colostrum ($n = 10$ per group).

Items	CON	MET	*P*-value
Serum prolactin, ng/L	220.85 ± 37.20	283.32 ± 52.70	0.034
Colostrum prolactin, ng/L	102.46 ± 35.01	137.11 ± 9.60	0.099

Data are reported as means ± standard deviation. CON, control diet; MET, methyl-donor micronutrient diet.

TABLE 7 | Effect of maternal MET supplementation during gestation on the concentration of S-adenosylmethionine (SAM), S-adenosyl-L-homocysteine (SAH), homocysteine (Hcy), 5-methyl-tetrahydrofolate (5-MTHF), and betaine in sows and offspring umbilical vein serum ($n = 10$ per group).

Items	CON	MET	*P*-value
Sows			
SAM, μmol/mL	41.98 ± 10.80	73.63 ± 15.86	0.004
SAH, μmol/mL	23.76 ± 5.83	37.17 ± 7.06	0.006
Hcy, nmol/mL	6.66 ± 1.57	4.42 ± 0.66	0.005
5-MTHF, ng/mL	19.45 ± 8.51	30.75 ± 6.93	0.030
Betaine, ng/L	1.01 ± 0.29	2.13 ± 0.45	0.001
Offspring umbilical vein			
SAM, μmol/mL	20.16 ± 1.06	22.67 ± 1.89	0.032
SAH, μmol/mL	11.19 ± 0.69	12.24 ± 0.36	0.001
Hcy, nmol/mL	17.15 ± 1.53	15.01 ± 1.26	0.033
5-MTHF, ng/mL	3.70 ± 0.22	3.99 ± 0.35	0.037
Betaine, ng/L	0.37 ± 0.03	0.42 ± 0.05	0.003

Data are reported as means ± standard deviation. CON, control diet; MET, methyl-donor micronutrient diet.

in colostrum prolactin between these two groups ($P > 0.05$; **Table 6**).

Serum Methyl Metabolite Profile

There was a significantly higher concentration of SAM in the sows and offspring umbilical vein of the MET group compared with the CON group ($P < 0.05$; **Table 7**). Similarly, serum concentrations of SAH, 5-MTHF, and betaine in the sows and offspring umbilical vein were higher in the treated group than in the control group ($P < 0.05$). However, the Hcy concentration in the sows and offspring umbilical vein serum was significantly decreased in the MET group ($P < 0.05$).

Serum Hormone Profile

Maternal MET supplementation during gestation increased serum IGF-1 concentration in the offspring at birth ($P < 0.05$; **Table 8**). However, there was no significant difference in serum IGF-1 concentration in the weaning pigs ($P > 0.05$). There was a significantly higher concentration of serum T3 and T4 in the weaning offspring of the MET group compared with the CON group ($P < 0.05$). The serum T3 concentration in piglets at birth and weaning was increased in the MET group ($P = 0.066$, $P < 0.05$, respectively). Furthermore, the maternal MET diet increased the ratio of T3 to T4 in the offspring serum at birth and weaning ($P < 0.05$).

TABLE 8 | Effects of maternal MET supplementation during gestation on serum concentration of insulin-like growth factor 1 (IGF-1), triiodothyronine (T3), and thyroxine (T4) in offspring ($n = 12$ at birth; $n = 8$ at weaning).

Items	CON	MET	*P*-value
Birth			
IGF-1, μg/mL	23.76 ± 2.02	25.37 ± 1.37	0.048
T3, ng/mL	0.22 ± 0.02	0.36 ± 0.06	0.066
T4, ng/mL	0.99 ± 0.27	1.50 ± 0.20	0.001
T3/T4	0.20 ± 0.04	0.16 ± 0.02	0.035
Weaning			
IGF-1, μg/mL	26.17 ± 1.80	26.75 ± 2.06	0.569
T3, ng/mL	0.27 ± 0.03	0.38 ± 0.06	0.010
T4, ng/mL	1.15 ± 0.27	2.10 ± 0.24	<0.001
T3/T4	0.24 ± 0.05	0.16 ± 0.02	0.033

Data are reported as means ± standard deviation. CON, control diet; MET, methyl-donor micronutrient diet.

Muscle Histological Analysis

To determine whether MET exposure altered offspring skeletal muscle fiber size, we evaluated the cross-sectional fiber area of the longissimus dorsi muscle. H&E staining showed that the diameter of the muscle fiber of the weaning pigs in the MET group was thicker than that of the CON group (**Figure 1**). The number of muscle fibers was decreased, and the muscle fiber cross-sectional area was increased in the weaning pigs with maternal MET dietary supplementation ($P < 0.05$). However, there was no variation in the offspring at birth.

Expression of Muscle IGF Signaling Gene

To further explore why maternal MET supplementation increased serum IGF-1 concentration in offspring at birth, we measured muscle IGF system genes mRNA expression and t-AKT and p-AKT protein expression in offspring at birth and weaning (**Figure 2**). Maternal dietary treatment had a significant effect on the mRNA abundance of the IGF signaling genes, including IGF-1 ($P < 0.05$), IGF-1r ($P < 0.05$), AKT1 ($P < 0.05$), and AKT2 ($P < 0.05$). A similar pattern was also demonstrated for gene expression of the longissimus dorsi muscle IGF-1 ($P < 0.05$) and AKT2 ($P < 0.05$) in the weaning pigs. Although the expression of total AKT protein level was not different, its phosphorylation was higher ($P < 0.05$) in MET supplemented offspring when compared to the CON group; the MET offspring had a significantly higher p-/t-AKT ratio ($P < 0.05$).

Expression of Muscle Growth-Related Genes

The mRNA abundance of the longissimus dorsi muscle growth-related genes is shown in **Figures 3A,B**. Dietary MET supplementation increased the mRNA expression of myogenic factor 6 (Myf6; $P < 0.05$), MyoD1 ($P < 0.05$), and paired box gene 7 (Pax7; $P < 0.05$) and reduced the mRNA expression of myostatin (MSTN; $P < 0.05$). The mRNA expression of Pax3 ($P < 0.05$) and Myog ($P = 0.057$) was increased in the longissimus dorsi muscle of MET compared to the CON newborn piglets. Maternal MET supplementation significantly upregulated the mRNA expression of Myf5 ($P < 0.05$) and

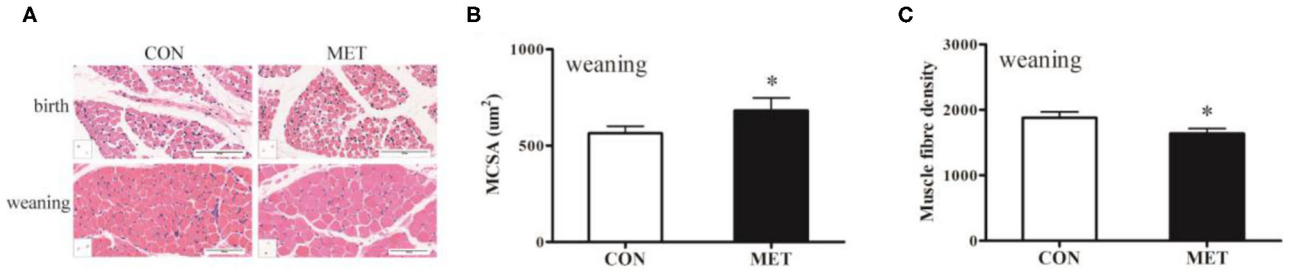

FIGURE 1 | Effects of maternal MET supplementation during gestation on histological properties in offspring longissimus dorsi muscle. Data are reported as means ± standard deviation, $n = 12$ at birth; $n = 8$ at weaning. **(A)** Representative hematoxylin and eosin (H&E) staining of newborn and weaning piglets. **(B)** Number of muscle fibers per mm^2 of weaning offspring. **(C)** Muscle fiber cross-sectional area of weaning offspring. CON, control diet; MET, methyl-donor micronutrients diet. *$P < 0.05$ (significant differences between MET vs. CON).

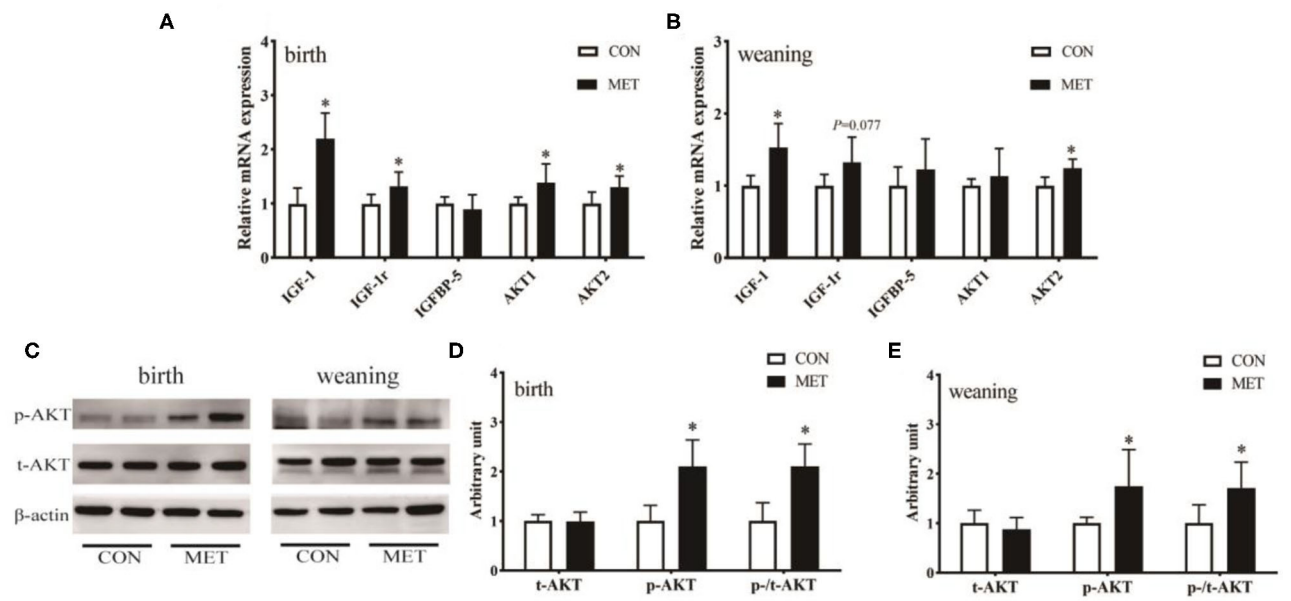

FIGURE 2 | Effects of maternal MET supplementation during gestation on IGF system gene mRNA expression and t-AKT and p-AKT protein expression in offspring longissimus dorsi muscle. Data are reported as means ± standard deviation, $n = 12$ at birth; $n = 8$ at weaning. **(A)** IGF system gene mRNA expression in newborn piglets. **(B)** IGF system gene mRNA expression in weaning piglets. **(C)** Representative images of immunoblotting. **(D)** Immunoblotting analysis of t-AKT and p-AKT protein expression in newborn piglets. **(E)** Immunoblotting analysis of t-AKT and p-AKT protein expression in weaning piglets. t-AKT, protein kinases; p-AKT, phosphorylated protein kinases; IGF1, insulin-like growth factor 1; IGF1r, insulin-like growth factor 1 receptor; IGFBP5, insulin growth factor-binding protein 5; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; t, total content, p, phosphorylated; CON, control diet; MET, methyl-donor micronutrient diet. *$P < 0.05$ (significant differences between MET vs. CON).

muscle regulatory factor 4 (MRF4; $P < 0.05$) in the weaning offspring. Western blotting further indicated that the protein expression of MyoD1 and Myog ($P < 0.05$) was significantly increased in the weaning offspring with maternal exposure to MET (**Figures 3C–E**). In addition, Myog protein expression of the newborn offspring tended to be increased in the MET group ($P = 0.063$).

Expression of Muscle Fiber Type-Related Genes

The mRNA abundance of MYH7 ($P < 0.05$), MYH2 ($P < 0.05$), and MYH1 ($P < 0.05$) was increased in the longissimus dorsi muscle in the MET compared to the CON newborn piglets (**Figure 4A**). Prenatal MET exposure increased the mRNA expression of MYH2 ($P < 0.05$), MYH1 ($P < 0.05$), and MYH4 ($P < 0.05$) in the weaning offspring (**Figure 4B**). Consistent with their mRNA expression, there was no significant difference in the protein expression of the fast MyHC isoform in the newborn piglets ($P > 0.05$). However, the protein expression of the fast MyHC isoform in weaning piglets was upregulated in the treated group than in the control group ($P < 0.05$; **Figures 4C–E**).

DISCUSSION

In our study, maternal MET exposure during pregnancy increased protein, T3, and T4 concentrations in colostrum

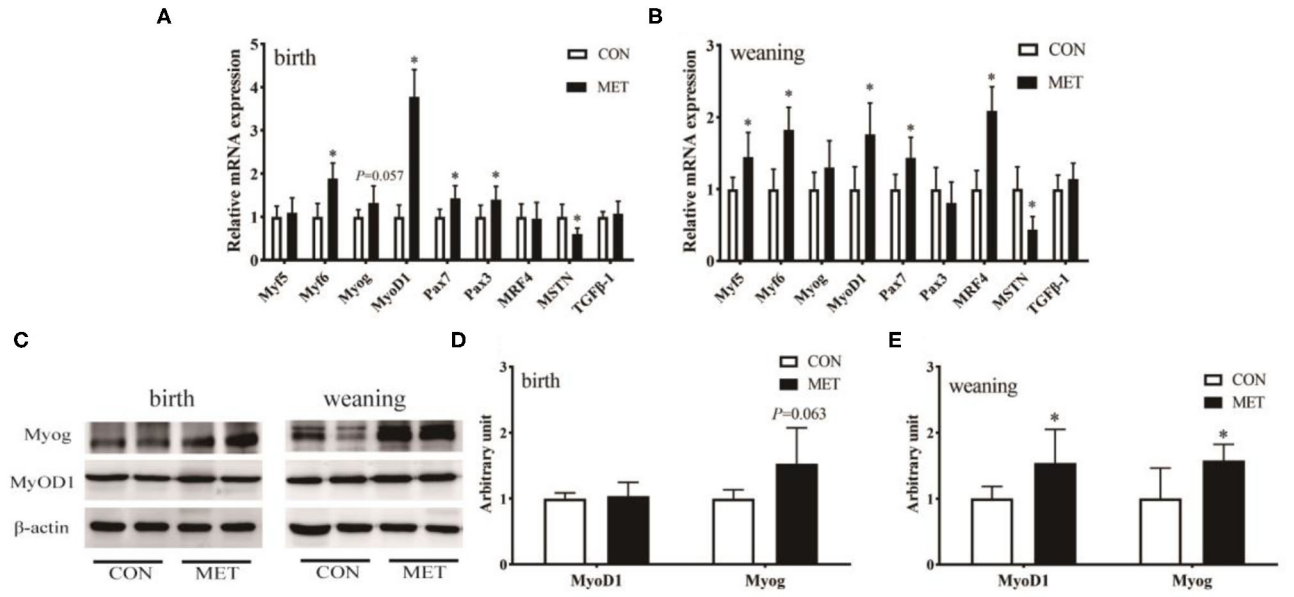

FIGURE 3 | Effects of maternal MET supplementation during gestation on longissimus dorsi muscle growth-related genes mRNA expression and MyoD1 and Myog protein expression in offspring. Data are reported as means ± standard deviation, $n = 12$ at birth; $n = 8$ at weaning. **(A)** Growth-related gene mRNA expression in newborn piglets. **(B)** Growth-related gene mRNA expression in weaning piglets. **(C)** Representative images of immunoblotting. **(D)** Immunoblotting analysis of MyoD1 and Myog protein expression in newborn piglets. **(E)** Immunoblotting analysis of MyoD1 and Myog protein expression in weaning piglets. MyoD1, myogenic differentiation factor 1; Myog, myogenin; Myf5, myogenic factor 5; Myf6, myogenic factor 6; Pax3, paired box gene 3; Pax7, paired box gene 7; MRF4, muscle regulatory factor 4; MSTN, myostatin; TGFβ-1, transforming growth factor-β 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CON, control diet; MET, methyl-donor micronutrient diet. *$P < 0.05$ (significant differences between MET vs. CON).

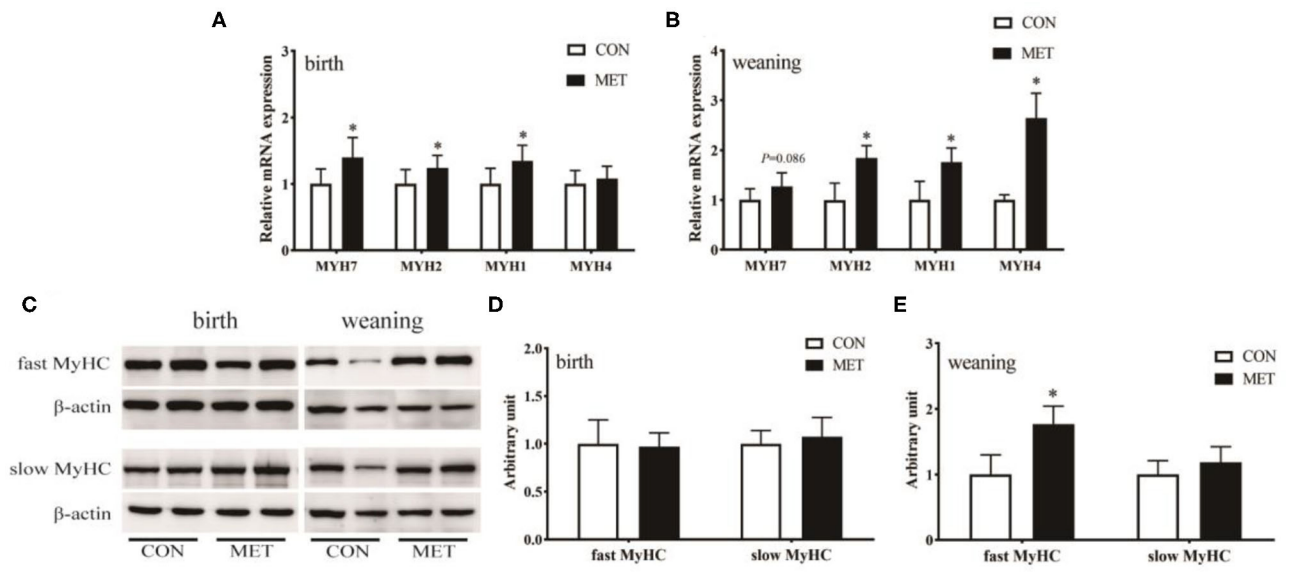

FIGURE 4 | Effects of maternal MET supplementation during gestation on longissimus dorsi muscle fiber type-related genes mRNA expression and fast MyHC and slow MyHC protein expression in offspring. Data are reported as means ± standard deviation, $n = 12$ at birth; $n = 8$ at weaning. **(A)** Muscle fiber type-related gene $n = 12$ at birth; $n = 8$ at weaning. **(A)** Muscle fiber expression in weaning piglets. **(C)** Representative images of immunoblotting. **(D)** Immunoblotting analysis of fast MyHC and slow MyHC protein expression in newborn piglets. **(E)** Immunoblotting analysis of fast MyHC and slow MyHC protein expression in weaning piglets. MYH/MyHC, myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CON, control diet; MET, methyl-donor micronutrients diet. *$P < 0.05$ (significant differences between MET vs. CON).

and methyl metabolites, including SAM, SAH, 5-MTHF, and betaine, in maternal and offspring umbilical vein serum. Similar findings were recorded in offspring BW gain and myofiber diameters. Meanwhile, maternal MET supplementation significantly increased the concentration of serum IGF-1, T3, and T4 in the offspring, decreased the concentration of maternal and offspring umbilical vein serum Hcy, and upregulated the mRNA expression of IGF-1 and IGF-1r and the phosphorylation level of AKT in the longissimus dorsi muscle of the offspring. Furthermore, maternal exposure to MET during pregnancy promoted skeletal muscle differentiation and maturity in pig offspring. We found no differences in the reproduction performance of the sows, which was similar to findings from a study by Zhao et al. (9). Some studies also found either a negative or no relationship between MET and fetal growth (26, 27). In betaine-supplemented dams, the first filial generation litter weights were smaller at birth and weaning, whereas the second filial generation had exactly the reverse effect in weaning (6). To date, the effect of maternal MET exposure during gestation on the growth performance of pig offspring has been controversial. However, maternal nutrition during pregnancy has been found to affect offspring development and has a profound effect throughout the life of the offspring via epigenetic modifications (28, 29). Thus, we speculate that the main reason for the different results is the different types and doses of MET.

Colostrum plays a major role in the survival of piglets during farrowing by not only providing heat and metabolic energy but also preventing infections through passive immunity. However, there are many factors affecting colostrum production, including genotype, parity, age, nursing behavior, and litter characteristics (30). To minimize the potential impact of sow condition on lactation performance, we used primiparous sow with similar genetic background and BW. Previously, it was reported that low-colostrum-producing sows have greater concentrations of progesterone and colostrum production in primiparous sows was positively associated with prolactin concentration (31). In our study, serum obtained from MET-fed sows had higher prolactin content than that obtained from CON-fed sows, which means sows could produce more milk to meet the needs of piglets. Meanwhile, colostrum obtained from MET-fed sows had higher protein content than that obtained from CON-fed sows. Because colostrum components are synthesized as early as a few weeks before farrowing, milk composition in colostrum is largely dependent on maternal body conditions (32). Previous studies have reported that methionine is an essential amino acid that may play a major role in swine protein structure and metabolism (33) and maintain the health of the sows (34). Methionine is a potential regulator of milk protein synthesis; it may easily restrict mammary protein synthesis (35). Furthermore, MET participates in the synthesis of nucleotides, proteins, and lipids by integrating glucose, amino acid status, and vitamins and feeds into epigenetic mechanisms by methyl group donation or transfer (8). These are also the main reasons for the increase in colostrum protein. Colostrum contains both nutrients and bioactive molecules. The concentration of the bioactive molecule in colostrum is closely related to the metabolic status of the sow during the perinatal and postnatal periods. We found higher T3, T4, and IgM in colostrum from MET-fed sows. Maternal MET exposure during pregnancy may promote mammary gland development of gilt and further increase the amount and quality of lactation, which lead to the offspring getting more adequate nutrition, promote skeletal muscle differentiation and maturity, and, ultimately, improve the growth performance of the offspring.

Previously, it was reported that an imbalance of methionine content in maternal diet will reduce the postnatal growth in rats (36). If the supply of methionine exceeds the maximum amount the animal can withstand, it may cause toxicities, such as hyperhomocysteinemia (a thrombogenic non-proteinogenic amino acid) and endothelial dysfunction. Hcy is a demethylated derivative of methionine that is produced during the one-carbon metabolism cycle. SAM is the most important metabolic substrate in methylation and participates in most methylation in animals (37). We found significantly higher serum SAM and SAH concentrations in sows and offspring umbilical vein of the MET group compared with the CON group. Folic acid supplementation was effective in reducing the total homocysteine levels (38). Reduced levels of serum Hcy in MET sows and the newborn pigs may be related to the increase in serum 5-MTHF concentration, which is the substrate of Hcy remethylation to methionine (39).

Dietary supplementation of methyl donors has been shown to promote Hcy metabolism. Moreover, changes in serum Hcy have been negatively correlated with IGF-I (40). Growth hormone (GH) stimulates protein synthesis, and IGFs are essential for the rapid proliferation and differentiation of most tissues in animals (41). Furthermore, skeletal muscle is a major insulin target. The thicker muscle fiber diameter of the weaning offspring demonstrates that maternal MET exposure may improve the growth and formation of offspring fibers. We speculated that the offspring growth might be associated with muscle IGF signaling because maternal MET supplementation significantly increased the serum IGF-1 level of the offspring at birth. Additionally, we found that maternal exposure to MET increased offspring serum T3 and T4 concentrations, which are major factors in piglet muscle development (42). Moreover, we found a significantly upregulated mRNA expression of IGF-1 in offspring longissimus dorsi muscle in the MET group. Protein expression indicated that MET offspring had a significantly higher p-/t-AKT ratio. AKT activation negatively regulates numerous pro-apoptotic factors and positively regulates the transcription factor, MyoD, in myoblast cells, inducing terminal differentiation into myocytes and then fusion to regenerate myofibers (43, 44).

Myogenesis is a multistep process, including myoblast proliferation and differentiation, from precursor determination to patterning, differentiation, fusion, and maturation of muscle fiber types (45). It is crucial for muscle growth and regeneration after delivery. Pax3 and Pax7 are important regulators of myogenic progenitor cells, which can directly act upstream of myogenic regulatory factors (MRFs) to further promote muscle differentiation and growth (46, 47). MRFs include MyoD1, Myf5, Myog, and MRf4 (48). MyoD1 and Myf5 are involved in muscle determination, while Myog plays a predominant role in muscle cell differentiation to form muscle fibers. MRF4 is essential in the maintenance of postnatal muscles (49). MSTN

is a negative regulator of skeletal muscle growth (50). After birth, muscle growth and development are mainly based on the increase of the muscle fiber area (51), Myogenesis promotes muscle fiber hypertrophy and growth by maintaining the proliferation and differentiation of the muscle satellite cells (3). The present results showed that the diameter of muscle fiber in weaning pigs in the MET group was thicker than that in the CON group. Furthermore, the mRNA expression of Myf5, MyoD1, MRF4, Myog, and Pax7 was increased and the protein expression of Myog was upregulated in weaning pigs in the MET group, suggesting that MET supplementation during gestation improved myogenesis and promoted muscle fiber hypertrophy and growth. We also noted that maternal MET supplementation during gestation increased MRF4 mRNA in weaning offspring. This was similar to findings from a previous study, which showed that MRF4 expression increased during hypertrophy induced by a high level of methionine administration in mice (52). However, although we did not measure the levels of MRF4 protein, it is tempting to infer that the increased level of MRF4 mRNA was probably associated with postpartum skeletal muscle hypertrophy and growth upon MET administration in sows.

Generally, skeletal muscle fiber types are characterized in part by the expression of multiple MYH genes (53). Muscle fiber types can be classified into slow MyHC and fast MyHC. We found that gestational MET increased MYH2, MYH1, and MYH4 mRNA expression and upregulated fast MyHC protein expression in weaning offspring, indicating that gestational MET caused early muscle differentiation leading to a higher degree of muscular maturity during weaning. Consistent with Senesi's et al. (54) findings, betaine supplementation enhanced skeletal muscle differentiation via IGF activation. The increased fast-twitch myofiber isoform in MET weaning piglets was also linked to serum T3 and T4 in our study. The thyroid hormone is a major determinant of myofiber composition, which can promote the uptake and utilization of glucose by muscle tissue and regulate the conversion of embryonic or prenatal MyHC isoforms to adult fast MyHC isoforms (42). Muscle differentiation is a process from slow-twitch myofibers to fast-twitch myofibers. MET regulates the expression of myogenesis-related genes through DNA methylation, thereby regulating the proliferation and differentiation of myoblast (55). It can significantly increase the concentrations of 5-mC and 6-mA than affect DNA methylation levels in C2C12 myoblasts (18, 56). However, we failed to measure the degree of methylation of the gene promoter. Nonetheless, according to the number of methyl metabolites and gene expression, we speculate that maternal MET exposure during pregnancy may promote skeletal muscle differentiation and maturity in offspring through the upregulation of the muscle gene expression of IGFs.

CONCLUSION

In summary, maternal MET exposure during pregnancy promoted the differentiation and maturity of skeletal muscle in pig offspring, which was associated with improved colostrum quality (protein, Ig, T3, and T4); a higher concentration of IGF-1, T3, SAM, SAH, and 5-MTHF; and lower Hcy level in offspring serum and upregulated expression of myogenic genes and fast MyHC in offspring skeletal muscle. These results suggest that supplementing sows with higher MET during gestation can promote skeletal muscle differentiation and maturity and improve the skeletal muscle mass of piglets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the experiment was conducted following the Chinese guidelines for animal welfare. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Jiangxi Agricultural University (Ethics Approval Code: JXAUA01).

AUTHOR CONTRIBUTIONS

JY and TZ contributed to experimental concepts and design. QH, LJ, and JH performed the animal feeding experiment and sample analysis. ZW, FX, and YX assisted with immunoblotting analysis and the detection of the serum level of hormones. QH analyzed the data and wrote the manuscript. JY, TZ, and JC finalized the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Key Research and Development Program of China (2018YFD0500401) and Project funded by the China Postdoctoral Science Foundation (2019M662270).

ACKNOWLEDGMENTS

We would like to thank Freescience (https://www.home-for-researchers.com) for the English language editing.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 14 January 2021
doi: 10.3389/fnut.2020.628405

LAT1 Protein Content Increases Following 12 Weeks of Resistance Exercise Training in Human Skeletal Muscle

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Introduction: Amino acid transporters are essential for cellular amino acid transport and promoting protein synthesis. While previous literature has demonstrated the association of amino acid transporters and protein synthesis following acute resistance exercise and amino acid supplementation, the chronic effect of resistance exercise and supplementation on amino acid transporters is unknown. The purpose herein was to determine if amino acid transporters and amino acid metabolic enzymes were related to skeletal muscle hypertrophy following resistance exercise training with different nutritional supplementation strategies.

Methods: 43 college-aged males were separated into a maltodextrin placebo (PLA, $n = 12$), leucine (LEU, $n = 14$), or whey protein concentrate (WPC, $n = 17$) group and underwent 12 weeks of total-body resistance exercise training. Each group's supplement was standardized for total energy and fat, and LEU and WPC supplements were standardized for total leucine (6 g/d). Skeletal muscle biopsies were obtained prior to training and ∼72 h following each subject's last training session.

Results: All groups increased type I and II fiber cross-sectional area (fCSA) following training ($p < 0.050$). LAT1 protein increased following training ($p < 0.001$) and increased more in PLA than LEU and WPC ($p < 0.050$). BCKDHα protein increased and ATF4 protein decreased following training ($p < 0.001$). Immunohistochemistry indicated total LAT1/fiber, but not membrane LAT1/fiber, increased with training ($p = 0.003$). Utilizing all groups, the change in ATF4 protein, but no other marker, trended to correlate with the change in fCSA ($r = 0.314$; $p = 0.055$); however, when regression analysis was used to delineate groups, the change in ATF4 protein best predicted the change in fCSA only in LEU ($r^2 = 0.322$; $p = 0.043$). In C2C12 myoblasts, LAT1 protein overexpression caused a paradoxical decrease in protein synthesis levels ($p = 0.002$) and decrease in BCKDHα protein ($p = 0.001$).

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *11 November 2020*
Accepted: *11 December 2020*
Published: *14 January 2021*

Conclusions: Amino acid transporters and metabolic enzymes are affected by resistance exercise training, but do not appear to dictate muscle fiber hypertrophy. In fact, overexpression of LAT1 *in vitro* decreased protein synthesis.

Keywords: amino acid metabolism, ATF4, protein supplementation, protein synthesis, BCKDH

INTRODUCTION

Skeletal muscle possesses the unique ability to adapt to metabolic and loading demands. For example, disuse and physical inactivity contribute to muscle atrophy (1, 2), while both aerobic and resistance training can improve skeletal muscle quality and promote hypertrophy (3, 4). To enhance exercise training adaptations it has been suggested to ingest upwards of 1.6–2.0 g/kg of dietary protein (5–7). While it remains to be fully determined how greater consumption of amino acids promotes muscle growth, essential amino acids (and leucine in particular) are capable of simulating increases in muscle protein synthesis suggesting a positive role for amino acids in promoting skeletal muscle hypertrophy (8–10).

In skeletal muscle, amino acid transporters function by transporting amino acids that are unique to specific transporters from the interstitial fluid to the sarcoplasm of muscle fibers. For instance, the L-type amino acid transporter 1 (LAT1) primarily transports leucine into the sarcoplasm while co-transporting glutamine out of the cell, whereas the sodium-coupled neutral amino acid transporter 2 (SNAT2) co-transports both sodium and glutamine into the sarcoplasm. These two transport proteins work in tandem such that SNAT2 brings in glutamine for LAT1 to pump back out of the cell while concomitantly transporting leucine into the cell. Leucine has been deemed as an essential regulator of protein synthesis (11), and independently increases protein synthesis through activation of the mechanistic target of rapamycin in complex 1 (mTORC1) (12–14). Another important amino acid transporter, proton-assisted amino acid transporter 1 (PAT1), lies within the lysosomal membrane and is speculated to be important for sensing lysosomal amino acids as well as signaling mTORC1 to the lysosomal membrane to promote the complex's activation (15). These intricate mechanisms illustrate that amino acid transporters are likely critical for facilitating skeletal muscle hypertrophy.

The previously mentioned amino acid transporters share a common transcription factor known as activating transcription factor 4 (ATF4) (16, 17). It could be posited that an increase in ATF4 protein would induce the transcription of the aforementioned amino acid transporters and, thereafter, promote an increase in protein synthesis by increasing intracellular amino acid concentrations. Indeed, several acute human studies have demonstrated that increases in protein synthesis occur in conjunction with an increase in ATF4 protein levels (18–21). However, recent literature suggests that inhibition or knockdown of ATF4 protein promotes protein synthesis, improves muscle strength and quality, and increases muscle fiber diameter in mice (22–24). It has yet to be determined how ATF4 protein is altered following exercise and/or nutrition chronic interventions in human skeletal muscle.

Metabolism of branched-chain amino acids (BCAAs) also plays a role in regulating protein synthesis. Notably, BCAA catabolism begins in the mitochondria with the reversible transamination of BCAAs by the metabolic enzyme branched-chain aminotransferase 2 (BCAT2) which produces branched-chain α-keto acids (BCKAs). BCKAs then undergo irreversible oxidative decarboxylation by the branched-chain α-keto acid dehydrogenase (BCKDH) complex. Importantly, the BCKDH reaction is the rate-limiting enzyme and the commitment step for BCAA metabolism. Regulation of the BCKDH complex is tightly controlled by BCKDH kinase and BCKDH phosphatase. BCKDH kinase phosphorylates the BCKDH complex on the alpha/E1 component (BCKDHα) to inactivate it (25), while BCKDH phosphatase dephosphorylates the BCKDH complex to activate it, resulting in BCAA catabolism. The literature involving the BCKDH complex with regard to humans, exercise, and nutrition is scarce and discrepant. For instance, in rat skeletal muscle it was reported that endurance training decreases skeletal muscle BCKDH kinase protein content (26); however, in human skeletal muscle, endurance training increases BCKDH kinase protein content (27). Interestingly, exercise increases activation of the complex to promote oxidation of BCAAs (28, 29). Furthermore, BCKDH kinase is inactivated by protein or amino acid supplementation promoting increased BCAA oxidation (29). Research on the complex involving exercise has utilized various forms of endurance exercise; however, the effects of resistance exercise and nutrition on regulation of the complex is unknown.

Resistance exercise independently, and with essential amino acid or protein supplementation, has been shown to improve skeletal muscle size, function, and quality in both young and old humans (30–36). Furthermore, acute resistance exercise has been shown to independently increase the mRNA expression and protein content of amino acid transporters (19, 37). Researchers have also demonstrated that amino acid transporter protein content is further improved when a supplement containing amino acids is ingested following exercise (18, 21, 38). Studies in this area, however, have only examined the acute response following resistance exercise, and the chronic response following resistance exercise training has yet to be determined. Therefore, the purpose of this study was to determine how skeletal muscle ATF4 protein levels, amino acid transporters, and metabolic enzymes involved with BCAAs catabolism are altered following resistance exercise training with different nutritional supplementation strategies, and if these changes are related to skeletal muscle fiber hypertrophy. Furthermore, based on these

in vivo findings, *in vitro* experiments were performed to elucidate potential mechanisms.

METHODS

Ethical Approval and Participant Screening

Prior to commencing this study, this protocol was reviewed and approved by the Auburn University Institutional Review Board and was in compliance with the Helsinki Declaration (approved protocol #: 15-320 MR 1508). Participants read and signed an informed consent prior to participation. Males that were apparently healthy, free of medical or orthopedic conditions, 19–23 years old, and unaccustomed to resistance training were recruited for participation. Participants stated they had not resistance trained within 6 months, used anabolic steroids, consumed dietary protein in excess of 2.0 g/kg/day regularly, or been consuming dietary supplements.

Experimental Design

This study is a follow-up analysis from a prior study, and readers are encouraged to read Mobley et al. (39) for complete methods including participant assignment to supplement groups according to whole-body lean tissue mass (assessed by dual-energy x-ray absorptiometry). Importantly, certain groups from that investigation were omitted (i.e., SOY and WPH), given that we felt the included groups (i.e., PLA, LEU, and WPC) would better delineate supplementation-related effects related to leucine and all amino acids (further described in "Supplementation and Dietary Intake"). Briefly, for baseline testing (PRE), participants refrained from physical activity for 96 h, reported to the laboratory hydrated (urine specific gravity <1.020), and 4 h fasted. Participants were then analyzed for body composition via DEXA (General Electric Lunar Prodigy enCORE, version 10.50086; Madison, WI, USA) and donated a skeletal muscle biopsy sample from the vastus lateralis. Two to three days following PRE, initial strength testing was conducted, and these baseline values were used for program prescription. Upon PRE completion, supplementation including placebo (PLA), leucine (LEU), and whey protein concentrate (WPC) was assigned to participants. Participants were instructed to consume supplements twice daily throughout the resistance exercise training protocol, which was completed 3x/week for 12 weeks. Approximately 72 h following each participant's last training session, the participant arrived similar to PRE conditions, underwent DEXA analysis, and donated a second skeletal muscle biopsy sample (POST).

Skeletal Muscle Biopsy

Skeletal muscle biopsy procedures have been described by our laboratory elsewhere (40, 41). Briefly, participants were instructed to lay supine on a treatment table. Excision of vastus lateralis tissue using a 5 mm Bergstrom Biopsy needle with suction was performed using local anesthesia (1% Xylocaine) and aseptic technique. Tissue was immediately blotted and then distributed for immunohistochemistry (IHC) and molecular analyses. The IHC section was placed in a cryomold with optimal cutting temperature (OCT) media (Electron Microscopy Sciences; Hatfield, PA, USA), slowly frozen in liquid nitrogen-cooled isopentane, and then stored at −80°C until staining procedures. Leftover tissue following IHC partitioning was flash-frozen in liquid nitrogen and stored at −80°C until further analyses. PRE and POST muscle biopsies were taken from the same leg at least 5 cm apart.

TABLE 1 | Macronutrient information per serving.

	PLA	LEU	WPC
Energy (kcal)	204	200	184
Total carbohydrate (g)	44.4	43.1	12.0
Dietary fiber (g)	1.6	1.8	1.8
Sugars (g)	6.0	5.1	5.9
Protein (g)	**0.4**	**2.3**	**26.3**
Total fat (g)	2.8	2.0	3.5
Leucine (mg)	**15**	**2,871**	**2,794**

Bold lettering highlights differences between supplementation groups.

Supplementation and Dietary Intake

Participants were counterbalanced into one of five groups although as previously mentioned only three groups were utilized: placebo (PLA), leucine (LEU), and whey protein concentrate (WPC). Group counterbalancing was based on DEXA lean body mass. Groups differed based on the supplementation provided, and the nutritional breakdown for each supplement is provided in **Table 1**. Notably, each PLA serving contained 44.4 g of carbohydrate with little leucine and protein, each LEU serving contained 43.1 g of carbohydrate with 2,871 mg of leucine and little protein, and WPC serving contained little carbohydrate with 2,794 mg of leucine and 26.3 g of protein. Each supplement was formulated to contain a similar amount of total energy (kcal) and fat (g) and was blinded to laboratory personnel and participants. Supplements were similar in appearance, taste, texture, and packaging; however, supplement packages only contained a serial number and were double-blinded to the investigators as well as the participants. The serial numbers corresponding to the individual supplements were kept blind to the staff until the conclusion of the study.

Participants were instructed to consume two servings per day of their respective supplement. On training days, one serving was consumed immediately following resistance exercise, and the other serving was consumed prior to bed. On non-training days, one serving was consumed between meals, and the other serving was consumed prior to bed. Supplements were mixed in shaker bottles with 500 mL of water, and participants were instructed as to how to blend their supplement. Individual packets were distributed in a 3-week supply, and empty packets were returned to ensure compliance. Participants that did not consume 80% or more of packets were excluded from analyses.

Food logs were filled out by participants at baseline (PRE) and week 12 (POST) of training. Participants were given detailed instructions on how to determine food proportions and thoroughness of meal description needed for accurate assessment. Four daily food records (2 weekdays [Mon–Fri]

and 2 weekend days [Sat and Sun]) were submitted by each subject at both PRE and POST. Dietary intake was analyzed using "MyFitnessPal" (MyFitnessPal, Inc., Baltimore, MD, USA). Participants were instructed to not add supplementation to their food logs. Upon completion of the study, supplement nutrition was added to existing totals and then averaged to represent participants' eating habits.

Resistance Training Program

Strength testing was completed at PRE whereby participants performed a 3-repetition maximum for squat and bench press. Following PRE testing, participants completed 12 weeks of resistance exercise training prescribed 3x/week. Exercises included squat, bench press, deadlift, and bent-over row prescribed at 4 sets of 10 repetitions on Sunday or Monday, 6 sets of 4 repetitions on Tuesday or Wednesday, and 5 sets of 6 repetitions on Thursday or Friday. Relative training intensities were based on PRE testing 3-repetition maximum values and increased weekly with the exception of 2 weeks (weeks 7 and 12) which were dedicated for "deloading" (i.e., a reduction in training volume) to prevent injury and promote recovery. More information regarding progression through the training period can be found within Mobley et al. (39). Participants who missed more than 4 exercise sessions were not included in analyses due to lack of training compliance. POST strength testing was performed following 12 weeks of resistance exercise training whereby participants performed a 3-repetition maximum for squat and bench press.

Western Blot Analysis

For protein analyses, ∼50 mg from each muscle biopsy sample was placed in 1.7 mL microcentrifuge tubes containing 500 μL of ice-cold cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton; Cell Signaling, Danvers, MA, USA] pre-stocked with protease and Tyr/Ser/Thr phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin). Samples were then homogenized by hand via micropestle manipulation, insoluble proteins were removed with centrifugation at 500 *g* for 5 min and obtained sample lysates were batch process-assayed for total protein content using a BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were then prepared for Western blotting using 4x Laemmli buffer and standardized for protein content at 1.0 μg/μL. Samples were equally loaded onto 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis (150 V for 60 min) using pre-made 1x SDS-PAGE running buffer (Ameresco). Proteins were then transferred (200 mA for 2 h) to polyvinylidene difluoride membranes (Bio-Rad), Ponceau S stained, and imaged to ensure equal protein loading between lanes. Membranes were then blocked for 1 h at room temperature with 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST; Ameresco, Solon, OH, USA). Following blocking, membranes were incubated in primary antibodies including: ATF4 (1:2,000; Abcam, ab1371, Cambridge, MA, USA), LAT1 (1:1,000; Cell Signaling, #5347, Danvers, MA, USA), PAT1 (1:200; Santa Cruz Biotechnology, sc-368553, Dallas, TX, USA), SNAT2 (1:1,000; Santa Cruz Biotechnology, sc-514037), BCAT2 (1:1,000; Abcam, ab95967), BCKDHα (1:1,000; Genetex, GTX45109, Irvine, CA, USA), eGFP (1:1,000; Bioss Inc.; bs-2194R, Boston, MA, USA), and Puromycin (1:1,000; MilliporeSigma; MABE342; Burlington, MA, USA). Primary antibody was incubated with membranes overnight at 4°C in TBST with 5% bovine serum albumin (BSA). The following day, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:2,000, Cell Signaling) in TBST with 5% BSA at room temperature for 1 h. Membrane development was performed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; MilliporeSigma). Band densitometry was performed using a gel documentation system and associated densitometry software (UVP, Upland, CA, USA). Densitometry values for all protein targets were normalized to Ponceau S densities. Each participant's PRE and POST sample was run in continuous lanes and on the same gel. All values for a given protein target were normalized to each participant's PRE value to provide fold change scores.

Notably, membranes for various targets were cut into upper and lower sections for antibody incubations; for example, one set of membranes were cut in half ∼55 kDa and the top half membrane was incubated with ATF4 primary antibody solution (target band ∼63 kDa), whereas the bottom half membrane was incubated with LAT1 primary antibody solution (target band ∼48 kDa). Additionally, bottom half membranes were re-probed for BCKDHα by exposing them to commercial stripping buffer following LAT1 quantification (ThermoFisher Scientific; 21059), and we ensured the stripping buffer removed all visible LAT1 bands prior to reprobing for BCKDHα. In these situations, the same Ponceau images were used to normalize ATF4, LAT1, and BCKDHα data (this can be seen in **Figures 2A,B,F** *where the same subjects were used for representative images*).

Immunohistochemical Analysis

Immunohistochemistry (IHC) was performed to determine muscle fiber cross-sectional area (fCSA) and LAT1 protein content, and these procedures have been previously described (42). Briefly, skeletal muscle biopsy samples frozen in OCT media were sectioned to 10 μm using a cryotome (Leica, Biosystems; Buffalo Grove, IL, USA). All samples were sectioned and stored at −80°C until staining occurred. To determine fCSA, sections were air-dried at room temperature for 30 min, fixed with 10% formalin for 10 min, permeabilized in phosphate-buffered saline (PBS) that contained 0.5% Triton X-100, and blocked with 100% Pierce Super Blocker (ThermoFisher Scientific; #37515) for 25 min. Sections were then washed with PBS and incubated for 1 h in primary antibody solution containing rabbit anti-dystrophin (ThermoFisher Scientific; PA5-32388) at 1:100 dilution and mouse anti-myosin II (Hybridoma Bank; SC71) at 1:100 dilution in blocking solution. Sections were then washed for 5 min in PBS and incubated with secondary antibody solution containing Texas Red anti-rabbit (Vector Laboratories; Burlingame, CA, USA) at 1:100 dilution and Alexa Flour 488 anti-mouse (ThermoFisher Scientific) at 1:100 dilution in blocking solution. Notably, it was possible to capture fiber-type distribution using this technique. As a result, the "mean fCSA" metric used as a dependent measure herein is the change

in fiber cross sectional area relative to that individual's fiber type distribution. Alternatively stated, the change in Type I fibers was made relative to the participant's respective fiber type distribution and was then added to the change in Type II fibers relative to the respective fiber type distribution for a "mean fCSA."

To determine LAT1 protein content, methods from Hodson et al. (43) were utilized with the exception of the fixation step as this step was found to confound the LAT1 signal during pilot staining. This alteration may explain the difference in staining intensity between their investigation and the present investigation. Briefly, sections were air-dried for 10 min, washed 3 times in PBS for 5 min each, and then blocked using Superblocker (ThermoFisher Scientific; #37515) for 30 min. Sections were then incubated with primary antibody solution containing mouse anti-dystrophin (ThermoFisher Scientific; PA5-32388) at 1:100 dilution and rabbit anti-LAT1 (Abcam, #85226) at 1:100 dilution (43). Notably, these experiments were duplicated with the same LAT1 primary antibody used during western blotting (Cell Signaling, #5347); however, this antibody was not specifically designated for IHC per manufacturer recommendations. As a result, only data yielded from the working antibody (Abcam, #85226) are presented herein. Sections were incubated in primary antibody solution for 2 h, washed in PBS, and incubated for 1 h with secondary antibody containing Texas Red anti-rabbit (Vector Laboratories; TI-1000) and Alexa Fluor 488 anti-mouse (Thermo Fisher Scientific; A-11001). Sections were then washed with PBS, mounted, and stored in the dark at 4°C until fluorescent images were taken. Notably, preliminary experimentation determined the antibodies used did not display cross-reactivity.

Digital 20x and 40x images were obtained using a fluorescence microscope (Nikon Instruments; Melville, NY, USA). Four sections (3 experimental and 1 negative control) per participant were mounted on each slide. Each section was separated by ∼100 μm. Two images were taken per experimental section for a total of 6 images per participant per timepoint, and at least 50 fibers were quantified per image. The counts from the 6 images were averaged and used during statistical analysis. Approximate exposure times were 600 ms for red and green imaging. This staining method allowed the identification of cell membranes (detected by the FITC filter; green) and LAT1 protein (detected by the TRITC filter; red). Measurements for type I fCSA, type II fCSA, and mean fCSA and LAT1 protein were conducted using the open-sourced software CellProfiler™ (44) whereby the number of pixels counted within the border of each muscle fiber were converted to total area. Threshold settings for quantifying LAT1 protein were first validated by manually counting the number of LAT1 protein particles, and then adjusting the threshold settings to meet the manually counted LAT1 protein particles.

Cell Culture

C2C12 myoblasts, passage number 5–8, were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco; #11965092) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, and 0.1% gentamycin. Myoblasts were used instead of myotubes given the well-known difficulty in transfecting myotubes without a virus. Cells were seeded in 6-well plates (Corning; #3335) at 0.3 x 10^6 in 3 mL of growth medium per well. Once myoblasts reached 60–70% confluence, myoblasts were transfected with a LAT1 plasmid (Genecopoeia, Rockville, MD, USA; EX-Mm05301-M98) via Lipofectamine 2000 (Invitrogen; #11668019) using manufacturer's protocols. Control cells were transfected with an empty vector (Genecopoeia, EX-NEG-M98) using identical methods. Both plasmids utilized a CMV promoter within the pReceiver-M98 vector and contained an eGFP reporter. Twenty-four hours later cells were pulse-labeled with 1 μM puromycin hydrochloride (Ameresco) in PBS to assess relative protein synthesis levels. Puromycin was incubated with cells for 30 min, medium was removed, cells were washed with ice-cold PBS, and harvested in ice-cold cell lysis buffer that was previously described. Western blotting methods for cell culture samples was identical to methods previously described. Proteasome activity assays on cell lysates were completed using a commercially available fluorometric kit according to manufacturer's instructions (MilliporeSigma; APT280).

Liquid Chromatography-Mass Spectrometry

Intracellular amino acid concentrations from C2C12 myoblasts were determined by using a liquid chromatography–electrospray ionization–mass spectrometry system (QTrap 5500 MS; AB Sciex; Framingham, MA, USA) with liquid chromatography device ExpressHT Ultra LC (Eksigent Div; AB Sciex). Samples were standardized with 0.1 N HCl that contained a stable isotopomer of every amino acid as an internal standard. Samples were transfer pipetted onto strong-cation-exchange drip columns. Columns were washed with water and eluted with 2.5 mol/L ammonia. Eluates were desolvated in a centrifugal evaporator. Solid-residue tubes were capped for storage in the dark at room temperature. Within 3 d of liquid chromatography–electrospray ionization–mass spectrometry analysis, samples were derivatized with 9-fluorenylmethoxycarbonyl (FMOC) and subsequently neutralized, after which sample solutions were injected onto a 0.5 × 100 mm HALO C18 column (Eksigent Div; AB Sciex) and kept at 35°C. Analytes were eluted with a segmentally linear gradient from 35 to 85% acetonitrile in water supplemented with ammonium acetate to 10 mmol/L and 5% isopropanol. Detection was performed by using electrospray triplequadrupole–tandem mass spectrometry in multiple-reaction monitoring mode. FMOC amino acid derivatives were fragmented in the collision cell for detection of either free aminoacyl anions or a fragment larger by 26 atom mass units (coming from the FMOC derivative), whichever gave the highest sensitivity. Thus, monitoring occurred for each amino acid, and internal standards. We used the SignalFinder algorithm in MultiQuant software (version 2.1; ABScieix) to quantify peaks.

Statistical Analyses

Certain data (e.g., participant characteristics, muscle fCSA, nutrition information) have been previously analyzed with additional supplementation groups, different sample sizes, and different statistical methods (39); however, these data have been reanalyzed herein using the three groups of interest, sample sizes

TABLE 2 | Participant characteristics prior to and following resistance training.

	PLA ($n = 12$)		LEU ($n = 13$–14)		WPC ($n = 15$–17)		ANOVA p-value		
	PRE	POST	PRE	POST	PRE	POST	Group	Time	G × T
Age (years)	21 ± 1		20 ± 1		21 ± 2		0.336		
Height (cm)	182.2 ± 9.1		179.4 ± 4.8		179.0 ± 6.4		0.444		
Total volume lifted (kg)	3,07,020 ± 34,937		3,24,637 ± 60,633		3,30,086 ± 51,791		0.414		
Fat mass (kg)	17.4 ± 4.9	17.8 ± 4.7	15.3 ± 5.8	15.8 ± 6.4	19.5 ± 8.6	18.7 ± 8.3	0.366	0.930	0.120
Fat free mass (kg)	57.3 ± 6.5	60.2 ± 6.4	56.8 ± 5.1	59.3 ± 4.9	59.0 ± 7.0	61.3 ± 6.9	0.625	**<0.001**	0.664
Total mass (kg)	74.8 ± 10.0	78.0 ± 9.5	72.1 ± 9.5	75.1 ± 10.3	78.5 ± 13.0	80.0 ± 13.4	0.387	**<0.001**	0.212
VL thickness (cm)	2.28 ± 0.30	2.74 ± 0.32	2.41 ± 0.24	2.77 ± 0.30	2.49 ± 0.23	2.96 ± 0.29	0.080	**<0.001**	0.446
3RM–Squat (kg)	67 ± 12[a]	108 ± 15*	85 ± 20[b]	112 ± 22*	83 ± 21[b]	118 ± 23*	0.177	**<0.001**	**0.031**

** <0.050 compared to PRE within group only if the G × T interaction was significant.*
Differing letters represent significant differences between groups within each time point. Bold lettering denotes a significant p-value.

related to this investigation, and alternate statistical methods which are described below.

Various participant characteristics were compared between groups using one-way ANOVAs. All dependent variables related to IHC, Western blotting, certain participant characteristics, and nutritional data were assessed using two-way repeated measures ANOVAs to test differences between groups (PLA, WPC, and LEU) and over time (PRE and POST). Fold-change was utilized for protein expression and IHC whereby POST was divided by PRE within each participant. This method was chosen based on past literature (18–20). Fold changes from all participants in a given group were then averaged. Given PRE was normalized to 1 with no standard deviation, the main effect for group p-value and group by time interaction p-value were identical; therefore, only the main effect for group and the main effect for time p-values were reported. Unlike this investigation, the aforementioned studies utilized 3 or more time points which allowed for numerical differences between the group main effect and group by time interaction p-values. Again, this method was chosen *a priori* based on the previous literature. Pearson correlations were also performed between all participant's individual change in fCSA and their fold change in western blot proteins to determine if relationships existed between these variables and, if trending toward significance or significant, was followed up with a simple linear regression analysis for each group to discern potential group differences. Cell culture data were analyzed using independent samples t-tests between conditions (LAT1 overexpression or control). Cell culture data fold change values were generated by dividing each sample value by the CTL average.

Normality was tested for all dependent variables using a Shapiro-Wilk test. Outliers were removed if they were ±3 times the interquartile range. When appropriate, Levene's test for equality of variances was determined. Assumptions testing was seldom violated; however, data were not manipulated due to the robustness of the ANOVA test, and the majority of the groups within the analysis did not violate this assumption. If a significant f-value was found following an ANOVA test, further statistical analysis was conducted using a LSD *post-hoc* comparison given the exploratory nature of this investigation. For regression analysis, heteroscedasticity, and residuals were analyzed. Sample sizes are noted in each figure legend and vary due to tissue and sample limitations. All data are presented as mean ± standard deviation values, trending statistical significance was established as $0.050 < p < 0.100$, and statistical significance was established as $p < 0.050$. All statistical analyses were performed using SPSS version 24.0 (Chicago, IL, USA).

RESULTS

Participant Characteristics and Nutrition Information

Participant characteristics are reported in **Table 2**. As noted in the parent publication by Mobley et al. (39), 18 participants were enrolled in the PLA group, and 15 participants finished the study due to supplement and workout compliance. The LEU group had 17 initial enrollees, and 15 participants finished the study due to supplement and workout compliance. The WPC group had 19 initial enrollees, and 17 participants finished the study due to supplement and workout compliance. Due to tissue limitations, however, samples sizes are reported in each figure legend.

Notably, differences did not exist between groups for age, height, or total volume lifted ($p > 0.050$). Moreover, fat mass, fat free mass, total mass, or vastus lateralis (VL) thickness were not different between groups at the beginning of the study ($p > 0.050$). Fat free mass, total mass, and VL thickness increased following resistance training ($p < 0.001$). Performance measured by squat demonstrated a group by time interaction ($p = 0.031$) whereby PLA squatted less than LEU and WPC at PRE; however, no differences existed at POST. All groups increased squat performance from PRE to POST ($p < 0.001$).

Self-reported nutrient intake data are reported in **Table 3**. All groups increased nutrient intake shown by a significant time effect for consumption of absolute and relative carbohydrates, protein, fat, and total energy following training ($p < 0.005$). WPC consumed less absolute and relative carbohydrates ($p < 0.050$) and more absolute and relative protein ($p < 0.050$) compared to PLA and LEU; albeit, all groups consumed statistically similar amounts of absolute and relative total energy.

TABLE 3 | Nutrition information prior to and following resistance training.

	PLA ($n = 12$)		LEU ($n = 14$)		WPC ($n = 17$)		ANOVA p-value		
	PRE	POST	PRE	POST	PRE	POST	Group	Time	G × T
Carbohydrates (g)	225 ± 92	348 ± 123*[a]	206 ± 62	310 ± 79*[a]	215 ± 59	244 ± 64[b]	0.100	**<0.001**	**0.009**
Carbohydrates (g/kg)	2.9 ± 1.1	4.3 ± 1.3*[a]	2.8 ± 1.1	4.1 ± 1.4*[a]	2.8 ± 1.0	3.0 ± 1.0[b]	0.143	**<0.001**	**0.010**
Protein (g)	80 ± 20	112 ± 49*[a]	87 ± 24	108 ± 36*[a]	88 ± 26	145 ± 24*[b]	0.094	**<0.001**	**0.009**
Protein (g/kg)	1.1 ± 0.2	1.4 ± 0.5[a]	1.2 ± 0.3	1.4 ± 0.5[a]	1.1 ± 0.3	1.8 ± 0.4[b]	0.294	**<0.001**	**0.009**
Fat (g)	78 ± 21	111 ± 52	73 ± 18	92 ± 24	71 ± 22	93 ± 51	0.354	**0.002**	0.744
Fat (g/kg)	1.0 ± 0.3	1.4 ± 0.5	1.0 ± 0.3	1.2 ± 0.4	0.9 ± 0.4	1.2 ± 0.7	0.480	**0.005**	0.851
Total energy (kcal)	1,969 ± 631	2,830 ± 1,006	1,835 ± 435	2,488 ± 495	1,866 ± 475	2,389 ± 729	0.347	**<0.001**	0.519
Total energy (kcal/kg)	25.4 ± 7.7	34.8 ± 10.1	24.9 ± 7.3	32.6 ± 8.7	23.7 ± 8.1	29.6 ± 11.0	0.454	**<0.001**	0.631

** <0.050 compared to PRE within group only if the G × T interaction was significant.*
Differing letters represent significant differences between groups within each time point. Bold lettering denotes a significant p-value.

Fiber Cross Sectional Area

Following 12 weeks of total-body resistance exercise training, type I fibers increased in fiber cross-sectional area (fCSA) from 3,618 ± 919 μm^2 to 4,052 ± 982 μm^2 which was demonstrated by a significant time effect ($p = 0.002$; **Figure 1A**). Likewise, Type II fibers increased in fCSA following training from 4,648 ± 1,170 μm^2 to 5,661 ± 1,768 μm^2 shown by a significant time effect ($p < 0.001$; **Figure 1B**). Lastly, growth of Type I and II fibers relative to each participant's fiber type composition (mean fCSA) increased following training from 4,297 ± 932 μm^2 to 5,063 ± 1,292 μm^2 demonstrated by a significant time effect ($p < 0.001$; **Figure 1C**). Neither type I, type II, or mean fCSA revealed a group effect ($p > 0.050$) or group x time interaction ($p > 0.050$).

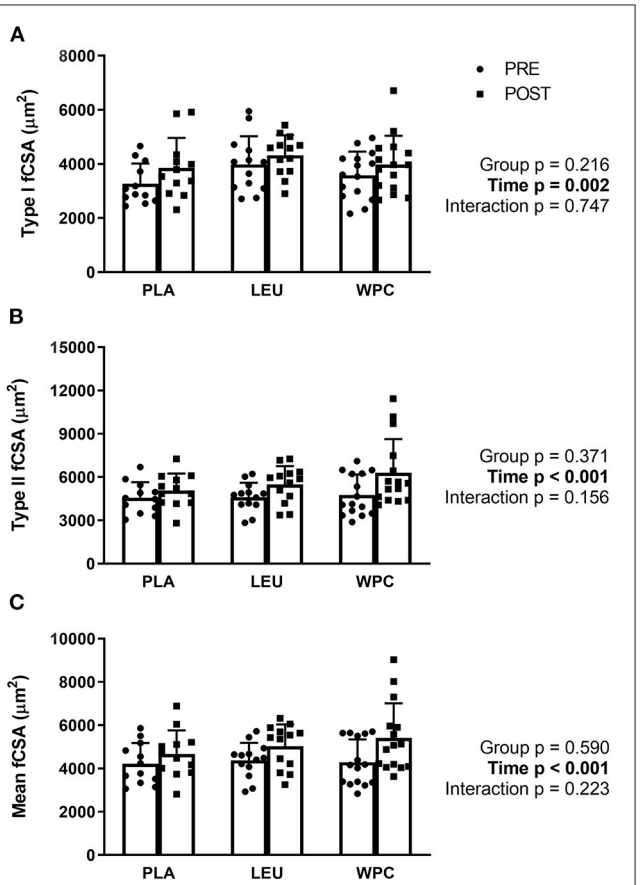

FIGURE 1 | Muscle fiber cross-sectional area (fCSA) increases with resistance exercise training independent of supplementation group. Skeletal muscle type I **(A)**, type II **(B)**, and mean **(C)** fCSA increased following 12 weeks of total-body resistance exercise training independent of consuming placebo (PLA), leucine (LEU), or whey protein concentrate (WPC). Open columns designate pre-training values (PRE), and closed columns designate post-training values (POST). Data are represented as mean ± standard deviation. Sample size for each group is PLA = 12, LEU = 13, and WPC = 15.

Protein Abundance

ATF4 protein (**Figure 2A**) demonstrated a group effect ($p = 0.030$) whereby the fold change in PLA (0.77 ± 0.09) was less than LEU (1.04 ± 0.29; $p = 0.011$), but not different than WPC (0.87 ± 0.27; $p = 0.308$), although WPC trended to be lower than LEU ($p = 0.061$). ATF4 protein decreased following training which was demonstrated by a significant time effect ($p = 0.029$; 1.00 to 0.91); albeit, only PLA significantly decreased from PRE to POST (1.00 to 0.77 ± 0.09) while LEU and WPC were unaltered ($p > 0.050$).

LAT1 protein (**Figure 2B**) revealed a group effect ($p = 0.043$) whereby the fold change in PLA (5.01 ± 5.50) was higher than LEU (1.87 ± 1.49; $p = 0.023$) and WPC (2.15 ± 2.43; $p = 0.031$), but LEU and WPC were not significantly different from one another. LAT1 protein also increased following training which was demonstrated by a significant time effect ($p = 0.001$; 1.00 to 2.86). PLA (1.00 to 5.01 ± 5.50) and LEU (1.00 to 1.87 ± 1.49) significantly increased from PRE to POST while WPC was unaltered ($p > 0.050$).

SNAT2 protein (**Figure 2C**) was unaltered following training or by supplementation shown by a non-significant group effect ($p = 0.399$) and time effect ($p = 0.216$). Similarly, BCAT2 protein (**Figure 2E**) was unaltered by training (time $p = 0.170$) or supplementation (group $p = 0.808$). PAT1 protein (**Figure 2D**) increased with training shown by a significant time effect ($p = 0.040$; 1.00 to 1.37) but not with supplementation shown by a non-significant group effect ($p = 0.993$).

BCKDHα protein (**Figure 2F**) demonstrated a group effect ($p = 0.044$) whereby the fold change in PLA (2.35 ± 1.36) was greater than LEU (1.44 ± 0.88; $p = 0.035$) and WPC (1.38 ± 0.95;

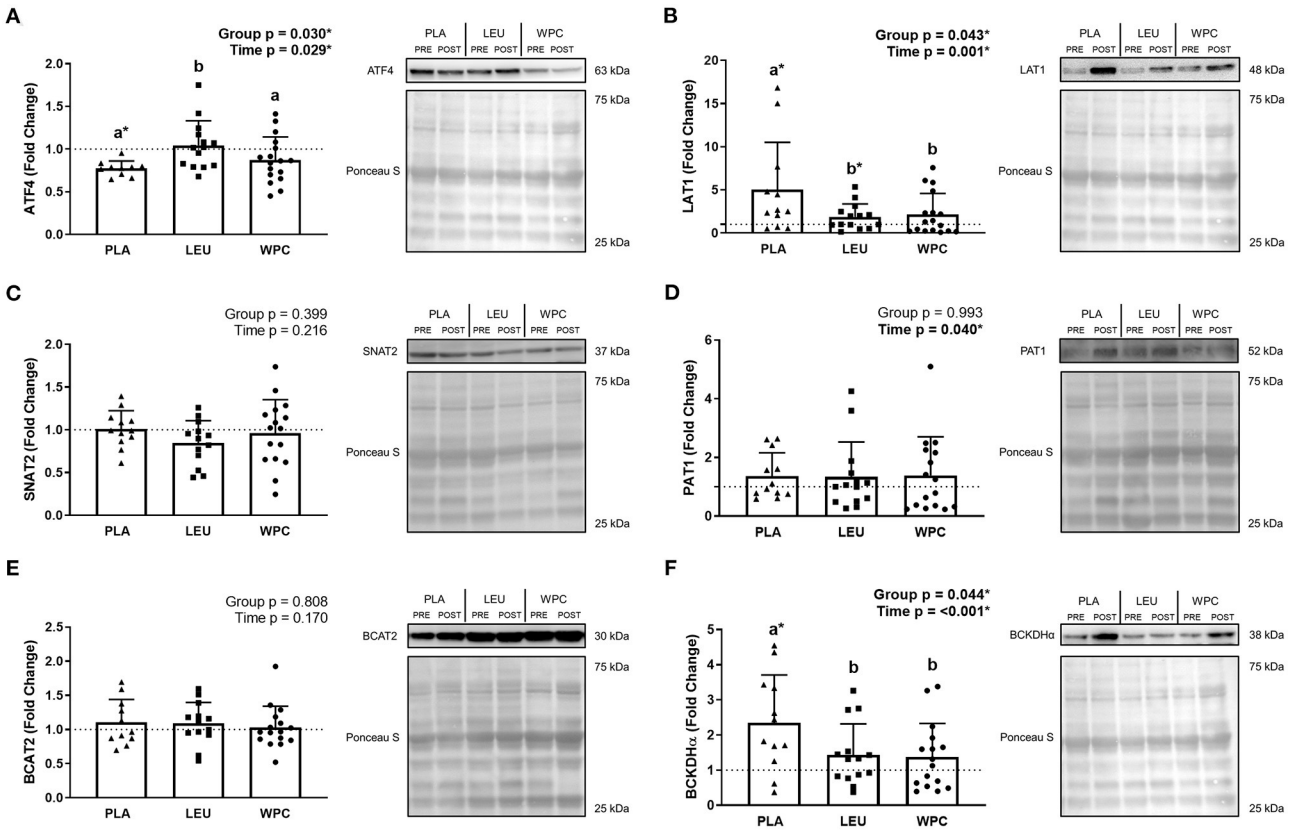

FIGURE 2 | The protein content of amino acid transporters and metabolic enzymes are altered following 12 weeks of resistance exercise training. The fold change from PRE, designated as the dashed line at 1.00, for ATF4 **(A)**, LAT1 **(B)**, SNAT2 **(C)**, PAT1 **(D)**, BCAT2 **(E)**, and BCKDHα **(F)** protein content for each group. A representative image for each protein and loading control (i.e., Ponceau S stain) accompanies each panel. Differing letters represent significant differences between groups within each time point. *$p < 0.050$ compared to PRE within group only if the G × T interaction was significant. Data are represented as mean ± standard deviation. Sample size for each group is PLA = 10–12, LEU = 13–14, and WPC = 16–17.

$p = 0.022$), but LEU and WPC were not significantly different from each other. BCKDHα protein also increased following training which was demonstrated by a significant time effect ($p < 0.001$; 1.00 to 1.78), although only PLA significantly increased from PRE to POST (1.00 to 2.35 ± 1.36) while LEU and WPC were unaltered ($p > 0.050$).

Immunohistochemistry

To confirm our LAT1 western blot data, IHC for LAT1 was performed. Two separate antibodies for LAT1 (Abcam, #85226; Cell Signaling, #5347) were used and analyzed identically but did not produce congruent results. In fact, no correlation existed for the same metric between the two different antibodies ($p > 0.050$) (*data not shown)*. In this regard, one antibody (Abcam, #85226) was designated for immunohistochemistry per manufacturer's recommendations and is presented herein (**Figure 3**), while the other antibody was not designated for immunohistochemistry (Cell Signaling, #5347). Results from this antibody (Cell Signaling, #5347) are shown in **Supplementary Figure 1**. Using the same dependent metrics for immunohistochemistry listed below, there were no significant findings using this antibody (Cell Signaling, #5347) ($p > 0.050$).

Using the appropriate antibody (Abcam, #85226), staining for total LAT1 protein (sarcoplasmic and membrane LAT1; **Figure 3A**), membrane LAT1 protein (**Figure 3C**), or membrane LAT1/fiber (**Figure 3D**) did not reveal a group effect ($p > 0.050$) or time effect ($p > 0.050$). Total LAT1/fiber did not demonstrate a group effect ($p = 0.599$) but did show a time effect whereby Total LAT1/fiber increased from PRE to POST ($p = 0.003$; 1.00 to 1.09 ± 0.14; **Figure 3B**).

Correlations and Regression

To determine if the change in amino acid transporters or metabolic enzymes was indicative of hypertrophy a correlation table was created and is shown in **Table 4**. Notably, changes in measured proteins were not indicative of the change in Type I fCSA, Type II fCSA, or mean fCSA; however, the change in ATF4 protein trended toward a significant ($0.050 < p < 0.010$) weak correlation ($r = 0.287$–0.314) for changes in measures of fCSA. Moreover, PRE raw-value protein content for each measured protein was not correlated with the change in Type I fCSA, Type II fCSA, or mean fCSA ($p > 0.100$) (*data not shown*).

To delineate if the trending correlation between the change in ATF4 protein content and the change in fCSA was skewed by a

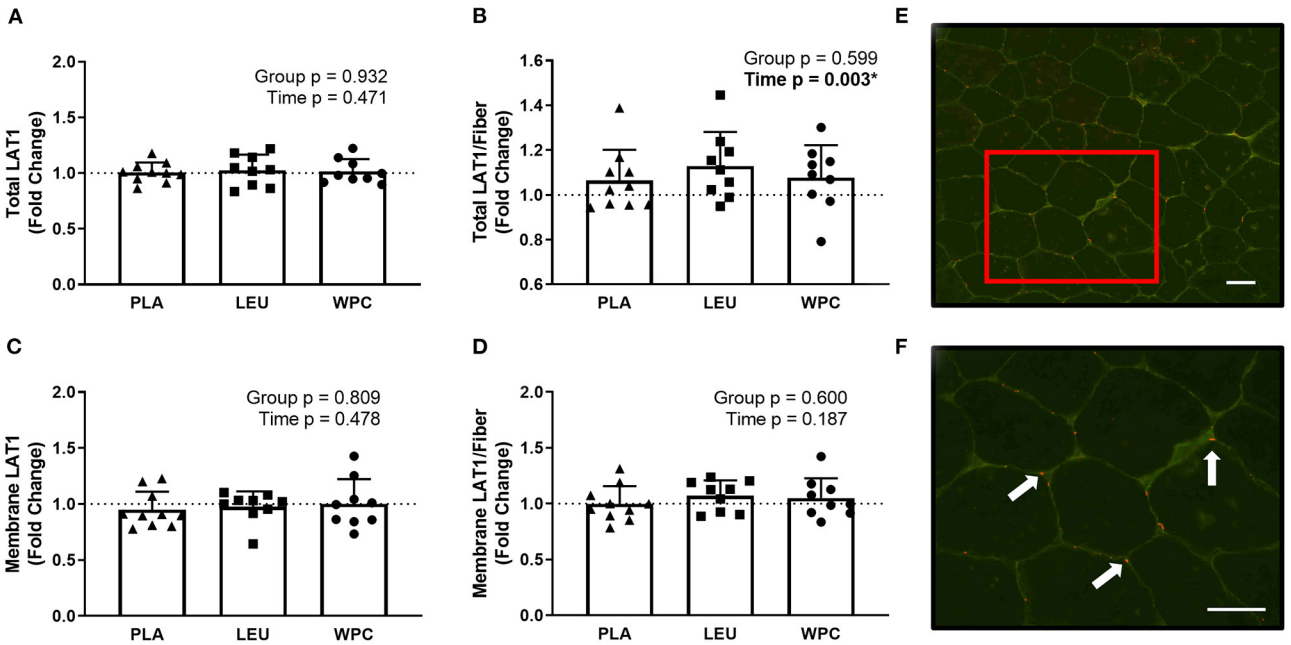

FIGURE 3 | LAT1 protein increases per skeletal muscle fiber with resistance exercise training but the increase in protein is not located on the membrane. The fold change from PRE, designated as the dashed line at 1.00, for Total LAT1 **(A)**, Total LAT1 per fiber **(B)**, Membrane LAT1 **(C)**, and Membrane LAT1 per fiber **(D)** protein content for each group using immunohistochemistry. A representative image showing green-stained dystrophin and red-stained LAT1 protein using 20x magnification **(E)**; the inset in E is magnified using 40x magnification **(F)** and the arrows point toward LAT1 protein. The scale bar in both images is 100 μm. *A significant effect. Data are represented as mean ± standard deviation. Sample size for each group is PLA = 10, LEU = 9, and WPC = 9.

certain group, a simple linear regression analysis was performed for each group. Indeed, when the change in ATF4 protein was used to predict the change Type I fCSA, a significant ($p = 0.035$) coefficient of determination ($r^2 = 0.344$) was found for LEU but not for PLA or WPC (**Figure 4A**). The change in Type II fCSA was not significantly predicted when using the change in ATF4 protein as the predictor ($p = 0.079$; **Figure 4B**). When the change in ATF4 protein was used to predict the change in mean fCSA, LEU demonstrated a significant ($p = 0.043$) coefficient of determination ($r^2 = 0.322$; **Figure 4C**).

Cell Culture

LAT1 protein was overexpressed (OvEX) in C2C12 myoblasts compared to controls (CTL) ($p = 0.026$; **Figure 5A**). Given the plasmid construct contained eGFP, this protein was also quantified using western blotting and was not different between CTL and OvEX ($p = 0.977$) suggesting transfection efficiency was equal between groups (*data not shown*). BCKDHα protein decreased with LAT1 protein overexpression compared to CTL ($p = 0.001$; **Figure 5B**). Using LCMS to determine amino acid concentrations within cell lysates, LAT1 protein overexpression did not alter the concentration of Thr, Val, Met, Ile, Leu, Phe, His, Lys, total BCAAs, or total EAAs (**Figure 5C**). Protein synthesis measured by puromycin incorporation was lower when LAT1 protein was overexpressed ($p = 0.002$; **Figure 5E**); however, proteasome activity was not altered ($p = 0.347$; **Figure 5D**).

TABLE 4 | Correlations for the change in fCSA and the fold change in proteins.

	Δ Type I fCSA		Δ Type II fCSA		Δ Mean fCSA	
	r-value	*p*-value	*r*-value	*p*-value	*r*-value	*p*-value
Δ LAT1	−0.079	0.627	−0.163	0.313	−0.165	0.309
Δ SNAT2	0.023	0.891	0.167	0.316	0.104	0.533
Δ PAT1	−0.066	0.690	−0.068	0.681	−0.065	0.692
Δ ATF4	0.287	0.081	0.305	0.063	0.314	0.055
Δ BCAT2	0.164	0.331	0.199	0.238	0.160	0.343
Δ BCKDHA	0.021	0.899	−0.043	0.796	−0.071	0.667

Sample size for each correlation was 37–40.

DISCUSSION

Resistance exercise training is a potent anabolic stimulus for promoting skeletal muscle growth and when paired with protein or amino acid supplementation, skeletal muscle growth is optimized (39, 45). Both resistance exercise and protein supplementation independently, and in concert, alter skeletal muscle amino acid transporters and enzymes involved with amino acid metabolism (29, 38). The current investigation demonstrates that amino acid transporters and enzymes involved with BCAA metabolism are altered following 12 weeks of total-body resistance exercise training. Our *in vitro* findings demonstrate that perturbations in LAT1 protein levels can

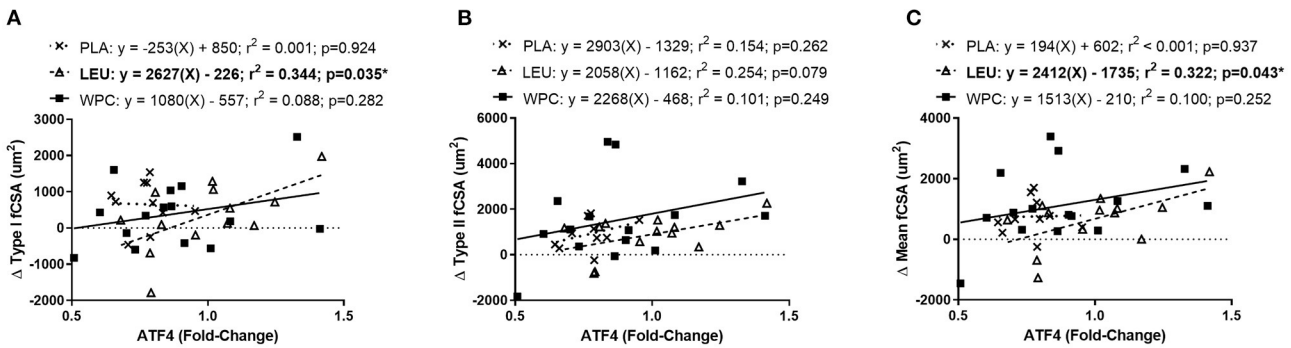

FIGURE 4 | The change in ATF4 protein predicts the change in fCSA only in LEU following 12 weeks of resistance exercise training. Regression analysis was completed using the change in ATF4 protein to predict the change in Type I **(A)**, Type II **(B)**, and mean **(C)** fCSA. Each panel contains each group's predictive equation, coefficient of determination (r^2), and *p*-value. Best fit lines are displayed in each graph. *A significant prediction. Sample size for each group is PLA = 12, LEU = 13, and WPC = 15.

lead to appreciable alterations in protein synthesis and amino acid metabolism.

Each group utilized in the present study consumed a unique supplement and, as expected, PLA and LEU ingested more carbohydrates, but less protein, than WPC. Importantly, absolute and relative total energy consumption was not different between groups given increased energy consumption provides more energy to meet metabolic demand or energy-intensive processes such as protein synthesis. Furthermore, the total amount of weight lifted (i.e., training volume) was not different between groups. Even though supplementation was different between groups, all groups equally increased muscle strength and hypertrophy which was measured by 3-RM squat and ultrasound, respectively. Furthermore, all groups equally increased type I, type II, and mean fCSA with training. Taken together, these results suggest the differences in molecular markers are specific to the supplementation provided.

Several studies have demonstrated skeletal muscle LAT1 protein levels acutely increase following amino acid ingestion, resistance exercise, or both together (18, 19, 38). LAT1 protein is also influenced by bed rest as 7 d of bed rest attenuated the increase in LAT1 protein compared to control (20). The acute increase in amino acid transporters in response to resistance exercise appears transient given that LAT1 protein is not different 72 h following 3 bouts of endurance or resistance exercise compared to basal levels (37) suggesting the findings herein are a resistance exercise training effect. Following 12 weeks of resistance exercise training, LAT1 protein increased and, unexpectedly, increased the most in PLA. This unexpected result may be a result of the significant downregulation of ATF4 protein in the PLA group. These findings regarding the ATF4 protein data expand and corroborate prior literature. First, ATF4 expression is upregulated during periods of amino acid or energy deficiency such as exercise (24). In this regard, Drummond et al. found an increase in ATF4 protein following resistance exercise (19); however, an unexpected and similar increase in ATF4 protein has also been observed following amino acid ingestion (18). Second, ATF4 has been implicated in promoting muscle atrophy (22, 24) and, therefore, a reduction in ATF4 protein content would promote hypertrophy, which is corroborated herein by the significant reduction in ATF4 protein seen in PLA. It is plausible to suspect an initial increase in ATF4 protein during the first weeks of training promoted an increase in LAT1 protein (as well as PAT1 protein); however, as training progressed, ATF4 protein content may have become downregulated from the improved transport capacity of amino acids.

Although other leucine transporters exist (e.g., LAT4), LAT1 is suggested to be most important for leucine transport, and it is essential for LAT1 to reside on the cellular membrane to fulfill transport (46, 47). This study extends the findings of Hodson et al. (43) who optimized a procedure for LAT1 IHC, and found LAT1 protein was greater in Type II muscle fibers. The present investigation found total LAT1 protein per fiber increased equally in all groups with training, which partly agrees with the western blot data. Total LAT1 protein was unaltered following training, which is likely due to the increased fCSA and less countable fibers in the field of view as well as the sensitivity differences between IHC and western blotting. Interestingly, we did not find LAT1 protein to increase along the sarcolemma following training. This notion suggests LAT1 may be internalized within the cell and translocated to the sarcolemma acutely in response to an anabolic stimulus similar to how LAT1 can be recruited to the lysosomal membrane (48). Moreover, Hyde et al. suggests internalization of SNAT2 with ceramide treatment (49) which suggests amino acid transporters have altered subcellular distribution following a given stimulus. Under basal conditions, LAT1 may be internalized and shuttled to the sarcolemma in response to resistance training or amino acid ingestion, although this is speculation.

To better understand if any of the molecular targets were related to changes in muscle fCSA, a simple linear regression analysis was performed. Following a correlation analysis, it was determined the change in ATF4 protein, albeit trending significance, was the only plausible candidate from measured markers to predict the change in muscle fCSA. Intriguingly, a relationship existed for LEU, but not PLA and WPC, and

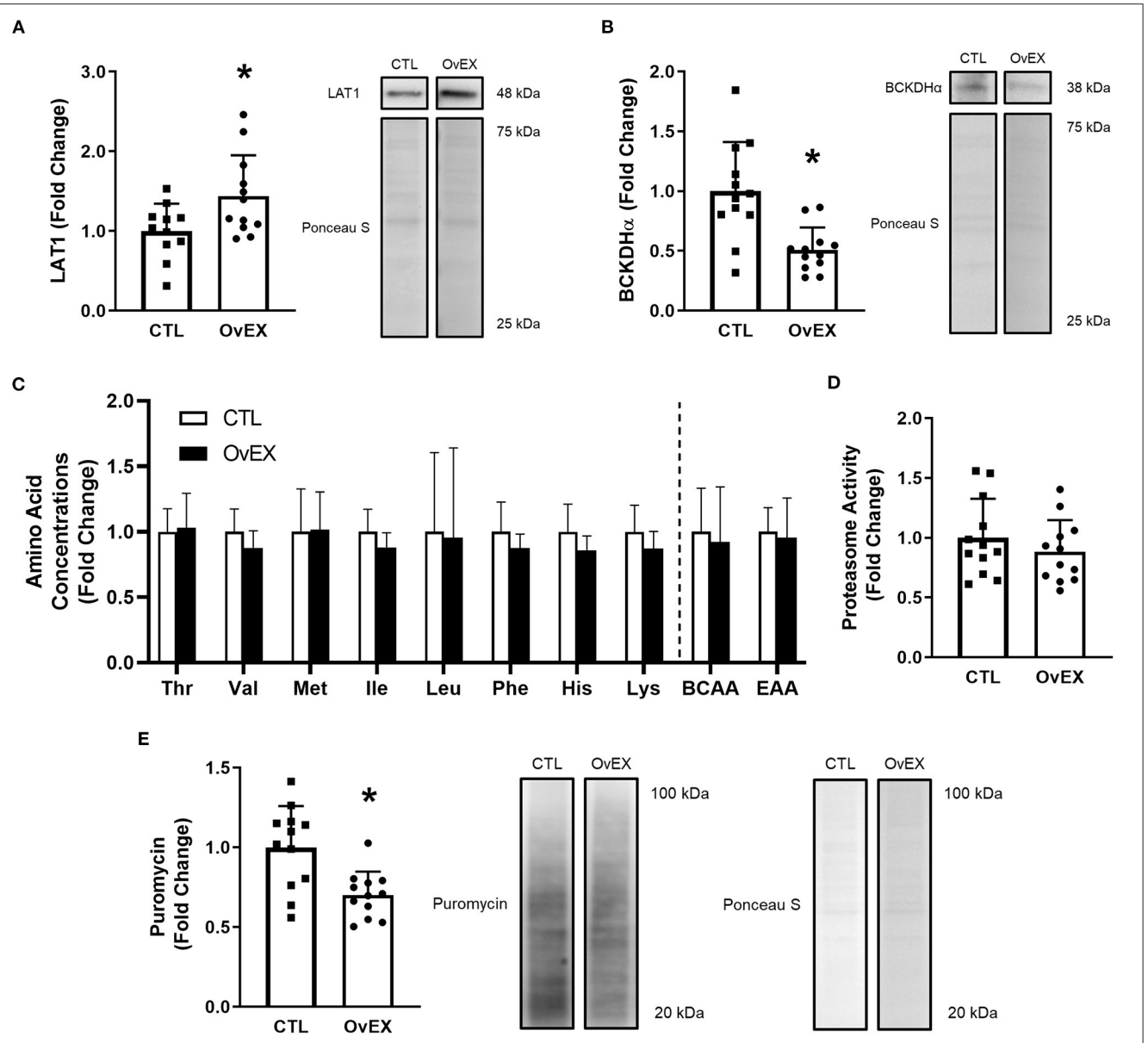

FIGURE 5 | LAT1 protein overexpression in C2C12 myoblasts decreases protein synthesis. Overexpression of LAT1 protein (OvEX) increases LAT1 protein **(A)** but decreases BCKDHα protein **(B)** compared to control (CTL). Amino acid concentrations from whole cell lysates **(C)** as well as proteasome activity **(D)** were unaltered by LAT1 protein overexpression. Puromycin incorporation, a surrogate for protein synthesis, decreased following LAT1 overexpression **(E)**. Representative images for each measured protein accompany each panel. In panel C open columns represent CTL and closed columns represent OvEX. *$p < 0.050$ compared to control. Sample sizes for each treatment group was CTL = 11–12 and OvEX = 12 with the exception of **(C)** as each group's sample size was 8.

the significant regression equations were positive suggesting an increase in ATF4 protein was associated with skeletal muscle fiber hypertrophy. These findings are in direct conflict with previous literature suggesting ATF4 promotes muscle atrophy (22–24); however, it is paramount to note these findings are only respective to the changes seen following 12 weeks of resistance exercise training and do not reflect acute alterations and the ensuing responses that could have occurred during training. Importantly, the downregulation found in ATF4 protein following resistance exercise training supports that an attenuation in ATF4 protein promotes skeletal muscle hypertrophy, but the regression analysis suggests that acute alterations in ATF4 protein may be more important for determining skeletal muscle hypertrophy seen with exercise training. As noted, the change in ATF4 protein only predicted the change in muscle fCSA in the LEU group suggesting a unique relationship may exist between ATF4 protein and leucine supplementation. Moreover, it is possible the absence of the other BCAAs or amino acids in the presence of high leucine concentrations is causing this effect. Future investigations are

needed to determine the precise relationship of ATF4 and leucine as well as other amino acids.

Amino acid metabolism, and specifically BCAA metabolism, is foundational for anaplerotic reactions. These reactions serve to meet metabolic demands while indirectly stimulating protein synthesis by limiting oxidation of amino acids (e.g., leucine). In spite of the fact that BCAT2 protein levels have been shown to be involved with metabolism and endurance performance (50, 51), our results suggest resistance exercise training or different supplementation paradigms do not impact this marker. The BCKDH complex is critical for BCAA metabolism given that it is the rate-limiting enzyme and is the commitment step toward generation of ATP from BCAA catabolism. BCKDHα protein levels increased with training herein, where a significantly greater increase occurred in the PLA vs. the LEU and WPC groups. The alpha-subunit of the complex is critical as phosphorylation of this subunit by the BCKDH kinase inactivates the complex (25). Unfortunately, due to limited resources and tissue, this investigation was unable to explore the protein content of other subunits of the BCKDH complex or associated regulatory enzymes such as the BCKDH kinase. Similarly, activity and phosphorylation of the BCKDH complex was also not measured, and these are limitations herein and warrant further investigation.

Given that resistance training was found to increase skeletal muscle LAT1 protein levels, *in vitro* experiments were performed to determine if LAT1 overexpression affected various molecular outcomes associated with anabolism (e.g., muscle protein synthesis and proteasome activity). While LAT1-tranfected cells possessed more LAT1 protein, intracellular amino acid concentrations (and leucine in particular) were not altered in LAT1-transfected vs. control cells. As mentioned above, this may be related to the lack of an anabolic stimulus required to localize LAT1 to the sarcolemma. Notwithstanding, a provocative and unexpected finding was that the LAT1-trasfected cells experienced an attenuation in puromycin incorporation suggesting rates of protein synthesis were lower. Notably, our laboratory has also observed that when LAT1 protein was knocked down in C2C12 myotubes, there was an increase in protein synthesis [(52); *unpublished observations*]. These findings are puzzling given the presumption that increased LAT1 protein would lead to increased intracellular leucine and, thus, increased protein synthesis. Interestingly, amino acid concentration nor proteasome activity was different between control and overexpression cells, but BCKDHα protein was lower in LAT1 overexpressed cells compared to control. The findings from cell culture in conjunction with the findings from humans regarding the different alterations in the BCKDHα protein suggests the BCKDH complex is not only important for metabolism but also affects protein synthesis. Indeed, dysregulation of the BCKDH complex results in maple syrup urine disease and is also associated with skeletal muscle atrophy (53, 54). Based on these findings, cellular transport of amino acids appears secondary to amino acid metabolism or, perhaps, overall metabolism given high glucose concentrations can limit amino acid oxidation (55). In attempt to reconcile the findings in this investigation in concert with findings from previous studies, increased LAT1 protein may promote greater efflux of amino acids from the sarcoplasm effectively lowering intracellular concentration and, perhaps, protein synthesis (48, 56). It is also possible the production of the BCKDH complex subunits turnover more slowly than transport proteins leading to a buildup of alpha-ketoacids which are toxic in excess. It is important to note these hypotheses are speculative and underpin the need for more research in this area.

In conclusion, the current investigation demonstrates total-body resistance exercise over 12 weeks affects amino acid metabolism and transporter content. While acute exercise was known to affect amino acid transporters, these data provide context to long-term adaptations in amino acid transporters in response to chronic resistance exercise. Indeed, resistance exercise training increases skeletal muscle LAT1, PAT1, and BCKDHα protein and decreases ATF4 protein. These markers are also affected by nutritional supplementation during training as shown by PLA participants experiencing the most pronounced alterations in these markers. The alterations in measured proteins do not appear to be associated with regulating skeletal muscle hypertrophy; however, regression analysis suggests a unique relationship between ATF4 and leucine. Lastly, overexpression of LAT1 protein decreased BCKDHα protein and protein synthesis without altering amino acid concentrations or proteasome activity, which suggests enzymes related to amino acid metabolism may affect protein synthesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Auburn University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PAR, MDR, and CM conceived and designed research. PAR analyzed data and prepared figures. PAR and MDR interpreted results of experiments and drafted manuscript. All authors edited and revised manuscript, approved final version of manuscript, and assisted with data collection and performed experiments.

FUNDING

Funding for participant compensation and reagents were provided by monetary donations to MDR through Hilmar Ingredients (Hilmar, CA, USA), Bionutritional Research Group (Irvine, CA, USA), and Lockwood, LLC (Belton, TX, USA). Funding for the data procured in the current manuscript as well as article publishing charges were provided by a discretionary laboratory fund of MDR. PAR is currently supported by

the National Institute of Health grant F32DK126312. Dr. Christopher Lockwood (Lockwood, LLC) was involved in the study design for the parent paper of this current manuscript (39). However, he had no involvement in the collection, analysis, interpretation of data, the writing of this current article or the decision to submit it for publication.

ACKNOWLEDGMENTS

The authors thank the participants for their dedication, time, and effort as well as Wes Kephart, Richard Anderson, Jessica Nendze, Casey Sexton, Chester Davidson, Romil Patel, David Baumohl, Coree Pledge, and Corey Chandler for assisting in overseeing participant workout sessions. We also thank Scot R. Kimball, Michael D. Dennis, John C. Quindry, John J. McCarthy, Andreas N. Kavazis, Jeffrey S. Martin, Kaelin C. Young, Darren T. Beck, and David D. Pascoe for insightful feedback prior to and throughout data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.628405/full#supplementary-material

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Conflict of Interest: Funding for participant compensation and reagents were provided by monetary donations to MDR through Hilmar Ingredients (Hilmar, CA, USA), Bionutritional Research Group (Irvine, CA, USA), and Lockwood, LLC (Belton, TX, USA). Funding for the data procured in the current manuscript as well as article publishing charges were provided by a discretionary laboratory fund of MDR. Dr. Christopher Lockwood (Lockwood, LLC) was involved in the study design for the parent paper of this current manuscript (39). However, he

had no involvement in the collection, analysis, interpretation of data, the writing of this current article or the decision to submit it for publication. The results of this study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The laboratory of MDR performs contracted research for nutritional supplement companies. Moreover, MDR has served as a paid consultant with various nutritional supplement companies. However, MDR has not served as a consultant for Hilmar Ingredients, Bionutritional Research Group, or Lockwood, LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Citation: Roberson PA, Mobley CB, Romero MA, Haun CT, Osburn SC, Mumford PW, Vann CG, Greer RA, Ferrando AA and Roberts MD (2021) LAT1 Protein Content Increases Following 12 Weeks of Resistance Exercise Training in Human Skeletal Muscle. Front. Nutr. 7:628405. doi: 10.3389/fnut.2020.628405

frontiers in Physiology

ORIGINAL RESEARCH
published: 03 March 2021
doi: 10.3389/fphys.2021.647743

Hypoxanthine Induces Muscular ATP Depletion and Fatigue via UCP2

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Hypoxanthine (Hx), an intermediate metabolite of the purine metabolism pathway which is dramatically increased in blood and skeletal muscle during muscle contraction and metabolism, is characterized as a marker of exercise exhaustion. However, the physiological effects of Hx on skeletal muscle remain unknown. Herein, we demonstrate that chronic treatment with Hx through dietary supplementation resulted in skeletal muscle fatigue and impaired the exercise performance of mice without affecting their growth and skeletal muscle development. Hx increased the uncoupling protein 2 (UCP2) expression in the skeletal muscle, which led to decreased energy substrate storage and enhanced glycolysis. These effects could also be verified in acute treatment with Hx through intraperitoneal injection. In addition, muscular specifically knockout of UCP2 through intra-muscle tissue injection of adenovirus-associated virus reversed the effects of Hx. In conclusion, we identified a novel role of Hx in the skeletal muscular fatigue mediated by UCP2-dependent mitochondrial uncoupling. This finding may shed light on the pathological mechanism of clinical muscle dysfunctions due to abnormal metabolism, such as muscle fatigue and weakness.

Keywords: hypoxanthine, exercise, skeletal muscle, UCP2, ATP, fatigue

OPEN ACCESS

Edited by:
Yan Huang,
University of Arkansas, United States

Reviewed by:
Yinshan Fang,
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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *30 December 2020*
Accepted: *11 February 2021*
Published: *03 March 2021*

Citation:
Yin C, Ma Z, Li F, Duan C, Yuan Y, Zhu C, Wang L, Zhu X, Wang S, Gao P, Shu G, Zhang H and Jiang Q (2021) Hypoxanthine Induces Muscular ATP Depletion and Fatigue via UCP2. Front. Physiol. 12:647743. doi: 10.3389/fphys.2021.647743

INTRODUCTION

Skeletal muscle, the largest metabolic tissue of the body, is the major source of many metabolites that are released primarily during muscle contraction but also in the resting state (Pedersen and Febbraio, 2012; Seiler et al., 2015; Hoffmann and Weigert, 2017). These intermediates are characterized as myometabolites because of the important determinant of their levels in the circulation of muscle. Besides their role as energy substrates for many other metabolic pathways, myometabolites have received considerable attention due to their potential positive signaling roles in maintaining physical homeostasis and pathological progression, including exercise adaptation, body weight regulation (Hoffmann and Weigert, 2017), inflammation and immunological disease (Korotkova and Lundberg, 2014), insulin sensitivity and glucose homeostasis (Rai and Demontis, 2016), cancer and tumor formation (Yang et al., 2017), and cognitive function (Moon and van Praag, 2014). However, most studies on myometabolites are focused on their beneficial functions, while the detrimental roles are poorly understood. Studies on the detrimental myometabolites are mostly about lactate, which is produced and accumulated during muscle contraction, and could

cause muscle fatigue and soreness. These detrimental myometabolites should also be paid attention, so that we could fully understand the physiological roles of them.

Hypoxanthine (Hx), an intermediate metabolite of purine nucleotides, is also an important myometabolite that mainly distributed in muscular tissue (Bone et al., 2015). During exercise or muscle contraction, Adenosine triphosphate (ATP) is catabolized to release high-energy phosphate for energy demand. Meanwhile, it produces numerous intermediate metabolites in this metabolic process, such as: adenosine monophosphate (AMP), inosine monophosphate (IMP), adenosine, inosine, and Hx (Zielinski et al., 2013a; Hoffmann and Weigert, 2017). Many studies on energy metabolism of exercise have identified the elevation of blood Hx level, which could be 2-10 times of the resting state in response to different exercise intensity (Zielinski et al., 2009, 2013a,b; Zieliński and Kusy, 2015). So Hx is also characterized as a marker of exercise exhaustion or fatigue. Moreover, Hx is involved in various physiological and pathological processes, for instance, cholesterol metabolism and atherosclerosis (Ryu et al., 2016), cell apoptosis (Kim et al., 2017), neural signaling presentation (Zamzow et al., 2008; Biasibetti et al., 2017), intestinal barrier protection (Lee et al., 2018), and embryonic development (Dienhart et al., 1997; Ma et al., 2003). However, the physiological roles of Hx in the skeletal muscle metabolism remain unknown.

The regulation of skeletal muscle metabolism involves changes in muscle mass, myofiber composition, and mitochondrial properties associated with appropriate metabolic modifications. Slow and fast myofibers in skeletal muscle exhibit different oxidative and glycolytic metabolic capacities, and can be converted from one fiber type to another in response to the metabolic demand (Frontera and Ochala, 2015). As the core element of energy metabolism, mitochondria determine the utilization of metabolic substrates and the transformation of metabolic pathways. Uncoupling proteins (U), located in the inner mitochondrial membrane, can dissipate the proton electrochemical gradient generated during electron transport (Bouillaud et al., 2016). Consequently, UCPs eliminate the reactive oxygen species (ROS) and protect the cell from oxidative stress. On the other hand, UCPs uncouple mitochondrial respiration from ATP formation, and the direct consequence of this process is a decrease in the oxidative phosphorylation efficacy.

In this study, we investigated the role of Hx in muscle physiological function. We found that Hx causes muscular weakness of mice both through chronic treatment (dietary supplement) and acute treatment (intraperitoneal injection). Hx induced the muscular ATP deficiency and enhanced glycolysis that involves the uncoupling protein 2 (UCP2). The expression of UCP2 was elevated with Hx-treated. Muscular specific knockout of UCP2 through adeno-associated viral vector injection blocked Hx-induced ATP deficiency and glycolysis enhancement, which rescued muscular weakness. These findings might be relevant to the mechanism of muscle fatigue and weakness.

MATERIALS AND METHODS

Animal Experiments

Animal experiments were approved by the College of Animal Science, South China Agricultural University. Wild-type C57BL/6J male mice were maintained in accordance with The Instructive Notions with Respect to Caring for Laboratory Animals (Ministry of Science and Technology, Beijing, China). Mice were housed in individual cages with a constant temperature of 25 ± 1°C, 55% relative humidity, and 12 h light/dark cycle.

For chronic Hx-treated experiment, 24 male mice of 7 weeks old were randomly divided into three groups according to body weight (8 mice per group), the mice were given *ad libitum* access to a standard chow diet with or without Hx (10 mg/g or 20 mg/g) (Sango Biotech, China, purity ≥ 98.0%) and water. The body weight and food intake were monitored for 4 weeks, and then the body composition was measured by a nuclear magnetic resonance system (Body Composition Analyzer MiniQMR23-060H-I, Niumag, China). Subsequently, the exercise capacity was measured. The mice were sacrificed 1 week after exercise measurement. Blood was collected for separation of serum. Different muscles in the hind limb (gastrocnemius, GAS; tibialis anterior, TA; soleus, SOL; and extensor digitorum longus, EDL) were separated for the subsequent detection and analysis.

For acute Hx-treated experiment, 16 male mice of 10 weeks old were randomly divided into two groups according to body weight (8 mice per group), the mice were given an intraperitoneal (i.p.) injection of Hx (30 mg/kg body weight) or saline. 30 min after injection, the mice performed exercise capacity assessment, energy metabolism measurements, or were sacrificed for collecting blood and muscle samples.

Adeno-Associated Viral Vector 9 (AAV9) Injection

Thirty-two male mice of 7 weeks old were randomly divided into four groups according to body weight (8 mice per group). AAV-GFP-*Cas9* combined with AAV9-GFP-*Ucp2*-sgRNA (abm, Canada) was injected into the GAS muscle of both hind limbs of the mice in two groups for knockout of *Ucp2* (m*UCP2*KO groups). AAV-GFP-*Cas9* combined with AAV9-GFP-*scramble*-sgRNA (abm, Canada) was injected into the GAS muscle of both hind limbs of the mice in the other two groups as negative control (control groups). Titer of the mixed virus was at least 10^{11}. The validated sgRNA sequence for knockout of *Ucp2* is (target 1: TGGTCTTTCAGATCCAAGGG, target 2: GCGCCCAGTACCGTGGCGTT, and target 3: TCCTAACCATGGTGCGCACT) (abm, Canada). 3 weeks after infection, the mice took part in the acute Hx-treated experiment as described above. In brief, the mice in the control groups or m*UCP2*KO groups were given an i.p. injection of Hx or saline. 30 min after injection, the mice performed exercise capacity assessment or were sacrificed.

Exercise Capacity Assessment

The exhaustion test was performed on a rodent treadmill (47303, Ugo, Italy) according to the following protocol: 8 m/min for 5 min, 10 m/min for 5 min, subsequently, velocity was increased by 2 m/min every 20 min until mice reached exhaustion at an inclination of 10°. Exhaustion was defined as the point at which the mice spent more than 5 s on the electric shocker without attempting to resume running. Adaption training session was performed three times before the experiment with 10 m/min for 10 min (10° incline) for each time.

For the grip strength test, muscle strength was measured by a grip test meter (BIO-GS3, Bioseb, France). The mice were held on to a metal grid with four paws and were gently pulled backward by the tail until the animals could no longer hold the grid. Each mouse was given eight trials, and the average values were used to represent the muscle grip strength of an individual mouse.

For weights lifting test, which was also performed to measure muscle strength that referred to research of Lahiri et al. (2019). Briefly, each mouse was grasped in the middle of the tail, and then grasped the first weight (26 g). After the mouse grasped the weight, the mouse was slowly raised until the weight was completely suspended. The criterion was met if the mouse could hold the weight for 3 s. If the mouse cannot hold for 3 s, rest for 10 s and repeat for five times maximum. If it successfully held the weight for 3 s, then it was allowed to progress to the next heaviest weight. The apparatus comprised six weights, weighing 26, 33, 44, 63, 82, 100, and 120 g respectively. 26 g is counted as 1 point, 33 g is 2 points, 44 g is 3 points, 63 g is 4 points, 82 g is 5 points, 100 g is 6 points, and 120 g is 7 points. The total score of each mouse is equal to the cumulative score of all weights that can be lifted for 3 s.

Biochemical Assays

Serum glucose, non-esterified fatty acid (NEFA), aspartate aminotransferase (AST), Alanine transaminase (ALT), and blood urea nitrogen (BUN), GAS muscle glycogen and lactate were measured by using the biochemistry detection kits (Nanjing Jiancheng Bioengineering Institute, China). The ATP contents in the GAS muscle tissues were measured using the ATP Assay Kit (S0026, Beyotime, China). The ROS contents of the GAS muscle tissues were measured using a tissue ROS Assay Kit (BB-470515-1, BestBio, China). All assays were performed according to the manufacturer's instructions.

Energy Metabolism Measurements

For metabolic studies *in vivo*, mice were housed individually in the Promethion Metabolic Screening Systems (Sable Systems International, North Las Vegas, NV, United States) with free access to corresponding food and water. After acclimatization to the systems for 24 h, energy expenditure (EE), respiration exchange ratio (V_{CO2}/V_{O2}), and locomotor activity were monitored for the following 48 h. Data were collected and analyzed by MetaScreen-Data Collection Software (V2.3.17) and Expedata-P Data Analysis Software (V1.9.17), respectively.

Immunofluorescence

The GAS muscles were separated as soon as the mice were sacrificed and frozen in liquid nitrogen, transverse 10-μm sections were generated by using the freezing microtome (CM1950, Leica, Germany). Sections were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton-X 100, and blocked in 5% BSA with 10% goat serum. Muscle slices were incubated with the primary antibodies overnight at 4°C, washed and incubated with the secondary AlexaFluor-conjugated antibodies for 1 h at room temperature. Slices were imaged using a Nikon Eclipse Ti-s microscope. Images were processed and analyzed with ImageJ (NIH). The following antibodies were used: rabbit anti-laminin antibody (1:500, A19970, ABclonal, China), mouse anti-MyHC I antibody (1:500, BA-D5-s, DSHB, United States), and mouse anti-MyHC IIb antibody (1:50, BF-F3, DSHB, United States).

RNA Isolation and Quantitative RT-PCR (qRT-PCR)

Total RNA of the GAS muscle tissues was extracted using the Hipure Universal RNA Mini kit (R4130-02, Magen, China) according to the manufacturer's instructions. A total 2 μg of RNA was converted into cDNA with random primers using the M-MLV enzyme (Promega, United States). qRT-PCR was performed with a QuantStudio 3 Flex Real-Time PCR system (7300HT, Applied Biosystems, United States) using SYBR Green master mix as per the manufacturer's instructions (Q711, Vazyme, China). Normalized mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method, β-*actin* was used as the reference gene. The list of primer sequences is presented in **Table 1**.

Protein Extraction and Western Blot

The GAS muscle tissues lysates were obtained using RIPA lysis buffer freshly supplemented with proteinase inhibitor (BB-3101-2, BestBio, China). The protein samples were separated by SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Germany), after blocking in 5% defatted milk dissolved in TBST. The membranes were incubated with primary antibodies overnight at 4°C and with HRP-conjugated secondary antibody for 1 h at room temperature. The signal was visualized by using ECL substrate (Merck Millipore, Germany). The protein band intensity was quantified by ImageJ (NIH). The following antibodies were used: rabbit anti-UCP2 antibody (1:1500, 162448, ZENBIO, China), rabbit anti-UCP3 antibody (1:1500, 162449, ZENBIO, China), mouse anti-MyHC I antibody (1:1000, BA-D5-s, DSHB, United States), mouse anti-MyHC IIb antibody (1:1000, BF-F3, DSHB, United States), and rabbit anti-β-actin antibody (1:5000; AP0060, Bioworld, China).

Statistical Analysis

All data were presented as means ± standard error of the mean (SEM) for experiments, including the numbers of mice in each group as indicated. Unpaired Student's *t*-test or one-way ANOVA analysis followed by *post hoc* Tukey's test was performed for statistical significance comparison by using the GraphPad Prism 8 software (GraphPad).

TABLE 1 | Primer sequences used for qRT-PCR.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
MyHC I	ACCAGGCCCTTTGACCTCAA GAAA	TCTTGTCGAACTTGGGTGGGT TCT
MyHC IIa	AGTCCCAGGTCAACAAGCTG	TTTCTCCTGTCACCTCTCAACA
MyHC IIb	AGTCCCAGGTCAACAAGCTG	TTTCTCCTGTCACCTCTCAACA
Hk2	GGACGGAATTCAGAAGGCCT	TCCTCGCCCTTGTTCTCTTT
Eno2	GAAGGAAGCCATCGACAAGG	TGGTCCCCAGTGATGTATCG
Gapdh	AGTGTTTCCTCGTCCCGTAG	GCCGTGAGTGGAGTCATACT
Ldha	GTCCAGCGAAACGTGAACAT	GCCACTGATTTTCCAAGCCA
Gck	GAAAGTACATGGGCGAGCTG	AAACGGGTCTCAAAAGCACC
Pkm	CTTCATTCAGACCCAGCAGC	CCGAGCCACATTCATTCCAG
Cpt1b	CTGGGATGCGTGTAGTGTTG	TCATGTATCGCCGCAAACTG
Crat	GGGCAGTTCTTCTAAGGCAG	TATGCCGCTATGTCTGGTCT
Acadm	ACACCCATACGCCAACTCTT	AGTACCCGTTCCCTCTCATC
Acads	GTAGGCCAGGTAATCCAAGCC	TGGCGACGGTTACACACTG
Nrf1	CCACGTTGGATGAGTACACG	CTGAGCCTGGGTCATTTTGT
Tfam	AGATATGGGTGTGGCCCTTG	AAAGCCTGGCAGCTTCTTTG
Cox6a1	TGCTCAACGTGTTCCTCAAG	TAAGGGTCCAAAACCAGTGC
Atp5b	GAGGGATTACCACCCATCCT	CATGATTCTGCCCAAGGTCT
Ndufa6	GTCACAGACCCCAGAGTGGT	TAACATGCACCTTCCCATCA
Ucp2	TCCTGCACCCCGATTTACTT	CTTTATGGGTGAAGGCTGGC
Ucp3	CCGTTTTGAACAAGGCAAGC	GTTTCTCGTGTCAGCAGCAG
Gss	GTTGCTGTGGTGTACTTCCG	CACCTTCTTAGTCCCAGCCA
Gpx4	TTGATAAGAACGGCTGCGTG	AGACCTTCATGAGTGCCGG
β-actin	TAGGCGGACTGTTACTGAGC	AATCCTGAGTCAAAAGCGCC

MyHC I, myosin heavy chain I; MyHC IIa, myosin heavy chain IIa; MyHC IIb, myosin heavy chain IIb; Hk2, hexokinase 2; Eno2, enolase 2; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ldha, lactate dehydrogenase A; Gck, glucokinase; Pkm, pyruvate kinase M1/2; Cpt1b, carnitine palmitoyltransferase 1B; Crat, carnitine O-acetyltransferase; Acadm, acyl-CoA dehydrogenase medium chain; Acads, acyl-CoA dehydrogenase short chain; Nrf1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A; Cox6a1, cytochrome c oxidase subunit 6a1; Atp5b, ATP synthase, H^+ transporting mitochondrial F1 complex, beta subunit; Ndufa6, NADH: ubiquinone oxidoreductase subunit A6; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3; Gss, glutathione synthetase; Gpx4, glutathione peroxidase 4.

RESULTS

Chronic Hx-Treated Induces Exercise Weakness of Mice

To investigate the physiological roles of Hx in the skeletal muscle and exercise, mice were treated with Hx through dietary supplement (0, 10, or 20 mg/g in diet). After 4 weeks of feeding, the body weight (**Figure 1A**) and accumulated food intake (**Figure 1B**) of the Hx-treated mice (both the 10 mg/g group and 20 mg/g group) were comparable to the control, and so were the lean mass, fat mass, and skeletal muscle weight (GAS, TA, EDL, and SOL) (**Figures 1C,D**). In addition, there was also no difference in mice growth and development between the 10 and 20 mg/g Hx-treated group (**Figures 1A–D**). Subsequently, the mice performed exercise capacity assessments including the strength test (weight lifting test score or grip strength) and the endurance test (treadmill running). We found that Hx induced exercise weakness depended on the dosage. The mice in the 20 mg/g Hx treated group showed lower weight lifting test scores ($P \leq 0.05$, **Figure 1E**), weaker muscle grip strength ($P \leq 0.05$, **Figure 1F**), and lower running endurance ($P \leq 0.05$, **Figure 1G**). There was no significant change in 10 mg/g Hx treated group compared to the control, although the weight lifting score and grip strength were slightly reduced after treatment with 10 mg/g Hx. Moreover, the weight lifting scores were significantly decreased in the 20 mg/g Hx-treated group compared to the 10 mg/g Hx-treated group ($P \leq 0.05$, **Figure 1E**), while there was no significant difference in the grip strength and running time (**Figures 1F,G**). Hence, we chose the additive concentration of 20 mg/g for the subsequent experiments.

Muscular plasticity is characterized as the muscle fiber development and myofiber type transformation in response to different internal or external stimuli (Allen et al., 2008). Then, we measured the muscle fiber size and fiber type composition. The mRNA and protein levels of myofiber type markers were comparable between the Hx-treated group and the control (**Figures 2A–C**). The immunofluorescence staining of Laminin (reflecting the boundary of myofiber) and myofiber type markers of muscle sections (slow-oxidative type, MyHC I; and fast-glycolytic type, MyHC IIb) also showed no differences in the myofiber type composition and fiber size between the two groups (**Figures 2D–F**). All these results show that chronic Hx intake induces exercise weakness of mice, without affecting the growth performance, myofiber size and fiber type composition.

Hypoxanthine Alters Muscular Energy Metabolism and Reduces ATP Content

We eliminated the influence of muscle fiber structure and composition on Hx-induced exercise fatigue. Consequently, we considered the role of skeletal muscle metabolism, which is also another key determinant in muscle function. Firstly, we measured the global energy metabolism of mice. **Figure 3A** showed the dynamic change of average EE per hour in the whole day. The EE was elevated in the Hx-treated group and was significantly different in the light cycle ($P \leq 0.05$, 3B), while the locomotor activity was similar between the two groups (**Supplementary Figure 1**), indicating that the enhanced metabolism was not due to the alteration of physical activity. Moreover, the respiratory exchange ratio (RER) was significantly lower in Hx-treated mice compare to the control ($P \leq 0.05$, **Figures 3C,D**), suggesting that Hx induced a metabolic tendency to use lipid as an energy substrate. Then, we investigated the main biochemical parameters related to muscle contraction metabolism of mice in circulation blood and muscle tissue. There was no difference in serum glucose between Hx-treated mice and controls (**Figure 3E**), but we found the significant decrease of NFFA of serum ($P \leq 0.05$, **Figure 3F**), which might show the increased mobilization of lipid substrate, which was consistent with the RER decreasing with the Hx-treated group. Moreover, decreased GAS muscle glycogen ($P \leq 0.05$, **Figure 3G**) and increased muscle lactate ($P \leq 0.001$, **Figure 3H**) was in response to Hx-treatment.

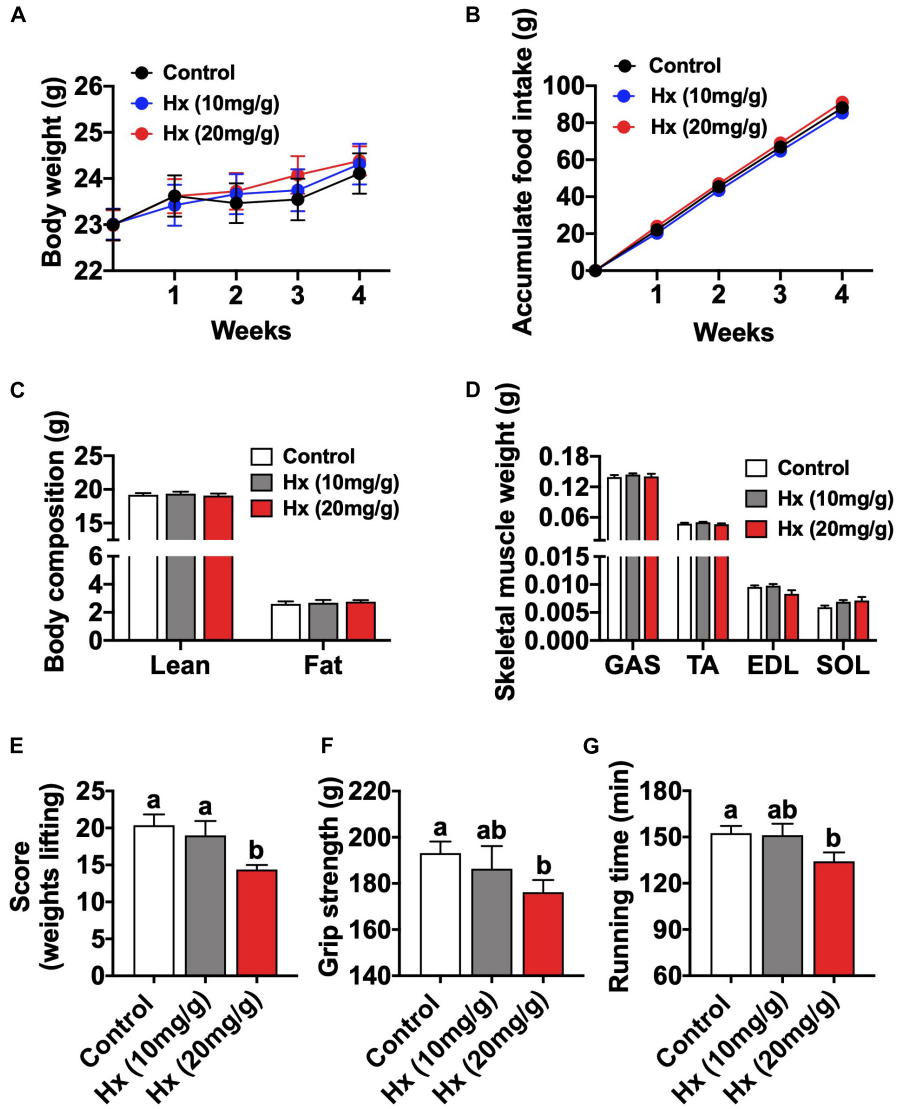

FIGURE 1 | Chronic Hx-treated induces muscular fatigue. **(A)** Body weight after 4 weeks Hx treatment (n = 8 mice per group). **(B)** Cumulative food intake after 4 weeks Hx treatment (n = 8 mice per group). **(C)** Body composition of mice after 4 weeks Hx treatment (n = 8 mice per group). **(D)** Skeletal muscle weight (GAS; tibialis anterior, TA; soleus, SOL; and extensor digitorum longus, EDL) (n = 8 mice per group). **(E)** Weights lifting score after Hx treatment (n = 8 mice per group). **(F)** Grip strength after Hx treatment (n = 8 mice per group). **(G)** Treadmill running time after Hx treatment (n = 8 mice per group). Values are presented as means ± SEM, different letters between bars mean $P \leq 0.05$ in one-way ANOVA analysis followed by *post hoc* Tukey's tests.

Based on the alteration of these metabolic indicators, we further measured the mRNA expression of genes related to glucose glycolysis (*Hk2*, *Eno2*, *Gapdh*, *Ldha*, *Gck*, and *Pkm*), fatty acid transport (*Cpt1b* and *Crat*) and catabolism (*Acadm* and *Acads*), and mitochondrial biogenesis (*Nrf1* and *Tfam*) and oxidative phosphorylation (*Cox6a1*, *Atp5b*, and *Ndufa6*). It showed the significant elevation of these pathways or processes ($P \leq 0.05$, **Figure 3I**), which was consistent with the previous results. ATP is the direct energy supply substance for muscle contraction. Interestingly, the muscular ATP was lower in the administration of Hx compared with the controls ($P \leq 0.05$, **Figure 3J**), this might be the key point in Hx-induced muscular weakness. Altogether, these data indicate that Hx caused an increase in cellular energy metabolism, but depressed ATP production in skeletal muscle.

Acute Hx-Treated Also Induces Muscular Metabolic Transformation and Weakness

Grip strength and weight lifting test both indicate the transient strength of muscle. Based on the effect of chronic Hx-treatment in skeletal muscle, we aimed to explore the acute effect of Hx in skeletal muscle. The mice were i.p. injected with Hx (30 mg/kg body weight) or saline, and then performed muscle grip test, weights lifting test, and treadmill running test, respectively (**Figure 4A**). Though treadmill running was not altered in

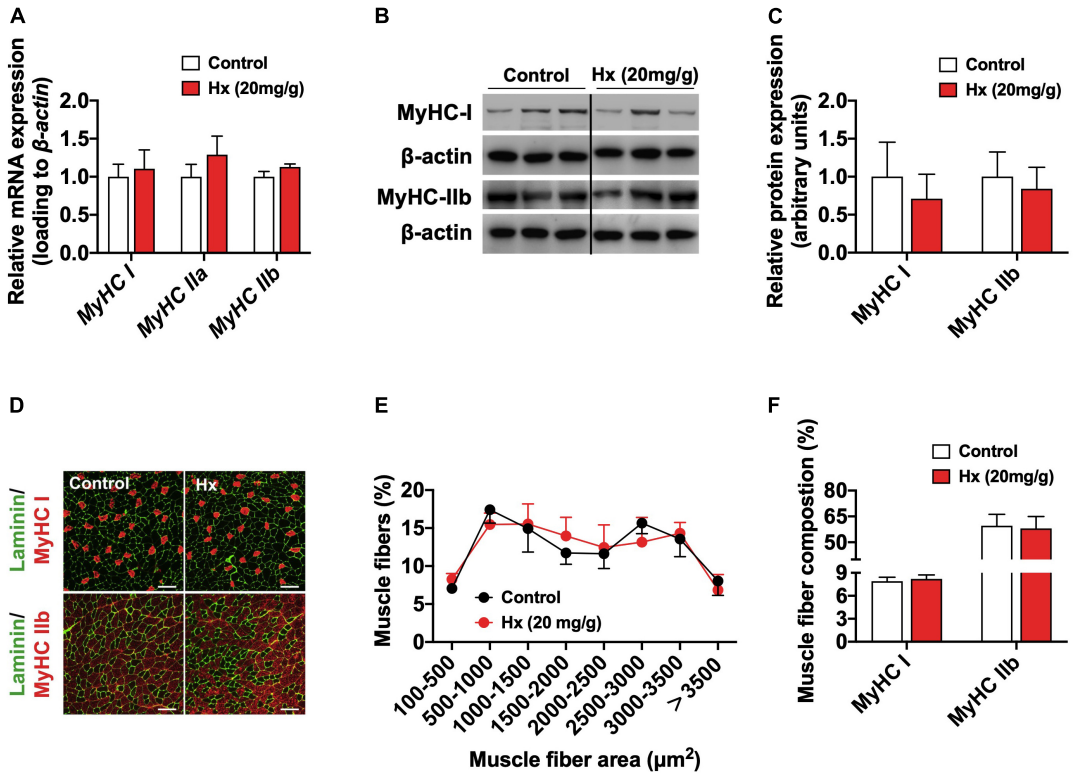

FIGURE 2 | Chronic Hx-treated does not alter muscle fiber area and fiber type composition. **(A)** mRNA expression of genes related to the myofiber types (n = 8–10 mice per group). **(B,C)** Western blot (B) and quantification **(C)** of MyHC I and MyHC IIb content in the GAS muscle (n = 6 per group). Dividing lines indicate spliced bands from the same gel. **(D)** Immunofluorescence staining of Laminin (green), MyHC I (upper, red), and MyHC IIb (lower, red) of GAS muscle section (bars = 50 μm). **(E,F)** Statistical analysis of muscle fiber area **(E)** and fiber type composition **(F)** according to immunofluorescence staining of muscle sections (n = 3 mice per group). β-actin was used as loading control both in qRT-PCR and western blot. Values are presented as means ± SEM.

Hx-treated mice compared with the control ones (**Figure 4D**). The weights lifting score of mice was significantly decreased after Hx-treatment ($P \leq 0.05$, **Figure 4B**), as well as the grip strength ($P \leq 0.01$, **Figure 4C**), which was consistent with the chronic treatment. The RER of mice was dramatically decreased within 6 h after Hx-treatment ($P \leq 0.05$, **Figure 4E**), which was similar to the chronic effect. But contrary to the chronic-treated, Hx decreased the EE of mice within 3 h after i.p. injection ($P \leq 0.05$, **Figure 4F**), which might be due to the reduced locomotor activity after Hx-treatment ($P \leq 0.05$, **Figure 4G**). The reduced locomotor activity was not due to the acute toxicity of Hx, it was because that there was no difference in the serum biochemical indicators, ASL, ALT, and BUN, which are related to the acute toxicity, with Hx-treatment compared with the control (**Supplementary Figures 2A–C**). We also measured the biochemical targets in serum and muscle. Consistently, we found the invariable serum glucose (**Figure 4H**), decreased serum NEFA ($P \leq 0.001$, **Figure 4I**), reduced muscular ATP ($P \leq 0.05$, **Figure 4J**) and glycogen ($P \leq 0.05$, **Figure 4K**), and elevated muscular lactate with Hx-treatment ($P \leq 0.05$, **Figure 4L**). Moreover, the mRNA expression of glucose glycolysis (*Hk2*, *Eno2*, *Gapdh*, *Ldha*, *Gck*, and *Pkm*) or fatty acid transport and catabolism genes (*Cpt1b*, *Crat*, *Acadm*, and *Acads*) were significantly increased as well ($P \leq 0.05$, **Figure 4M**). Together, these data show that acute Hx-treatment also induces muscular metabolic transformation and exercise weakness.

Hypoxanthine Induces the Activation of UCP2 in Skeletal Muscle

Generally, enhanced cellular energy metabolism should produce more energy substrate. But actually, the promotion of cellular energy metabolism induced by Hx was accompanied with a lack of ATP, that indicated that the metabolism was ineffective, caused by Hx. Hence, we focused on the UCPs that mediate the mitochondrial uncoupling process. It has reported that ATP production is suppressed by increased uncoupled respiration mediated through UCPs in any given mitochondria (Slocinska et al., 2016). Among the UCPs family, UCP1 is mainly expressed in brown adipose tissue, while the UCPs mainly expressed in skeletal muscle are UCP2 and UCP3 (Brand and Esteves, 2005). To validate the hypothesis related to UCPs, we detected the expressions of UCP2 and UCP3 in skeletal muscle. As expected, the mitochondrial UCP2, but not UCP3, was significantly increased with chronic Hx-treated both in the mRNA expression ($P \leq 0.05$, **Figure 5A**) and protein levels ($P \leq 0.05$, **Figures 5B,C**). Consistently, the mRNA expression ($P \leq 0.05$, **Figure 5D**) and protein levels ($P \leq 0.05$, **Figures 5E,F**)

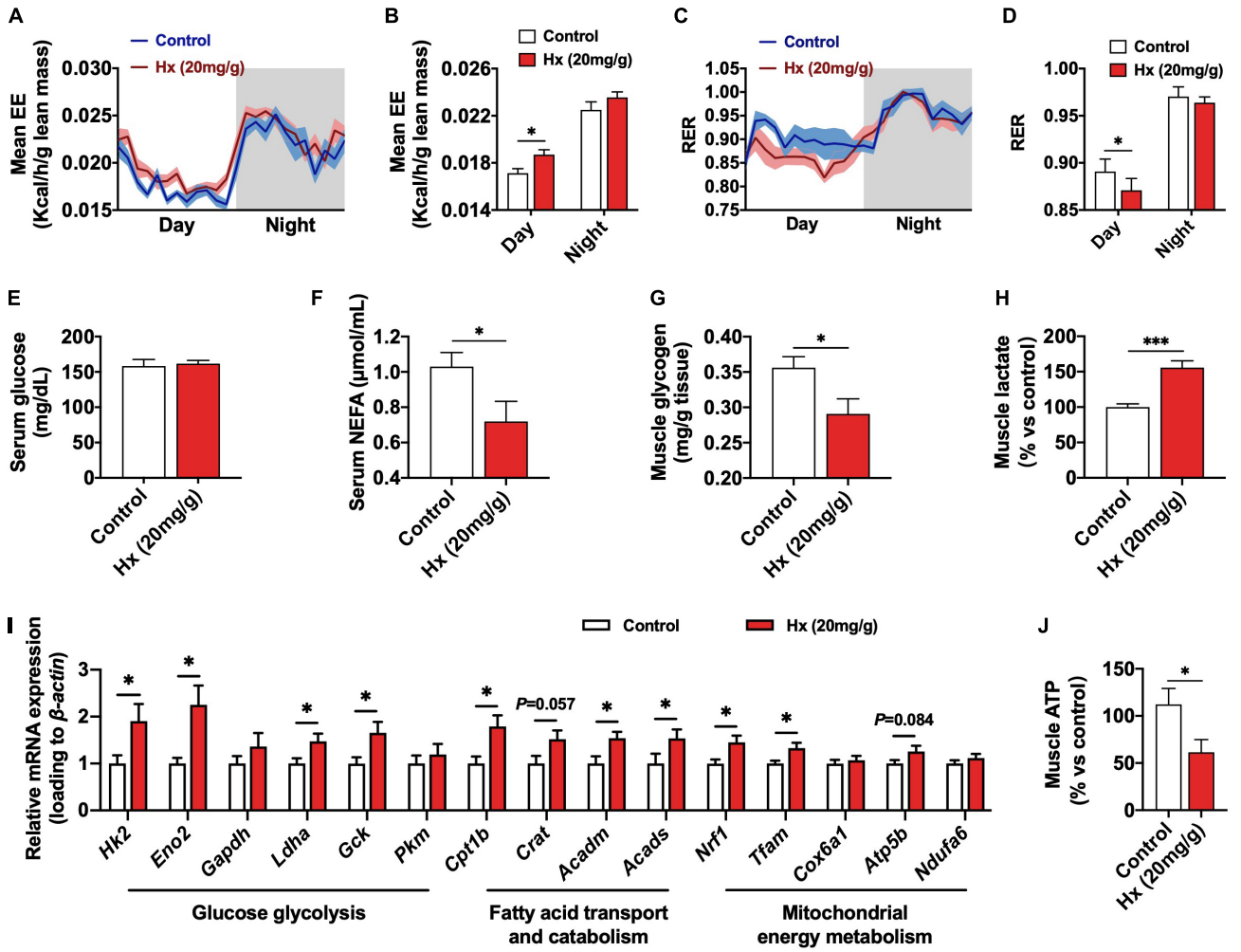

FIGURE 3 | Effects of Hx on the skeletal muscle energy metabolism. **(A)** EE of mice after 4 weeks Hx treatment. Grey areas indicate dark periods (n = 8 mice per group). **(B)** Statistics of mean EE values of mice in night or dark cycle after 4 weeks Hx treatment (n = 8 mice per group). **(C)** RER of mice after 4 weeks Hx treatment. Gray areas indicate dark periods (n = 8 mice per group). **(D)** Statistics of mean RER values of mice in night or dark cycle after 4 weeks Hx treatment (n = 8 mice per group). **(E–H)** Serum glucose **(E)** and NFFA **(F)** levels; GAS muscle glycogen **(G)**, and lactate **(H)** content of mice after 4 weeks Hx treatment (n = 8 mice per group). **(I)** mRNA expression of genes related to the glucose glycolysis, fatty acid transport and catabolism, and mitochondrial energy metabolism of GAS muscle after 4 weeks Hx treatment (n = 7–8 mice per group). **(J)** GAS muscle ATP content of mice after 4 weeks Hx treatment (n = 8 mice per group). β-*actin* was used as loading control in the qRT-PCR. Values are presented as means ± SEM, $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ according to non-paired Student's t-test between individual groups.

of UCP2 were also upregulated with acute Hx-treated. Enhanced mitochondrial metabolism could generate more oxidative free radicals that would be neutralized by UCPs and other peroxidase systems to maintain the intracellular redox homeostasis (Deyu et al., 2018). Moreover, ROS is also an important regulator of UCP2 (Mailloux and Harper, 2012). Herein, we observed no change in the muscle ROS content in the chronic Hx-treated group compared to the controls (**Supplementary Figure 3A**). However, Hx induced significantly increased mRNA expression of *glutathione synthetase* (*Gss) and glutathione peroxidase* (*Gpx4)*, which mediate the glutathione reduction system ($P \leq 0.05$, **Supplementary Figure 3B**). These indicated the enhanced cleaning capacity in response to activated UCP2 and glutathione reduction system might have kept the ROS homeostasis. All these results suggest that UCP2 might be a potential target in Hx-induced muscular weakness.

UCP2 Is Required for the Hx-Induced Muscular ATP Deficiency and Weakness

To further explore the functional importance of UCP2 in the Hx-induced muscular weakness, we used an intra-muscle tissue AAV injection strategy to knockout (KO) *Ucp2* in the GAS muscle through CRISPR-Cas9 system (**Figure 6A**). 3 weeks after injection, there was no difference in the body weight (**Supplementary Figure 4A**) and muscle mass (**Supplementary Figure 4B**) of mice between muscular *Ucp2* specifically KO (m*UCP2*KO) mice and the controls. Then, the mice were

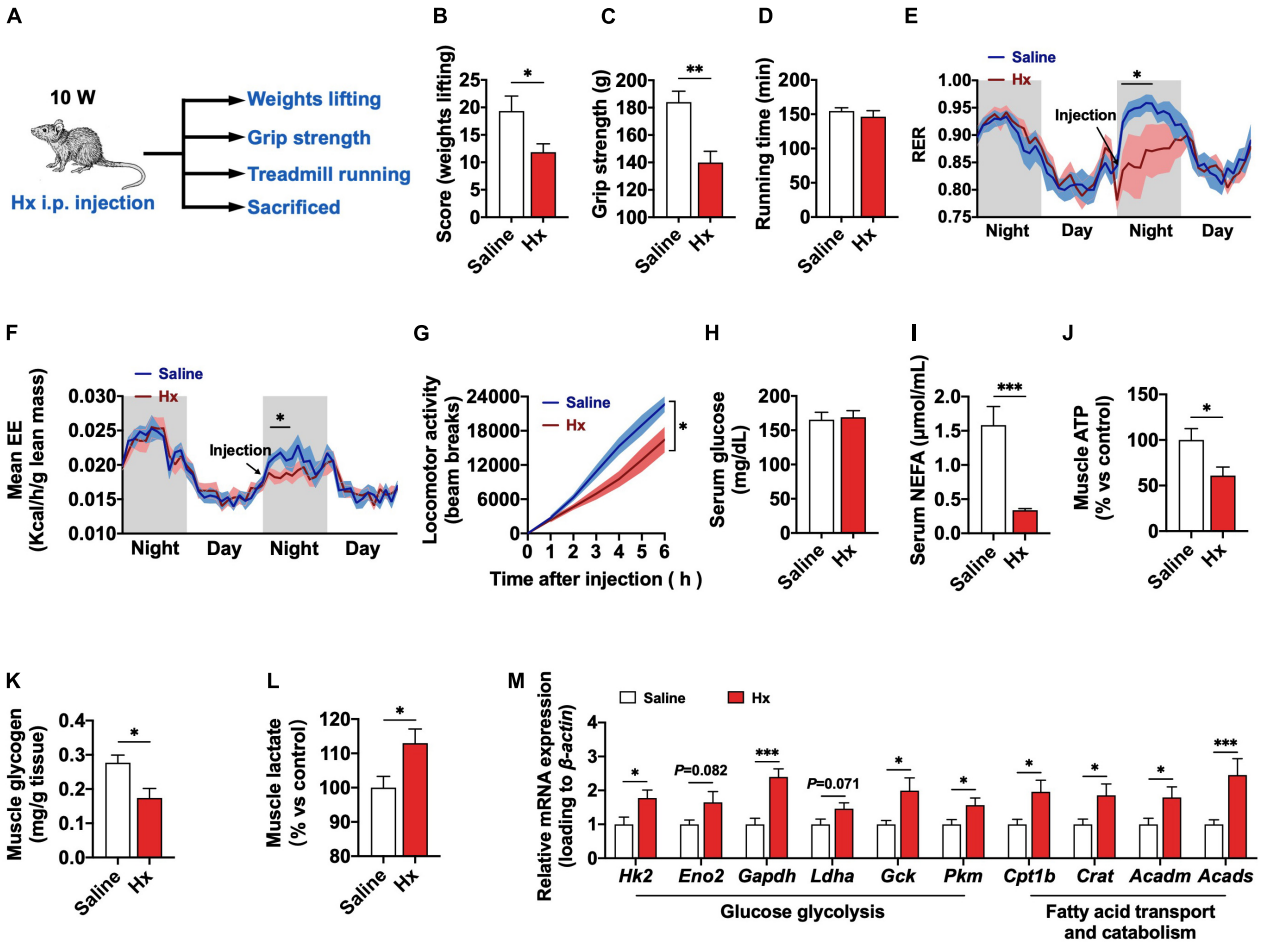

FIGURE 4 | Acute effects of Hx on the exercise capacity and metabolism of mice. **(A)** Experimental scheme for the acute Hx treatment of mice. **(B)** Weights lifting score after acute Hx treatment (n = 8 mice per group). **(C)** Grip strength after acute Hx treatment (n = 8 mice per group). **(D)** Treadmill running time after acute Hx treatment (n = 8 mice per group). **(E,F)** RER and EE of mice. The arrow pointed to the time of acute i.p. injection. Gray area indicated dark period (n = 8 mice per group). **(G)** Accumulated locomotor activity (bean breaks) of mice after acute Hx treatment (n = 8 mice per group). **(H–L)** Serum glucose **(H)** and NEFA **(I)** levels; GAS muscle ATP **(J)**, glycogen **(K)**, and lactate **(L)** content of mice after acute Hx treatment (n = 7–8 mice per group). **(M)** mRNA expression of genes related to the glucose glycolysis and fatty acid transport and catabolism of GAS muscle after acute Hx treatment (n = 7–8 mice per group). β-*actin* was used as loading control in the qRT-PCR. Values are presented as means ± SEM; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ according to the non-paired Student's t-test between individual groups.

given i.p. injection of Hx (30 mg/kg body weight) or saline, and measured the weights lifting score, grip strength, or sacrificed for sample collection, respectively (**Figure 6A**). First, the m*UCP2*KO model was established successfully through the validation of reduced UCP2 levels in protein expression ($P \leq 0.05$, **Figures 6B,C**). Acute Hx-treated induced significantly increased of UCP2 protein level in skeletal muscle ($P \leq 0.05$, **Figures 6B,C**), and m*UCP2*KO blocked the Hx-induced UCP2 activation (**Figures 6B,C**). As expected, Hx induced significantly decreased weight lifting scores ($P \leq 0.05$, **Figure 6D**), grip strength ($P \leq 0.05$, **Figure 6E**), and muscle ATP content ($P \leq 0.05$, **Figure 6F**) in mice, while m*UCP2*KO restored the exercise fatigability and ATP deficiency of mice (**Figures 6D–F**). The enhanced glycolysis in muscle which was presented by the reduced muscular glycogen ($P \leq 0.05$, **Figure 6G**), increased muscular lactate content ($P \leq 0.05$, **Figure 6H**), and elevation of mRNA expression of glycolysis genes (*Hk2*, *Eno2*, *Gapdh*, *Ldha*, *Gck*, and *Pkm*) with Hx-treatment ($P \leq 0.05$, **Figure 6I**), was also reversed by m*UCP2*KO (**Figures 6G–I**). These results suggest the roles of UCP2 in Hx induced muscular glycolysis, ATP depletion, and weakness.

DISCUSSION

Herein, we showed that Hx impaired the exercise capacity of mice both with chronic and acute treatment. Mechanistically, Hx altered muscle energy metabolism, mainly reflected in enhanced glycolysis, accelerated energy substrate consumption and mobilization, and depressed ATP flowage. Otherwise, Hx induced UCP2 activation in skeletal muscle, which mediated the mitochondrial uncoupling process that blocked ATP production.

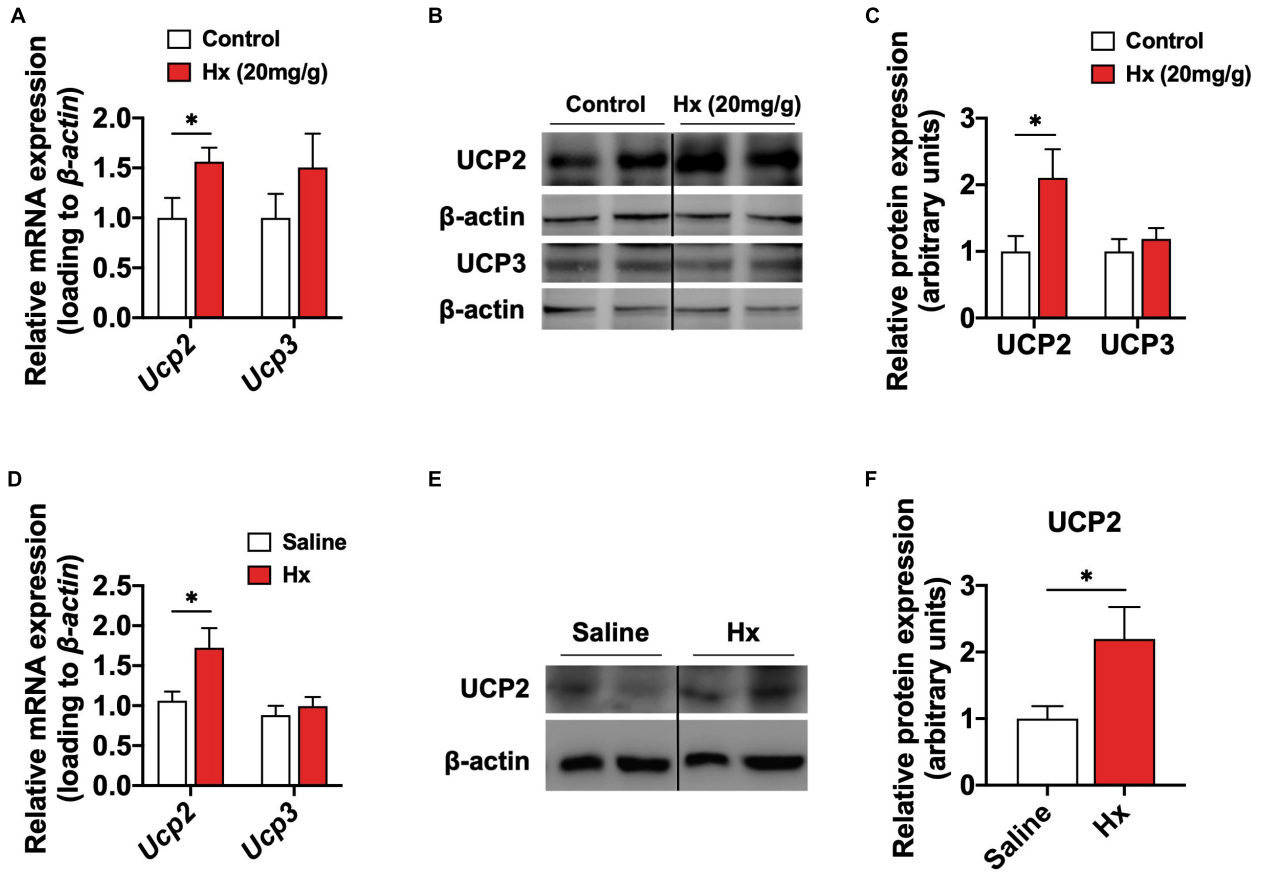

FIGURE 5 | Hx induces the activation of UCP2 in skeletal muscle. **(A)** mRNA expression of *UCP2* and *UCP3* in the GAS muscle after 4 weeks Hx treatment (n = 8 mice per group). **(B,C)** Western blot **(B)** and quantification **(C)** of UCP2 and UCP3 content in the GAS muscle after 4 weeks Hx treatment (n = 6 per group). Dividing lines indicate spliced bands from the same gel. **(D)** mRNA expression of *UCP2* and *UCP3* in the GAS muscle after acute Hx treatment (n = 8 mice per group). **(E,F)** Western blot **(E)** and quantification **(F)** of UCP2 content in the GAS muscle after acute Hx treatment (n = 6 per group). Dividing lines indicate spliced bands from the same gel. β-actin was used as loading control both in qRT-PCR and western blot. Values are presented as means ± SEM. $^*P \leq 0.05$ according to the non-paired Student's t-test between individual groups.

Muscle specifically knocked out of UCP2 could block glycolysis elevation and rescue energy insufficiency and muscle fatigue with Hx-treatment. These results shed a new light on the physiological role of Hx in skeletal muscle metabolism and function.

Physical exercise is widely accepted to prevent several pathogenic conditions, such as hypertension, coronary heart disease (Downing and Balady, 2011), obesity (Heo et al., 2017), type 2 diabetes mellitus (Fan et al., 2017), age-related muscle wasting (sarcopenia) (Bowen et al., 2015), and cancer (Hojman et al., 2018), largely due to metabolic adaptations induced by contraction in skeletal muscle. Yet how exercise induces the effects remains to be fully understood. It is generally believed that a cascade of events in skeletal muscle initiates the process. During physical exercise, skeletal muscle burns significant amounts of carbohydrates and lipids, or even protein and nucleic acid, contributing to most of the total EE, and producing lots of metabolites at the same time. Owing to the high throughput metabolomic techniques, numerous metabolites have been screened and identified. These muscle-derived metabolites (myometabolites) facilitate crosstalk with other tissues including the brain, adipose tissue, liver, gut, and cardiovascular. They likely play a role in the health of these tissues and associated diseases, which coincided with the beneficial roles of exercise. These findings supply the molecular mechanisms underlying the beneficial effects of exercise. However, due to the complex diversity of metabolites, more work needs to be explored in-depth, especially the detrimental aspects of myometabolites. So that we can fully understand the physiological role of the metabolites, avoiding their negative effects on the body homeostasis as well as pathological treatment.

Hypoxanthine is the principal purine nucleobase involved in the purine salvage pathway, and also the intermediate of nucleotides metabolism (Farthing et al., 2015). During exercise or muscle contraction, ATP consumption is pronounced, which is accompanied by the Hx formation via AMP and IMP. The accumulated Hx is released from the muscle into blood and then transferred to other tissues. Although Hx can also be produced and released by other tissues, muscle is an important determinant of its level, because it constitutes such a high percentage of body mass and is highly metabolically active during contraction.

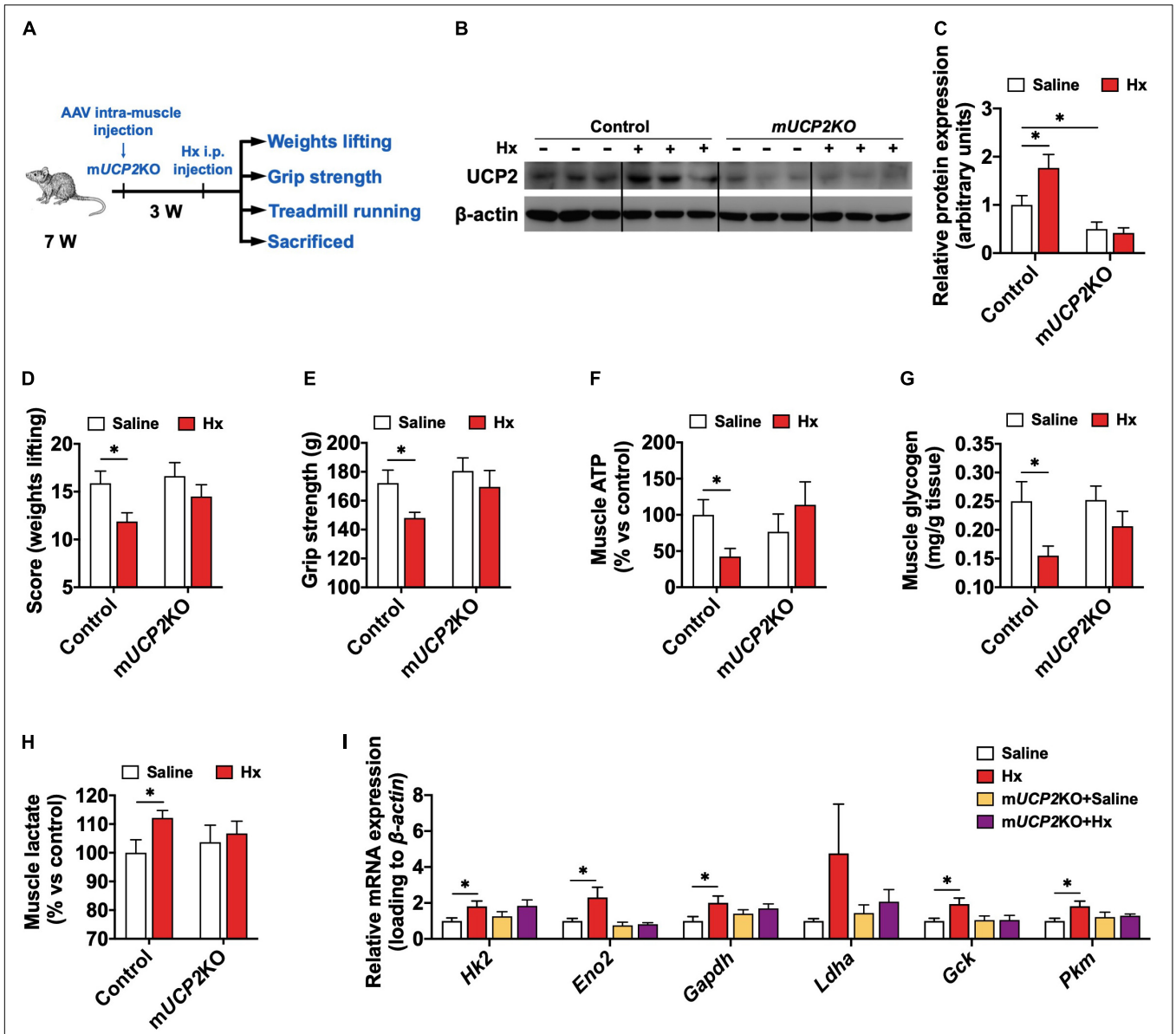

FIGURE 6 | UCP2 is required for the Hx-induced muscular ATP deficiency and weakness. **(A)** Experimental scheme for the acute Hx treatment on m*UCP2*KO mice. **(B,C)** Western blot **(B)** and quantification **(C)** of UCP2 content in the GAS muscle after acute Hx treatment (n = 6 per group). Dividing lines indicate spliced bands from the same gel. **(D)** Weights lifting score after acute Hx treatment (n = 8 mice per group). **(E)** Grip strength after acute Hx treatment (n = 8 mice per group). **(F–H)** GAS muscle ATP **(F)**, glycogen **(G)**, and lactate **(H)** content of mice after acute Hx treatment (n = 7–8 mice per group). **(I)** mRNA expression of genes related to the glycolysis of GAS muscle after acute Hx treatment (n = 7–8 mice per group). β-actin was used as loading control both in qRT-PCR and western blot. Values are presented as means ± SEM; $^*P \leq 0.05$ according to the non-paired Student's t-test between individual groups.

Indeed, Hx in the muscle tissue is the most abundant compared with other tissues (Bone et al., 2015). Though its paracrine physiological roles in other tissues have been reported, the roles of Hx in the autocrine regulation of the muscles *per se* remain unknown. In the present study, we showed the Hx-induced muscular metabolism alteration and fatigue even in acute treatment, this rapid and dramatic effect is closely related to the role of Hx as a marker of exercise exhaustion or fatigue.

In this study, two doses of Hx (10 and 20 mg/g diet) had been conducted in the chronic Hx treatment. The lower dose of 10 mg/g Hx-treatment did not have significant effect on the growth and exercise ability of mice, while 20 mg/g Hx-treatment induced exercise weakness. The doses of metabolites in diet, 10 mg/g or 20 mg/g, are almost the maximal for the study about the physiological functions of metabolites as several research reported (Tian et al., 2020; Yuan et al., 2020). Higher doses might be toxic to animals and certainly change the nutrients concentration. Herein, 20 mg/g Hx-treatment caused muscle fatigue, but it had no effect on the growth and food intake of mice, and we did not observe any abnormal behavior of mice during the

feeding experiment as well. In the acute Hx treatment, the effect on skeletal muscle was similar to that in chronic treatment, which appeared rapidly (30 min after i.p. injection), but without acute toxicity. These results exclude the toxicity of metabolite in Hx-induced muscle fatigue and further support the effect of Hx on muscle metabolism.

Uncoupling proteins, the mitochondrial anion carrier proteins, are located in the inner mitochondrial membrane and dissipate the proton electrochemical gradient generated during electron transport via the mitochondrial respiratory chain (Divakaruni and Brand, 2011; Bouillaud et al., 2016). UCPs uncouple mitochondrial respiration from ATP formation and the direct consequence of their activity is a decrease in the oxidative phosphorylation efficacy (Donadelli et al., 2014). UCP2 belongs to the UCP subfamily and is ubiquitously expressed in most cell types of different tissues (Donadelli et al., 2014). UCP2 expression variants were found to be associated with insulin sensitivity and diabetes mellitus (Jia et al., 2009; Diano and Horvath, 2012; Sreedhar and Zhao, 2017), obesity and metabolic syndrome (Li et al., 2003; Ricquier, 2007; Jia et al., 2009; Dinh et al., 2015; Sreedhar and Zhao, 2017), cardiac diseases (Brand and Esteves, 2005), immunity process (Emre et al., 2007; Emre and Nubel, 2010), tumorigenesis and cancer (Selimovic et al., 2008), aging (Brand and Esteves, 2005), and neurological diseases (Toda and Diano, 2014). Unlike its widely studied homolog UCP1, UCP2 protein level increases and decreases in a very short time, the half-life of UCP2 is short, approximately 30 min, which is about 30 h for UCP1 (Rousset et al., 2007). As expected, in the acute Hx-treated experiment, Hx worked in 30 min after i.p. injection, and we did not observe the decrease in muscle weight lifting and grip strength in 1 h after injection, which we did not show in this research. This highlights the importance of UCP2 in the cellular energy metabolism, which requires precise regulation. Recent advancements in the understanding of cellular glucose and lipid metabolism identified UCP2 as a critical regulator of cellular fuel utilization and the systemic glucose and lipid metabolism (Diano and Horvath, 2012). UCP2 overexpression enhanced glycolysis via activation of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) during skin cell transformation (Sreedhar et al., 2017). On the contrary, Vozza et al. (2014) showed that *UCP2*-silenced human hepatocellular carcinoma (HepG2) cells, cultured in the presence of glucose, showed a higher inner mitochondrial membrane potential and ATP/ADP ratio associated with a lower lactate release. As expected, the mRNA and protein levels of UCP2 were significantly elevated in the skeletal muscle after Hx-treatment, which was accompanied with the increased muscular glycolysis and decreased ATP production. Then, knocked out of *UCP2* blocked the effects of Hx in skeletal muscle, which showed the indispensable role of UCP2 in Hx-induced muscle fatigue. Several studies have demonstrated the negative effect of ROS and oxidative stress in muscle function (Mailloux and Harper, 2012; Nautiyal et al., 2012; Deyu et al., 2018). In fact, UCP2 is a regulator of mitochondrial ROS, which is produced during oxidative phosphorylation by the electron transport chain. Through eliminating the accumulation of ROS, UCP2 could protect the cells against oxidative damage. Herein, we did not find a significant difference with Hx-treated mice compared to the control, but Hx induced increased mRNA expression of *Gss* and *Gpx4*, which mediate the glutathione reduction system. Therefore, we inferred that Hx-induced mitochondrial oxidative phosphorylation elevation caused ROS formation, which activated the UCP2 and other redox systems to neutralize the ROS. Furthermore, UCP2 contains purine nucleotide binding site in the structure (Vozza et al., 2014). So, as an intermediate metabolite of purine nucleotide, whether Hx could directly bind to UCP2 and affect its activity, or other regulators might mediate the crosstalk between Hx and UCP2, which need the in-depth investigation. Otherwise, UCP3 is another UCP that mainly exists in skeletal muscle. Unlike UCP2 activation, no alteration was found in the UCP3, which is consistent with divergent physiological functions of UCPs.

Overall, our study showed a new role of Hx in the skeletal muscle metabolism and function. Hx induced the elevation of UCP2-dependent mitochondrial uncoupling and blocked the respiratory chain from the synthesis of ATP. In addition, UCP2 caused excessive consumption of glycogen into lactate through enhanced glycolysis. These cases all together caused the skeletal muscle fatigue. Our results demonstrate a new perspective on the physiological role of myometabolites. This finding might be the potential pathological mechanism of muscle fatigue that requires further confirmation. On the other hand, it might be identified as a new strategy for the protection of the body from damage caused by excessive exercise.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Instructive Notions with Respect to Caring for Laboratory Animals (Ministry of Science and Technology, Beijing, China).

AUTHOR CONTRIBUTIONS

QJ, HZ, and CY designed the project and experiments. CY and QJ wrote the manuscript. CY, ZM, FL, and CD performed the experiments. CY, ZM, and HZ collected, analyzed, and interpreted the data. XZ, PG, and GS provided the technical expertise and discussed the data. QJ, CY, HZ, LW, YY, CZ, and SW discussed, reviewed, and edited the manuscript. QJ and HZ supervised the work. All authors approved the final manuscript.

FUNDING

This work was supported by the Major Program of the National Natural Science Foundation of China (Grant No.

31790411 to QJ), Local Innovative and Research Teams Project of Guangdong Province (Grant No. 2019BT02N630 to GS), National Key Point Research and Invention Program (Grant No. 2018YFD0500403 to GS), and Innovation Team Project in Universities of Guangdong Province (Grant No. 2017KCXTD002 to GS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.647743/full#supplementary-material

Supplementary Figure 1 | Locomotor activity (bean breaks) of mice after 4 weeks Hx treatment (n = 8 mice per group). Values are presented as means ± SEM.

Supplementary Figure 2 | (A–C) Serum AST **(A)**, ALT **(B)**, and BUN **(C)** levels after acute Hx treatment (n = 8 mice per group). Values are presented as means ± SEM.

Supplementary Figure 3 | (A) ROS level in the GAS muscle (n = 8 mice per group). **(B)** mRNA expression of *Gss* and *Gpx4* in the GAS muscle (n = 8 mice per group). β-*actin* was used as loading control in the qRT-PCR. Values are presented as means ± SEM; $^*P \leq 0.05$ according to non-paired Student's t-test between individual groups.

Supplementary Figure 4 | Body weight **(A)** and body composition **(B)** of AAV injection mice before acute Hx treatment (n = 8 mice per group). Values are presented as means ± SEM.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 14 April 2021
doi: 10.3389/fphys.2021.660498

Vitamin D Promotes Skeletal Muscle Regeneration and Mitochondrial Health

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Vitamin D is an essential nutrient for the maintenance of skeletal muscle and bone health. The vitamin D receptor (VDR) is present in muscle, as is CYP27B1, the enzyme that hydroxylates 25(OH)D to its active form, 1,25(OH)D. Furthermore, mounting evidence suggests that vitamin D may play an important role during muscle damage and regeneration. Muscle damage is characterized by compromised muscle fiber architecture, disruption of contractile protein integrity, and mitochondrial dysfunction. Muscle regeneration is a complex process that involves restoration of mitochondrial function and activation of satellite cells (SC), the resident skeletal muscle stem cells. VDR expression is strongly upregulated following injury, particularly in central nuclei and SCs in animal models of muscle injury. Mechanistic studies provide some insight into the possible role of vitamin D activity in injured muscle. *In vitro* and *in vivo* rodent studies show that vitamin D mitigates reactive oxygen species (ROS) production, augments antioxidant capacity, and prevents oxidative stress, a common antagonist in muscle damage. Additionally, VDR knockdown results in decreased mitochondrial oxidative capacity and ATP production, suggesting that vitamin D is crucial for mitochondrial oxidative phosphorylation capacity; an important driver of muscle regeneration. Vitamin D regulation of mitochondrial health may also have implications for SC activity and self-renewal capacity, which could further affect muscle regeneration. However, the optimal timing, form and dose of vitamin D, as well as the mechanism by which vitamin D contributes to maintenance and restoration of muscle strength following injury, have not been determined. More research is needed to determine mechanistic action of 1,25(OH)D on mitochondria and SCs, as well as how this action manifests following muscle injury *in vivo*. Moreover, standardization in vitamin D sufficiency cut-points, time-course study of the efficacy of vitamin D administration, and comparison of multiple analogs of vitamin D are necessary to elucidate the potential of vitamin D as a significant contributor to muscle regeneration following injury. Here we will review the contribution of vitamin D to skeletal muscle regeneration following injury.

Keywords: vitamin D receptor, vitamin D, 25(OH)D, calcitriol, skeletal muscle injury, satellite cells, reactive oxygen species, skeletal muscle regeneration

OPEN ACCESS

Edited by:
Yan Huang,
University of Arkansas, United States

Reviewed by:
Huan Kang,
University of Kansas, United States
Dinghong Zhang,
University of California, San Diego, United States

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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *29 January 2021*
Accepted: *17 March 2021*
Published: *14 April 2021*

Citation:
Latham CM, Brightwell CR, Keeble AR, Munson BD, Thomas NT, Zagzoog AM, Fry CS and Fry JL (2021) Vitamin D Promotes Skeletal Muscle Regeneration and Mitochondrial Health. Front. Physiol. 12:660498. doi: 10.3389/fphys.2021.660498

INTRODUCTION

Vitamin D is a fat-soluble vitamin with critical roles for bone and skeletal muscle health. Dietary sources of vitamin D include fortified foods, such as bread and milk, fatty fish, some mushrooms, and dietary supplements. Additionally, upon exposure to ultraviolet B rays, human skin can convert 7-dehydrocholesterol to vitamin D. After ingestion or synthesis, vitamin D is activated by two hydroxylation reactions, occurring mainly in the liver and kidneys, to form biologically active 1,25-hydroxyvitamin D [1,25(OH)D]. In the liver, vitamin D-25-hydroxylase converts vitamin D to 25(OH)D. The second hydroxylation is carried about by CYP27B1-encoded 1α-hydroxylase (CYP27B1), which yields 1,25(OH)D (Bikle, 2000). Though most 1α-hydroxylase activity occurs in the kidneys, the enzyme is expressed in other cell types including macrophages, monocytes, and muscle fibers (Pojednic and Ceglia, 2014). Bioactive 1,25(OH)D is a transcription factor affecting the expression of hundreds of genes (Bikle, 2000). In this capacity, 1,25(OH)D first binds to its nuclear receptor, the vitamin D receptor (VDR). This complex binds to a retinoid X receptor (RXR) to form a VDR-RXR heterodimer. The heterodimer interacts with genomic vitamin D response elements (VDREs) to regulate transcription (Bikle, 2000).

Since serum 25(OH)D best reflects vitamin D exposure and absorption, and has a relatively long half-life, this metabolite is used to evaluate vitamin D status (Holick, 2009). Serum 25(OH)D < 30 nmol/L (12 ng/ml) is defined as vitamin D deficiency while 25(OH)D concentrations between 30 and 50 nmol/L (12–20 ng/ml) are categorized as vitamin D insufficiency (Ross, 2011). To support bone health, 25(OH)D concentrations > 50 nmol/L (20 ng/ml) are considered adequate (Houston et al., 2007). However, evidence indicates that full suppression of parathyroid hormone, which is released when vitamin D is needed, occurs at a concentration of about 100 nmol/L (40 ng/ml; Ginde et al., 2012), suggesting that optimal vitamin D status may be above cut-points established to prevent vitamin D deficiency symptoms. These discrepancies in estimates of sufficiency are reflected by a lack of consensus on vitamin D cut points in skeletal muscle literature (**Table 1**), making comparisons between studies difficult.

Since sunlight exposure often contributes an appreciable amount to overall vitamin D supply, those living in latitudes above 43°N are more likely to be vitamin D deficient (Ross, 2011). For example, participants living in Erie, Pennsylvania were more likely to be vitamin D deficient compared with those in Bradenton, Florida (Leary et al., 2017). People with deeper skin tones and higher body fat are also more likely to be deficient (Leary et al., 2017). A classical 1,25(OH)D function is to promote intestinal calcium absorption to maintain blood calcium and bone mineralization (Ross, 2011). Severe vitamin D deficiency causes rickets in children and osteomalacia in adults, which is characterized by a softening and weakening of the bones (Nair and Maseeh, 2012). Vitamin D insufficiency is associated with inadequate bone health leading to loss of bone density, fractures, muscle weakness, osteopenia, and osteoporosis (Holick and Gordon, 2011).

Vitamin D status is also associated with muscle strength outcomes across a broad range of age groups (Redzic et al., 2013). In older adults, plasma 25(OH)D concentrations < 25 nmol/L are associated with significantly lower grip strength (Ranathunga et al., 2019). In a study of younger adults, including participants with deficient and optimal vitamin D status, higher baseline 25(OH)D concentrations predicted the restoration of strength following an intense resistance exercise bout (Barker et al., 2013). Though vitamin D supplementation studies have methodological differences in dosing, length of intervention, and participant characteristics (including baseline vitamin D status), a wealth of data indicates that correcting vitamin D status through supplementation improves muscle strength. A 2015 meta-analysis including primarily younger participants with 25(OH)D concentrations < 25 nmol/L showed vitamin D supplementation ranging from 4,000 to 60,000 IU per week significantly improved both upper and lower body strength (Tomlinson et al., 2015). An in-depth review of vitamin D supplementation and skeletal muscle strength outcomes in human participants may be found elsewhere (Chiang et al., 2017). Here we will review the contribution of vitamin D to skeletal muscle regeneration following injury.

SKELETAL MUSCLE DAMAGE AND REGENERATION THROUGHOUT THE LIFESPAN

Skeletal muscle is a remarkably plastic tissue, capable of robust adaptation and regeneration in response to stress and damage (**Figure 1**). Muscle damage can occur as a result of crush injury, ischemia-reperfusion injury, and resistance exercise,

TABLE 1 | 25-hydroxyvitamin D [25(OHD)] cut points vary significantly across skeletal muscle studies.

	25(OH)D cut points in human vitamin D/skeletal muscle studies			
	Deficient	**Insufficient**	**Sufficient**	**Optimal**
National Academies/Institutes of Medicine standards	<30 nmol/L	30 to <50 nmol/L	≥50 nmol/L	N/A
Ranathunga et al., 2019	<25 nmol/L	25 to <50 nmol/L	N/A	N/A
Bang et al., 2018	<50 nmol/L	50 to <100 nmol/L	≥100 nmol/L	N/A
Dzik et al., 2018	30 to <50 nmol/L	N/A	≥50 nmol/L	N/A
Jang et al., 2020	<25 nmol/L	25 to <50 nmol/L	50 to <75 nmol/L	≥75 nmol/L
Kitsu et al., 2020	<50 nmol/L	50 to >75 nmol/L	>75 nmol/L	N/A
Montenegro et al., 2020	N/A	N/A	50 to 100 nmol/L	>100 nmol/L

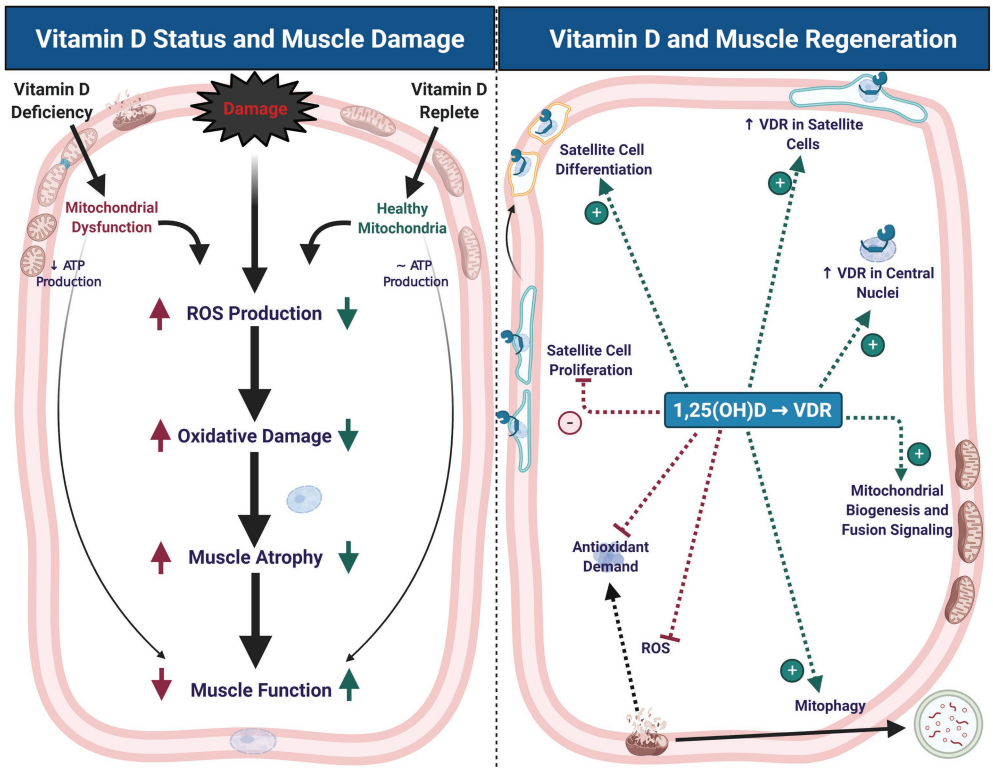

FIGURE 1 | Vitamin D status contributes to muscle damage and regeneration. Vitamin D deficiency leads to mitochondrial dysfunction, decreased adenosine triphosphate (ATP) production, increased reactive oxygen species (ROS) production, oxidative damage, muscle atrophy, and impaired muscle function. These symptoms of deficiency may exacerbate similar symptoms that typically occur with muscle damage **(left panel)**. During muscle regeneration **(right panel)**, hydroxylated, activated vitamin D [1,25(OH)D] stimulates an increase in vitamin D receptor (VDR) abundance in satellite cells and central myonuclei. These changes in VDR abundance are accompanied by inhibition of satellite cell proliferation and stimulation of differentiation, which may contribute to maintenance of satellite cell self-renewal capacity. Signaling at the VDR also increases mitochondrial biogenesis and fusion signaling, inhibit ROS production, and thereby mitigate antioxidant demand, which may contribute to a more competent regenerative phenotype. Created with BioRender.com

among other stimuli (McGeachie and Grounds, 1987; Roth et al., 1999, 2000; Mackey and Kjaer, 2017; Kuroda et al., 2020) and pre-clinical injury models in rodents offer reproducible and controlled experimental models (Hardy et al., 2016). The best-studied model of muscle damage in humans is unaccustomed resistance exercise, with high load eccentric muscle contractions being a significant driver of muscle damage (Roth et al., 1999, 2000; Damas et al., 2016; Mackey and Kjaer, 2017).

Severe muscle damage is characterized by disruption of muscle fiber integrity leading to impairments in structure and function of damaged muscle (Roth et al., 1999, 2000; Mackey and Kjaer, 2017). Muscle fiber damage is identified by Z-disc streaming and a smeared appearance of sarcomeres, indicating ultrastructural damage (Lieber and Fridén, 1999; Damas et al., 2016). This damage is further defined by severe disruption of the arrangement and structure of contractile proteins (Fridén et al., 1983a,b; Brown et al., 1997; Roth et al., 1999, 2000; Koh and McNally, 2007; Damas et al., 2016), leaving behind necrotic zones where regeneration of the muscle fiber can be initiated (Mackey and Kjaer, 2017). Necrotic zones in damaged muscle fibers serve as loci for neutrophil and macrophage accumulation (Mackey and Kjaer, 2017), a common theme of cellular damage (Uderhardt et al., 2019). In these damaged areas, cell proliferation is increased in response to heightened apoptosis and muscle fiber biogenesis, and this increased cellular turnover aids in the regeneration of viable muscle tissue (Mackey and Kjaer, 2017).

Muscle fiber damage is a unique form of cellular stress whereby the basement membrane of the damaged fiber is preserved, allowing for regeneration and adaptive remodeling of the fiber, as opposed to *de novo* fiber formation (Fridén et al., 1983a,b; Mackey and Kjaer, 2017). This regenerative potential of muscle fiber damage is unique, as comparable cellular stress in mitosis-competent cell types often necessitates *de novo* cell formation (Liao et al., 1997; Griffiths et al., 1999). The unique nature of muscle fiber damage and robust regenerative potential is owed to its resident stem cell population and multinucleation (Lepper et al., 2011). Skeletal muscle stem cells are termed satellite cells (SC), due to their residency on the periphery of the muscle fiber, between the basal lamina and sarcolemma (Katz, 1961; Mauro, 1961). SCs are indispensable for regeneration after severe muscle injury (McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011; Lepper et al., 2011), and are characterized by constitutive

expression of Pax7 (Seale et al., 2000). SCs are activated after damage to the surrounding muscle and undergo asymmetric division to generate a "sister" and a "daughter" cell. The sister cell is able to return to quiescence, maintaining the resident SC pool. The daughter cell differentiates, progresses through the myogenic program, and eventually fuses with the surrounding muscle tissue and donates its nucleus (Zammit et al., 2004).

Recent evidence suggests that vitamin D signaling also contributes to muscle regeneration. In skeletal muscle of mature and aged mice, protein expression of VDR is closely associated with serum concentration of 25(OH)D (Srikuea et al., 2020). The VDR and vitamin D-activating enzyme CYP27B1 are lowly expressed in homeostatic skeletal muscle *in vitro* and *in vivo*, evidenced by immunocytochemical and immunohistochemical visualization and immunoblotting in both C2C12 myoblasts and whole mouse muscle (Srikuea et al., 2012, 2020). After muscle injury, the minimal expression of VDR and CYP27B1 observed in homeostatic conditions is starkly augmented. Vitamin D receptor expression is marginally detectable in uninjured skeletal muscle but is highly expressed and localized to regenerating muscle fibers after muscular injury (**Figure 2**; Srikuea et al., 2012), and is colocalized with central myonuclei (Srikuea and Hirunsai, 2016). Furthermore, endogenous VDR is expressed at detectable levels in SCs in regenerating skeletal muscle after injury, substantiated by colocalization of the Pax7 transcription factor and VDR protein (Srikuea and Hirunsai, 2016). The role of the vitamin D system in muscle regeneration is further supported by rapidly increased Pax7 and VDR protein expression in skeletal muscle initiating a repair response after an acute bout of damaging high-intensity exercise (Puangthong et al., 2020), demonstrating that the myogenic repair system and vitamin D system are both rapidly and concurrently initiated after damage to skeletal muscle.

Although muscle regeneration is highly efficient, the regenerative process can be compromised in pathological conditions (myopathies) and in aged individuals. Compromised regenerative processes in skeletal muscle during aging provide a particularly interesting area of study, as they are multifactorial and complex. Skeletal muscle mass peaks around the third decade of life and gradually decreases over time, with observable loss starting in the fifth decade (Janssen et al., 2000). A hallmark of age-related muscle atrophy is an imbalance between muscle protein synthesis and degradation. During aging,

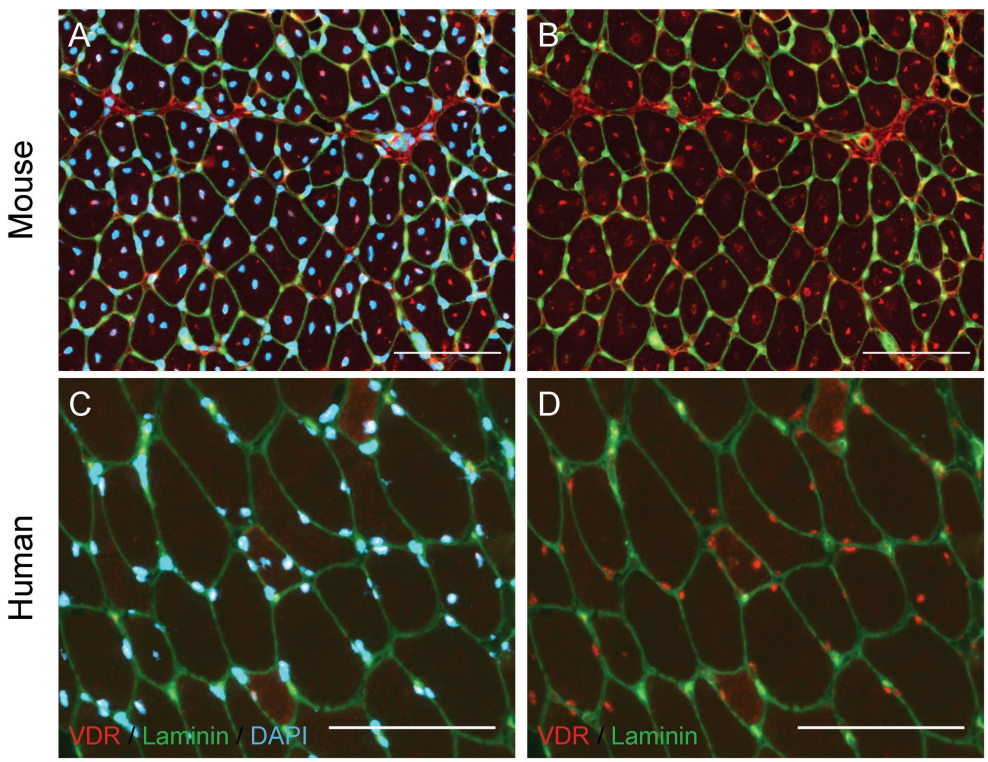

FIGURE 2 | Localization of Vitamin D Receptor (VDR) in regenerating mouse and human skeletal muscle. **(A,B)** Muscle regeneration was induced in the mouse tibialis anterior muscle by injecting $BaCl_2$ (1.2%), and the tibialis anterior muscle was harvested 7 days following injury. Immunohistochemical analysis revealed regenerating fibers with centrally-located myonuclei (**A**, DAPI) show strong positive VDR expression (**B**, red puncta) in myonuclei. **(C,D)** A vastus lateralis biopsy was obtained from a human research participant 7 days after severe thermal injury, which we have shown to induce significant skeletal muscle regeneration (Fry et al. 2016). Myonuclear localization of VDR (**D**, red puncta) is observed, with the strongest VDR intensity present within myonuclei (**C**, DAPI) of small, regenerating fibers. In all images, laminin (green) denotes the fiber border. In images **A** and **C**, DAPI (blue) denotes nuclei. In images **B** and **D**, DAPI is omitted to allow visualization of VDR staining within nuclei. Scale bar = 100 μm.

research has shown increases in protein degradation signaling (Cai et al., 2004) and anabolic resistance (Welle et al., 1993), which contribute to the observed loss of muscle mass. Additionally, impaired mitochondrial function and resultant excessive ROS production have been implicated in age-related muscle loss (Short et al., 2005). These declines in mitochondrial health have been attributed to impaired mitochondrial processes such as fusion and fission (Chabi et al., 2008; Gouspillou et al., 2014; Leduc-Gaudet et al., 2015; Liu et al., 2020), mitophagy (García-Prat et al., 2016), and biogenesis (Chabi et al., 2008). Further, the loss in skeletal muscle mass is coupled with decreases in the resident SC pool (Shefer et al., 2006) and SC activity (Conboy, 2003). Age-associated reductions in abundance and functionality of SCs induce significant decrements in the muscles' regenerative capacity, which is often associated with accumulation of fibrotic material. This increase in fibrosis may be due to SC transition to a fibrogenic phenotype (Stearns-Reider et al., 2017) and resultant decline in SC-mediated regulation of fibroblast activity (Fry et al., 2017).

In mice, VDR protein expression is increasingly elevated from development through maturation and aging (Srikuea et al., 2020); however, a study in human muscle showed an inverse relationship between skeletal muscle VDR expression and age (Bischoff-Ferrari et al., 2004). VDR expression is strongly associated with changes in circulating 25(OH)D concentrations following vitamin D_3 supplementation in older women with vitamin D insufficiency (25OHD of 46.3 ± 9.5 nmol/L; Ceglia et al., 2013). In mice, elevated VDR expression in aged skeletal muscle is also associated with central nucleation of muscle fibers (Srikuea et al., 2020) – typically indicative of muscle fibers recovering from a damaging stimulus. Further understanding the environment facilitating deficits in muscle regeneration, and perhaps the contribution of vitamin D signaling, could lead to the development of novel therapeutic strategies that support greater myogenic potential throughout the lifespan.

VITAMIN D AND MITOCHONDRIAL HEALTH

Vitamin D deficiency and insufficiency, when combined typically defined as 25(OH)D concentrations < 50 nmol/L, are associated with muscle atrophy and deficits in muscle strength in several clinical models (Sato et al., 2005; Tagliafico et al., 2010; van Langenberg et al., 2014; Almurdhi et al., 2017; Bang et al., 2018). While many studies of vitamin D deficiency and insufficiency focus on its impact on protein synthesis and degradation, there is also a growing body of evidence to suggest that vitamin D supplementation in deficient individuals improves measures of mitochondrial density and function (Sinha et al., 2013; Rana et al., 2014). These studies are supported by research in rodent models, where vitamin D supplementation in deficient animals improves the balance between muscle protein synthesis and degradation, as well as measures of mitochondrial density and function (Gogulothu et al., 2020).

Due to evidence implicating compromised mitochondrial health as a key factor in the pathology of vitamin D deficiency-induced atrophy, mechanistic action of vitamin D on skeletal muscle atrophy – particularly regarding mitochondrial health – has gained attention in recent studies. Overexpression of VDR in rat skeletal muscle results in increased skeletal muscle hypertrophy, driven by increases in anabolic signaling, ribosomal biogenesis, and protein synthesis (Bass et al., 2020). While the effect of VDR overexpression on mitochondrial dynamics has not been studied, recent studies have confirmed that vitamin D regulates oxidative capacity through binding of 1,25(OH) D to the VDR in skeletal muscle (Ashcroft et al., 2020). Furthermore, mitochondrial ATP production is significantly reduced in VDR-knock down C2C12 myoblasts, supporting the idea that the absence of 1,25(OH)D signaling at the VDR may significantly reduce ATP availability. This could have important consequences for muscle regeneration, which has shown to be hampered by loss of mitochondrial capacity (Jash and Adhya, 2012). Interestingly, reductions in ATP generation following VDR knock-down occurred independently of changes in many measures of mitochondrial machinery, including electron transport system (ETS) subunits I-V, citrate synthase, and cytochrome *c* oxidase (Ashcroft et al., 2020). These results were recapitulated by an *in vivo* experiment in vitamin D deficient mice, which showed decrements in maximum oxidative capacity in the absence of differences in protein expression of ETS complexes I-V (Ashcroft et al., 2020).

Studies of VDR ablation suggest that 1,25(OH)D regulation of oxidative capacity can occur independently of significant changes in mitochondrial density and ETS protein abundance. This may be explained in part by a study showing that VDR-knockdown in C2C12 myotubes results in an increase in optic atrophy 1 (OPA1) abundance, which was proposed to be a compensatory mechanism to rescue deficits in mitochondrial function resulting from vitamin D deficiency (Ashcroft et al., 2020). Optic atrophy 1 is a marker of fusion of the inner membrane on mitochondria, which results in larger mitochondria and increased oxidative capacity (Kushnareva et al., 2013). In contrast, an increase in OPA1 expression was observed after vitamin D supplementation in vitamin D deficient mice with statin-induced myopathy (Ren et al., 2020), as well as in human skeletal muscle cells treated with 1,25(OH)D (Ryan et al., 2016). While these studies all suggest that 1,25(OH) D treatment or VDR expression alter OPA1, it is unclear why both VDR knockdown and 1,25(OH)D treatment led to increased OPA1 expression. Additionally, few studies have explored the effect of different vitamin D analogs on oxidative capacity, but in human skeletal muscle cells, only 1,25(OH) administration increased oxygen consumption rate, while exposure to both 25(OH)D and vitamin D_3 significantly reduced it (Ryan et al., 2016). Given these disparate findings, future research should clarify the impact of VDR expression and different vitamin D analogs and doses on mitochondrial dynamics.

In addition to reductions in mitochondrial ATP production, another mechanism by which vitamin D deficiency may contribute to muscle atrophy is excess mitochondrial ROS production (Ricca et al., 2018). While normal levels of ROS

are important for skeletal muscle signaling following injury, excessive ROS production that overwhelms protective antioxidant systems can be deleterious to muscle health (Le Moal et al., 2017). Vitamin D deficiency has been shown to increase lipid (Cielen et al., 2016; Dzik et al., 2018) and protein (Bhat and Ismail, 2015; Dzik et al., 2018) oxidation in skeletal muscle. Additionally, vitamin D deficiency causes alterations in antioxidant enzyme activities (Bhat and Ismail, 2015; Dzik et al., 2018). Interestingly, while vitamin D deficiency was associated with increased superoxide dismutase (SOD) activity in humans experiencing chronic lower back pain (Dzik et al., 2018), a study of rats showed a decrease in SOD activity in the plantaris of deficient animals (Bhat and Ismail, 2015). However, both studies report that deficiency results in an increase in muscle glutathione peroxidase (GPx) activity. The difference between these two studies may result from species differences (rat vs. human), the presence of muscle pathology, or any number of other factors. Notwithstanding, vitamin D supplementation was associated with correction of alterations in SOD and GPx activities in both studies. There are clear opportunities for future studies to elucidate the impact of vitamin D status on antioxidant systems in human participants.

In several models, providing vitamin D analogs exerts a protective effect on skeletal muscle and cells undergoing oxidative stress. Administration of 1,25(OH)D *in vitro* resulted in a reduction in ROS production, lipid and protein oxidation, protein ubiquitination, muscle proteolysis, intracellular damage and gene markers for atrophy, and an increase in SOD activity and markers of mitochondrial biogenesis (Bhat and Ismail, 2015; Chang, 2019). There is a paucity of data regarding the protective effects of vitamin D analogs *in vivo*. However, one study of patients experiencing chronic lower back pain revealed that vitamin D_3 supplementation resulted in lower Cu/Zn SOD and GPx activity in paraspinal muscle compared to deficient individuals. These differences in antioxidant activity were mirrored by lower protein and lipid peroxidation. On the other hand, the same measures in un-supplemented, vitamin D replete patients did not differ from deficient patients (Dzik et al., 2018). These results not only underscore the importance of vitamin D signaling for optimal redox balance, but also highlight how the provision of vitamin D analogs promote muscle mitochondrial health during oxidative stress (**Figure 1**).

SATELLITE CELL MITOCHONDRIAL FUNCTION AND VITAMIN D

Adequate oxidative capacity is critical for skeletal muscle regeneration following injury. In addition to providing energy for protein synthesis, mitochondria play an important role in regulating SC activity. Quiescent SCs have less mitochondria and lower oxidative capacity than activated, differentiating SCs, and mitochondrial activity is a key process underlying SC activation (Latil et al., 2012). This lower oxidative capacity has been recapitulated *in vivo*, where reduced mitochondrial respiration of SCs was associated with a greater proportion of SCs expressing self-renewal markers in endurance-trained mice (Abreu and Kowaltowski, 2020). In addition to the importance of metabolic reprogramming, other mitochondrial processes are implicated in optimal SC function. For instance, competent mitophagy has been shown to be necessary for normal SC activity in a study that examined Parkin null mice (Esteca et al., 2020). Parkin is an E3 ubiquitin ligase that is essential for regulation of mitophagy. Parkin knockout resulted in increased SC proliferation and impaired differentiation in mouse skeletal muscle following injury with cardiotoxin. These changes in SC activity were accompanied by delayed skeletal muscle fiber repair and smaller muscle fibers during regeneration (Esteca et al., 2020).

Another means by which mitochondria influence SC activity is through production of ROS, which stimulate symmetric division, followed by terminal differentiation (Mohammad et al., 2019). Interestingly, a study of human skeletal muscle myoblasts showed that vitamin D treatment resulted in inhibition of myoblast proliferation that was accompanied by an increase in differentiation and mitochondrial oxygen consumption rate. The authors proposed that decreased proliferation induced by vitamin D may serve to maintain SC quiescence and thus maintain the stem cell population in muscle. Conversely, the increase in mitochondrial oxygen consumption rate likely serves to power the increase in synthesis of metabolic machinery that accompanies differentiation and mature myotube formation (Montenegro et al., 2019). Taken together, these data suggest that vitamin D may modulate SC activity *via* alterations in mitochondrial density or function. Since measures of mitochondrial density and ROS production were not assessed, it is difficult to speculate on the nature of the changes in mitochondria that caused increased oxygen consumption rate, and whether alterations in ROS production may have contributed to the changes in SC activity. More research is needed to determine how vitamin D signaling affects mitochondrial function and regulation of SC activity.

Taken together, the global effect of vitamin D on mitochondrial function and the presence of VDR in SCs of regenerating muscle (Srikuea et al., 2012) suggest an interplay between mitochondria and SCs during muscle repair. Indeed, CYP27B1 is expressed in mitochondria, suggesting an intrinsic link between vitamin D activation and mitochondria (Nakamura et al., 1997). However, more research is needed to fully understand the effects of vitamin D on the relationship between mitochondria and SC activity following skeletal muscle injury. Elucidation of the precise nature of signaling between the VDR, SCs, and mitochondria may provide avenues for treatment of skeletal muscle atrophy where one or more of these components in compromised.

VITAMIN D ANALOGS AND SKELETAL MUSCLE INJURY

A potential regenerative role of the vitamin D system in injured skeletal muscle is supported by improved cellular turnover and enhanced muscle function with subcutaneous administration of vitamin D_3 after crush injury in rats (Stratos et al., 2013).

With vitamin D_3 treatment initiated immediately after injury, proliferation of cells in the interstitium of injured skeletal muscle is elevated with a concurrent decline in necrotic cells (Stratos et al., 2013) – supportive of enhanced activity of mononuclear cells with potential roles in muscle repair, such as various immune cells, macrophages, and fibrogenic cells. Though vitamin D_3 administration enhanced activity of mononuclear cells within the injured skeletal muscle, Pax7+ SC abundance and muscle morphology were not clearly altered at any time point through 42 days post-injury. At the same time, vitamin D_3-exposed rats showed higher peak tetanic torque after the severe crush injury when compared with vehicle controls (Stratos et al., 2013). Interestingly, the timing, delivery, dose, and vitamin D analog may be crucial to optimize muscle regeneration after injury. When 1,25(OH)D (the bioactive form of vitamin D) is provided in a delayed manner by intramuscular injection 4 days after $BaCl_2$ muscle injury, no regenerative benefit is observed (Srikuea and Hirunsai, 2016). Both physiological and supraphysiological delayed intramuscular administration of 1,25(OH)D elevated VDR protein expression in injured muscle, but neither delayed dose produced larger muscle fibers 8 days after injury. In fact, mice receiving a supraphysiological delayed dose of 1,25(OH)D showed impaired SC differentiation and subsequent *de novo* myogenesis and presented with injured skeletal muscle having smaller muscle fibers and excessive accumulation of fibrotic materials (Srikuea and Hirunsai, 2016). In both the aforementioned studies where different vitamin D analogs were provided with different timing, routes of administration, and doses after muscular injury, muscle morphology was not positively altered toward an enhanced regenerative phenotype. This is despite improved muscle function with immediate systemic delivery of vitamin D analogs after injury (Stratos et al., 2013; Srikuea and Hirunsai, 2016). It's worth noting that rodents consumed standard diets formulated to provide sufficient vitamin D. Therefore, it's possible that vitamin D_3 or 1,25(OH)D administration would affect deficient animals differently. *In vivo* protocols including both deficient and replete animals would help determine the means by which treatment with different vitamin D analogs and dosing affect muscle recovery and function after injury.

Vitamin D_3 supplementation in young males with insufficient serum 25(OH)D concentrations resulted in improved knee extensor torque output 2 and 7 days after a damaging bout of exercise (Owens et al., 2015) suggestive of enhanced muscle regeneration to support superior muscle function. Here, insufficiency for recruitment purposes was defined as 25(OH)D < 75 nmol/L, above the Institutes of Medicine (IOM) cut point of 50 nmol/L. This study included men in both insufficiency and sufficiency ranges as defined by the IOM, which is common in human studies investigating vitamin D status and muscle phenotypes (**Table 1**). Nonetheless, in skeletal muscle-derived myoblasts isolated and cultured from these same young men, administration of 1,25(OH)D enhanced differentiation and myotube fusion and resulted in larger myotubes after an *in vitro* mechanical wound injury (Owens et al., 2015). These data suggest that supplementing vitamin D_3 in young people with marginally insufficient vitamin D status may optimize regenerative processes in skeletal muscle after muscular injury. This is particularly relevant given the high prevalence of vitamin D deficiency in athletes (Farrokhyar et al., 2015) – associated with a higher prevalence of muscle strain injuries (Rebolledo et al., 2018) – and the general population across the lifespan (Holick, 2017).

DISCUSSION

Within the last decade, it has become clear the vitamin D receptor is expressed in skeletal muscle and has integral roles in both recovery following injury and maintaining mitochondrial function. What remains to be determined are the precise mechanisms by which it exerts these actions and how varying forms, doses, and timing of vitamin D analogs affect the outcomes. Though mounting evidence indicates that vitamin D_3 supplementation supports mitochondrial health and oxidative capacity while reducing oxidative stress in deficient patients, it is unknown what circulating concentration of vitamin D promotes "optimal" mitochondrial health, especially in the context of injuries causing oxidative damage where it is established that some vitamins turn over more quickly (Kesavan et al., 2003; Barbosa et al., 2009). Moreover, different studies evaluating strength-associated outcomes have had overlapping sufficiency and insufficiency definitions and equivocal results, which makes interpretation exceedingly difficult. Though different countries and professional bodies recommend different cut points, future studies require consistent 25(OH)D cut points for analysis of the efficacy of specified outcomes and should evaluate the effect of dosing and timing. Specifically, it would be helpful to compare directly efficacy of a single megadose and sustained daily supplementation after muscle injuries. Though supplementation prior to injury may be realistic in some high-risk settings, it would also be helpful to establish how soon after injury doses should be administered since prior supplementation will not always be feasible.

Different natural vitamin D analogs and doses have been used to elucidate vitamin D's cellular functions (**Table 2**). Most *in vitro* studies administer the active vitamin D metabolite 1,25(OH)D, which is a hormone regulating about 3% of the genome. Though valuable mechanistic insight may be gained from this approach, concentrations of this hormone vary 10,000 fold across studies applying treatments to C2C12 myoblasts and human skeletal-derived muscle myoblasts (Ryan et al., 2016; Montenegro et al., 2019). Few *in vitro* studies include treatments with 25(OH)D, but it is the vitamin D metabolite that skeletal muscle cells would be exposed to *in vivo*. Moreover, it is established that 1,25(OH)D alters mineral metabolism and carries the risk of clinically dangerous hypercalcemia, so it is typically prescribed for patients with hyperparathyroidism, chemotherapies enhanced by calcitriol, and conditions promoting hypocalcemia (Quarles et al., 1988; Muindi et al., 2002; Tartaglia et al., 2005). Animal studies we reviewed utilized oral and injectable forms of vitamin D_3, 25(OH)D, 1,25(OH)D; however, no study compared more than one analog in the same experiment. Inclusion of both 25(OH)D and synthetic vitamin D analogs

TABLE 2 | Forms and doses of vitamin D analogs vary significantly across studies.

Author	Form	Dose	Model	Outcome
Bhat and Ismail, 2015	1,25(OH)D	1 or 10 nM	C2C12 myotubes	Reduced oxidative stress and proteolysis
Chang, 2019	1,25(OH)D	1, 10, or 100 nM	C2C12 myotubes	Reduced oxidative stress
Montenegro et al., 2019	1,25(OH)D	100 nM	Human skeletal muscle-derived myoblasts	Inhibited proliferation, increased differentiation and oxygen consumption
Owens et al., 2015	1,25(OH)D	10 or 100 nM	Human skeletal muscle-derived myoblasts	Improved muscle cell migration dynamics
Ryan et al., 2016	1,25(OH)D 25(OH)D Vitamin D_3	0.01, 0.1, or 1 nM 1 nM 1 nM	Primary human skeletal muscle cells	Only 1,25(OH)D increased oxygen consumption
Srikuea et al., 2012	1,25(OH)D 25(OH)D	20 nM 2,000 nM	C2C12 myoblasts and myotubes	1,25(OH)D and 25(OH)D inhibited myoblast proliferation
Chogtu et al., 2020	25(OH)D	0.1 μg/kg/day oral	Wistar rats 6–8 weeks old (unknown sex)	Attenuated statin-induced increases in plasma creatine kinase
Keskin et al., 2018	Vitamin D_3	8.3 mg/kg subcutaneous injection	Male Wistar rats, 8–12 weeks old	Increased nitric oxide levels in muscle following ischemia/reperfusion injury
Ren et al., 2020	Vitamin D_3	1,000 IU/kg/day oral	Male C57 BL/6 mice, 10–12 weeks	Reduced statin-induced myopathy and improved mitochondrial cristae shape
Srikuea and Hirunsai, 2016	1,25(OH)D	1 μg/kg TA muscle wet weight or 1 μg/kg mouse body weight intramuscular injection	Male C57BL/6 mice, 10 weeks	High dose decreased satellite cell differentiation, delayed regenerative muscle fiber formation, and increased muscular fibrosis
Stratos et al., 2013	Vitamin D_3	8.3 mg/kg body weight subcutaneous injection	Male Wistar rats, unknown age	Increased muscle cell proliferation after crash injury and did not alter VDR expression

that do not target vitamin D's classical mineral-regulating pathways (Brown and Slatopolsky, 2008) in future *in vitro* and *in vivo* studies would add considerably to the literature.

Furthermore, sport and muscle research has suffered from limited inclusion of female subjects and animals. The vast majority of animal studies we reviewed included only male rodents, and prior evidence indicates that vitamin D metabolism is affected by sex (Dam et al., 2009; Heidari and Haji Mirghassemi, 2012). Though the inclusion of both biological sexes creates variability within an experiment, future studies should include both sexes or, particularly for *in vivo* studies, include only female animals when funding and power analyses support the inclusion of a single-sex.

In summary, *in vitro* and *in vivo* models of 1,25(OH) exposure, VDR overexpression and knockdown, as well as supplementation in deficient rodents and humans, clearly indicate a role for vitamin D in the regeneration of muscle and supporting mitochondrial health. Deeper mechanistic inquiries are needed to illuminate how differing vitamin D analogs, timing, and initial vitamin D status affect skeletal muscle regeneration and mitochondrial function and how these processes may be inherently linked.

AUTHOR CONTRIBUTIONS

CL, CB, AK, BM, NT, AZ, and JF wrote the manuscript. CL, CF, and JF created the tables and figures. CL, CB, AK, BM, NT, AZ, CF, and JF edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Research reported in this publication was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under award number 3R01AR071398-04S1. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

BRIEF RESEARCH REPORT
published: 30 April 2021
doi: 10.3389/fnut.2021.654008

Drastic Effects on the Microbiome of a Young Rower Engaged in High-Endurance Exercise After a Month Usage of a Dietary Fiber Supplement

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Food supplements are increasingly used worldwide. However, research on the efficacy of such supplements on athlete's well-being and optimal sports performance is very limited. This study performed in junior academic rowing explores the effects of nutritional supplements to aid to the high energy requirements at periods of intense exercise. Herein, the effects of prebiotic fibers on the intestinal microbiome composition of an 18-year-old athlete exercising at high loads during an 8-month period in a "real-life" setting were examined using next-generation sequencing analysis. Results demonstrated that although the alpha diversity of the subject's microbiome drastically decreased [from 2.11 precompetition to 1.67 ($p < 0.05$)] upon fiber consumption, the *Firmicutes/Bacteroidetes* ratio increased significantly [from 3.11 to 4.55, as compared with population average ($p < 0.05$)]. Underlying these macrolevel microbial alterations were demonstrable shifts from acetate- to butyrate-producing bacteria, although with stable effects on the *Veillonella* species. To our knowledge, this a unique study that shows pronounced changes in the gut microbiome of the young athlete at the competition season and their favorable compensation by the dietary fiber intake. The data here expand the overall understanding of how the high energy needs in high-intensity sports like academic rowing could be supported by dietary fiber supplement consumption.

Keywords: microbiome, endurance sports, rowing, dietary fiber, athlete, junior, case study

OPEN ACCESS

Edited by:
Yan Huang,
University of Arkansas, United States

Reviewed by:
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Emory University, United States
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UC Davis Genome Center, United States

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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *02 February 2021*
Accepted: *22 March 2021*
Published: *30 April 2021*

Citation:
Jaago M, Timmusk US, Timmusk T and Palm K (2021) Drastic Effects on the Microbiome of a Young Rower Engaged in High-Endurance Exercise After a Month Usage of a Dietary Fiber Supplement. Front. Nutr. 8:654008. doi: 10.3389/fnut.2021.654008

INTRODUCTION

The microbiome contributes to thehomeostatic regulation of different tissues in our body (1) with the largest and most diverse cluster of microorganisms inhabiting the gut (2). These core functions are linked to the production of essential and extremely diverse metabolites such as vitamins (vitamin B_{12}, folic acid, or vitamin K), bile acids, neurotransmitters (serotonin, dopamine, acetylcholine), and short-chain fatty acids (SCFAs: acetic acid, propionic acid, and butyric acid) (3). Diet and the level of physical activity are the main determinates for altering the gut microbiota (4). Increases in bacterial diversity and a proliferation of taxa responsible for the production of SCFAs, such as butyrate, are among the most pervasively observed microbial alterations with exercise (5).

Athlete microbiomes have been found to contain distinct microbial compositions defined by elevated abundance of *Veillonellaceae*, *Bacteroides*, *Prevotella*, *Methanobrevibacter*, or *Akkermansia* (6). Cardiorespiratory fitness in exercising subjects was associated with higher abundance of butyrate-producing bacteria by the *Clostridiales*, *Erysipelotrichaceae*, *Lachnospiraceae*, and *Roseburia* families (7). Exercise type along with athlete diet patterns (bodybuilders: high protein, high fat, low carbohydrate, and low dietary fiber diet; distance runners: low carbohydrate and low dietary fiber diet) was significantly associated with the relative differential abundance of *Faecalibacterium*, *Sutterella*, *Clostridium*, *Haemophilus*, *Eisenbergiella*, *Bifidobacterium*, and *Parasutterella* in bodybuilders and distance runners (8). Variation in genera was suggested to be linked to the variance in species' composition across different types of sports (9). So, athletes participating in sports with high dynamic and static component like academic rowing displayed greater abundance of *Bacteroides caccae* (9). The effects of exercise on gut microbial microorganisms were concluded to depend significantly on its intensity and timing with the notion that the microbiota could also influence muscle mass, as reported by Ticinesi et al. (10). Excessive exercise among professional athletes disturbs the homeostasis of the gut microbiota [reviewed in (7)]. Physical exertion at a very high level for a prolonged time means that the whole body initiates a defense response because of oxidative stress, intestinal permeability, muscle damage, systemic inflammation, and immune responses (11). It has been observed that endurance athletes present a high prevalence of upper respiratory tract infections and gastrointestinal troubles, including a "leaky gut," disruption of mucous thickness, and higher rates of bacterial translocation (12, 13). Overall, all these studies suggest that the gut microbiome affects exercise performance and vice versa.

Diet has a major impact on gut microbiota composition, diversity, and richness. Dietary supplements that employ non-digestible dietary fibers have been developed for several decades as prebiotics to support growth of beneficial GI microbiota (14). Dietary fibers can be found in plants, bacteria, and fungi and can be chemically synthesized (15). The health effects of these dietary fibers have extensively been reviewed and accepted worldwide (14). It has been concluded that the extent by which different fiber types are utilized or fermented by the GI microbiota is structure dependent and relies on the metabolic capabilities of the individual's microbiome (16). Virtually, all fibers induce specific shifts in microbiota composition due to competitive interactions; however, which of these shifts contribute to health, or if at all, is not known (17, 18). The commensal bacteria ferment non-digestible fiber primarily into CO_2, H_2, and CH_4 and SCFAs (19). Most of the SCFAs produced in the intestine are absorbed by the host to contribute to energy and beneficial metabolites (20) that are also used as carbon and energy sources by other specialized bacteria including reductive acetogens, sulfate-reducing bacteria, and methanogens (21).

Multiple lines of evidence support the hypothesis that modification of the microbial community through diet could be an effective tool to improve athlete's health (14) performance and energy availability while controlling redox levels and inflammation (22). Endurance diets are rich in protein (1.2–1.6 g/kg/day), which produce a range of potentially harmful compounds in the intestine in addition to SCFA productions (22). There are only a few demonstrated studies in athletes consuming prebiotics (23). Research has suggested the validity of probiotics to improve training parameters and increase training capabilities (24).

Nutritional supplements are popular among athletes to improve performance and physical recovery. Long-term (10 weeks) protein supplement (whey isolate and beef hydrolysate) consumption by cross-country runners, however, decreased the presence of health-related taxa including *Roseburia*, *Blautia*, and *Bifidobacterium longum* and increased the abundance of the Bacteroidetes phylum (25). However, it appeared that protein overconsumption was an offset by a higher intake of indigestible polysaccharides (26).

Athletes with very high training and competition loads can have serious problems getting the necessary amount of energy from regular food. Clark and Mach (2016) reported that diets recommended for athletes likely influence gut microbiota by reducing diversity because these diets include insufficient dietary fiber (27). In addition, a recent study showed that *Veillonella atypica* has a beneficial impact on the performance of elite athletes (6). Based on these findings, we decided to look for dietary ways to increase beneficial bacteria for better athletic performance and faster recovery, in particular considering that nutritional supplements are popular among athletes. We hypothesized that microbial profiles of the young rower might share features of those previously described in endurance sports studies but that this might change in response to the dietary changes upon dietary fiber supplement intake.

MATERIALS AND METHODS

Case Presentation

At the time of the study, the male athlete was 18 years old and was studied over the course of a 31-week period during the 2019 race and training season preceding the world championship competition in U19 category of academic rowing. By this time, the subject had been undertaking rowing for 4 years and had not previously sought any conditioning or dietary advice. Furthermore, the athlete was not on any prescribed medication and was a non-smoker, and his usual diet was previously supplemented with whey protein [SiS (Science in Sport) Limited products] only. In the 7 months prior to the supplement intake, the subject held a normal diet that was alike on a daily basis, comprising mostly of meals that were high in carbohydrate and protein, medium in fat, and modest in dietary fiber. The athlete was fully informed of the study aims and confirmed participation in the study by signing a consent form, understanding that the parameters of health were not associated with the study and that the subject was not physically harmed by the study. This study was approved by Protobios (1-05/2019).

Goals of the Study

The primary goals of the support provided were to (a) get insights into the subject's intestinal microbial community during periods

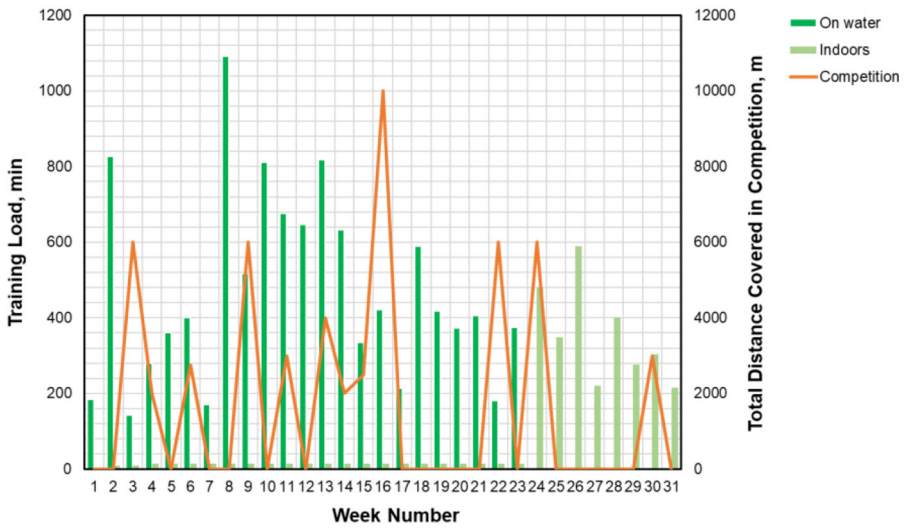

FIGURE 1 | Training program undertaken throughout the study period. Electronic exercise diary (www.sportlyzer.com) was kept for water and ergometer training hours throughout the study period. On the left *y*-axis—training load in minutes per week, min/week (Rowing on the *Water* and *Indoors*); on the right *y*-axis—distance in meters per week, m/week (*Competition/m*); on the *x*-axis—duration of the study period in weeks. Samples for microbiome analysis were taken at weeks 1, 27, and 31. *On water*, training on the water; *Indoors*, training on rowing ergometer; *Competition*, competition calendar of the rower.

of high exercise and (b) examine the effects of dietary fiber intake to the bacterial compositions associated with energy production.

Diet and Activity Recordings and Microbiome Sampling

The participant was informed to maintain the usual dietary habits throughout the study. Body composition estimates were made preseason (sample #1) and post study (sample #3) with no substantial change (BMI 23.3 ± 0.2, fat percentage of 8.4 ± 0.0) according to the medical sports health survey records. Based on diet recall, the usual macronutrient intake was assessed using image-based dietary assessment software (NutriData, National Institute for Health Development, Estonia). We used this tool to ascertain the usual eating patterns of the subject including type, frequency, and amount of foods consumed. Foods consumed were matched to the nutritional analysis for the specific menu items that had been coded in NutriData. If not consumed from the menu, the item was coded against the most appropriate matching food. On average, across the study period, the athlete consumed 2,560 ± 750 kcal/day, and the estimated macronutrient intake was 23 ± 7.12% protein, 52 ± 19.1% carbohydrate, 26 ± 19.3% fat, and 15 ± 4.5 g fiber. The consumption of the nutrient supplement (*Food, not only for thought*, Elsavie, Estonia) started on week 27 and continued on a daily basis until week 31 (altogether 30 days), with intake immediately after breakfast, and the recommended daily intake [1.5 tablespoons (1.5 tbsp/20 g) mixed with water] was not exceeded. The dietary supplement provided to the participant as the prebiotic mix (20 g) included dietary fiber (8.79 g), consisting of resistant starch (2.25 g), arabinoxylan (2.05 g), citrus fiber (2 g), beta-glucans (1.03 g), inulin (1.03 g), and rye fiber (0.57 g). The athlete kept the training diary in Sportlyzer (Sportlyzer, Estonia). The 31-week high-training and intensive competition program is presented in **Figure 1**. The mean physical activity (8 months duration) of the subject during the study was 472 min/week of water rowing, 354 min/week of indoor rowing, and 60 min/week of stretching exercises (**Figure 1**). Since the time the first microbial sample (#1) was taken, the subject was participating in a series of national and international competition activities including five international and 11 local competitions (53.25 km total of total race distance) following the planned program as in **Figure 1**. The second sample (#2) was taken at week 27, by the end of the water season and at early weeks of the indoor rowing season. Thereafter, the subject began to take the dietary fiber supplement as recommended for 30 days. A third sample (#3) was taken at week 31. The seasonality of sampling was spring (#1), autumn (#2), and winter (#3), respectively.

Microbiome Assessment

Microbiome composition was determined on three occasions (week 1, week 27, and week 31, **Figure 1**). Samples were self-collected from morning stool samples by using the commercially available kit (INTEST.pro, BIOMES NGS GmbH, Germany) in accordance with the specifications laid out by the manufacturer. The first two samples were taken at normal nutrition (at the start of and after the active competition period), followed by 1 month of dietary supplement intake to investigate the dynamics of the intestinal microbiome and the effects of the fiber supplement on the microbiota. Collected samples were transported to the lab according to the service provider's instructions where the microbiome composition was analyzed *via* 16S rRNA gene amplification and sequencing by BIOMES NGS GmbH (Germany). In brief, microbial genomic DNA from fecal material was extracted by the bead-beating technique,

the V3–V4 region of the 16S rRNA gene was amplified, and sequencing was performed on the Illumina MiSeq platform using a 2 × 300-bp paired-end protocol (Illumina, San Diego, CA, USA). These DNA sequencing techniques were then used to generate data outputs that provided a comprehensive bacterial taxonomic profile of the subject (28) in comparison with the average microbiome of the European population as the evidence indicates that microbiome may vary by geography (29).

Data Analysis and Statistics

Different packages of MS Excel (based on MS Excel 2011) and licensed MedCalc (version 19.1.6) statistical analysis programs were used for taxonomic, functional analysis, and visualization of bacterial composition data obtained from microbiome sample study reports. Spearman's correlation coefficients r_s were calculated for comparing abundances of genera at two time points. Statistical significance of differences of Shannon's indices (α-diversity) across time points was assessed using Hutcheson's modified t-test with a significance level of $p < 0.05$. Statistical significance of differences between the athlete's characteristics and the control group (general population) was assessed using the single mean t-test with a significance level of $p < 0.05$. Relative abundance values of bacteria on genus and species level at different time points were compared using non-parametric Wilcoxon rank sum tests (also named the Mann–Whitney U-test or Mann–Whitney–Wilcoxon test) with a significance level of $p < 0.05$.

RESULTS

General Diversity, Phylogenic Composition, and Core Gut Microbiota of the Athlete

The Shannon index indicating the diversity of bacterial families present in the samples of the subject ranged from 1.67 to 2.11 (**Figure 2**). At the beginning and end of the training period, the Shannon indices were similar (2.11 and 2.08, respectively), concluding that the microbiome diversity did not change significantly at times of high competition (Hutcheson's modified t-test, p-value > 0.05). After the dietary fiber mix intake, the Shannon index dropped to 1.67 with a significant decrease in community diversity (Hutcheson's modified t-test, p-value < 0.05). The Shannon index of the control group was 1.63 (data not shown).

The evenness of the distribution of species in communities showed that at high exercise, microbiome uniformity indices were similar (J1 = 0.54 vs. J2 = 0.53), whereas, after the fiber intake, J showed a substantial drop (to J3 = 0.42). Although, also the evenness values of the microbiota were the lowest after the fiber mix diet (changed from 0.53 to 0.42), the statistical significance of the reduction could not be concluded. The uniformity index for the control group was $J = 0.41$. These data allowed us to conclude that at the family level, the athlete's microbiome at high training and competition loads (samples #1 and #2) were more diverse and more balanced (even) than after the dietary fiber consumption (sample #3). Intense exercise accompanied by a high intake of dietary fiber did not lead to the increased diversity of gut microbiota as was initially expected.

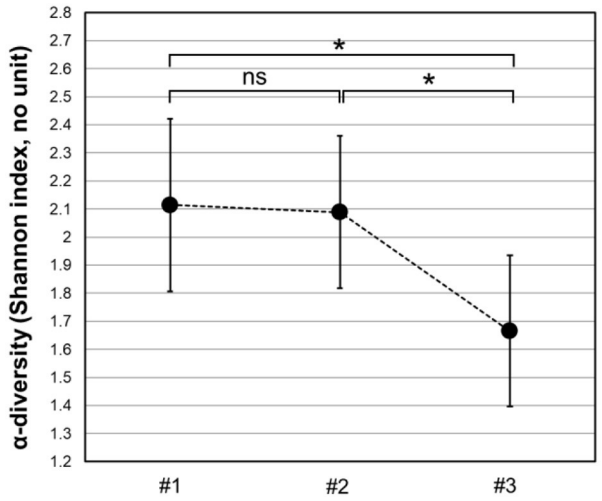

FIGURE 2 | Changes in the microbiome diversity over time and drastic effects on its relative diversity within bacterial families upon the dietary fiber consumption. The Shannon index expressed as a measure of the incidence and frequency of bacterial families in the test samples, ranged from 2.11 (#1) to 2.08 (#2), which was not a statistically significant difference (p-value > 0.05, Hutcheson's modified t-test, denoted *ns*—not significant). One month after consumption of the fiber mixture, the Shannon index dropped to 1.67, which was a significant decrease in the family diversity values (p-value < 0.05, Hutcheson's modified t-test, denoted by *). Error bars represent 95% CI for the calculated Shannon index. y-axis—Shannon index, without units. x-axis—sampling times. #1, time point week 1 (baseline); #2, time point week 27 (high training and competition period); #3, time point week 31 (after 30 days of dietary supplement intake).

As for the phylogenic composition, a total of nine phyla were present in all the samples with the dominance of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (**Figure 3**). The *Firmicutes* [67.8 ± 6.2 (mean abundance across three time points ± SD)] outranked *Bacteroidetes* (18.0 ± 1.8), *Actinobacteria* (8.8 ± 1.7), *Proteobacteria* (2.7 ± 1.6), *Verrucomicrobia* (1.6 ± 1.2), and *Cyanobacteria* (1.0 ± 1.4) phyla. Dietary fiber consumption had a positive effect on the abundance of *Firmicutes* (+20%), whereas, it showed drastic negative effects on *Verrucomicrobia* and *Cyanobacteria*, whose drop in abundance was almost 100%, and on *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* that declined by 75, 18, and 13%, respectively (**Figure 3B**). Analysis of *Firmicutes*/*Bacteroidetes* (*F*/*B*) ratio values showed relative stability during the study, where during the intense competition period, the reduction in *Firmicutes* [changing the *F*/*B* ratio by 17.9% (from 3.78 to 3.11)] was rescued upon fiber consumption by increases in abundance by 20% and resulting in the *F*/*B* ratio of 4.55 (**Figure 3C**). Compared with the control group, the *F*/*B* ratio of the athlete was significantly higher in all time points (t-test of one mean, p-value < 0.05, **Figure 3D**). Altogether, these results showed that dietary fiber along with high exercise loads affected the phylogenic composition by the microbiome of the athlete becoming relatively poorer at the phylum level. Overall, these results were in good agreement with data showing that training promoted relative increases in

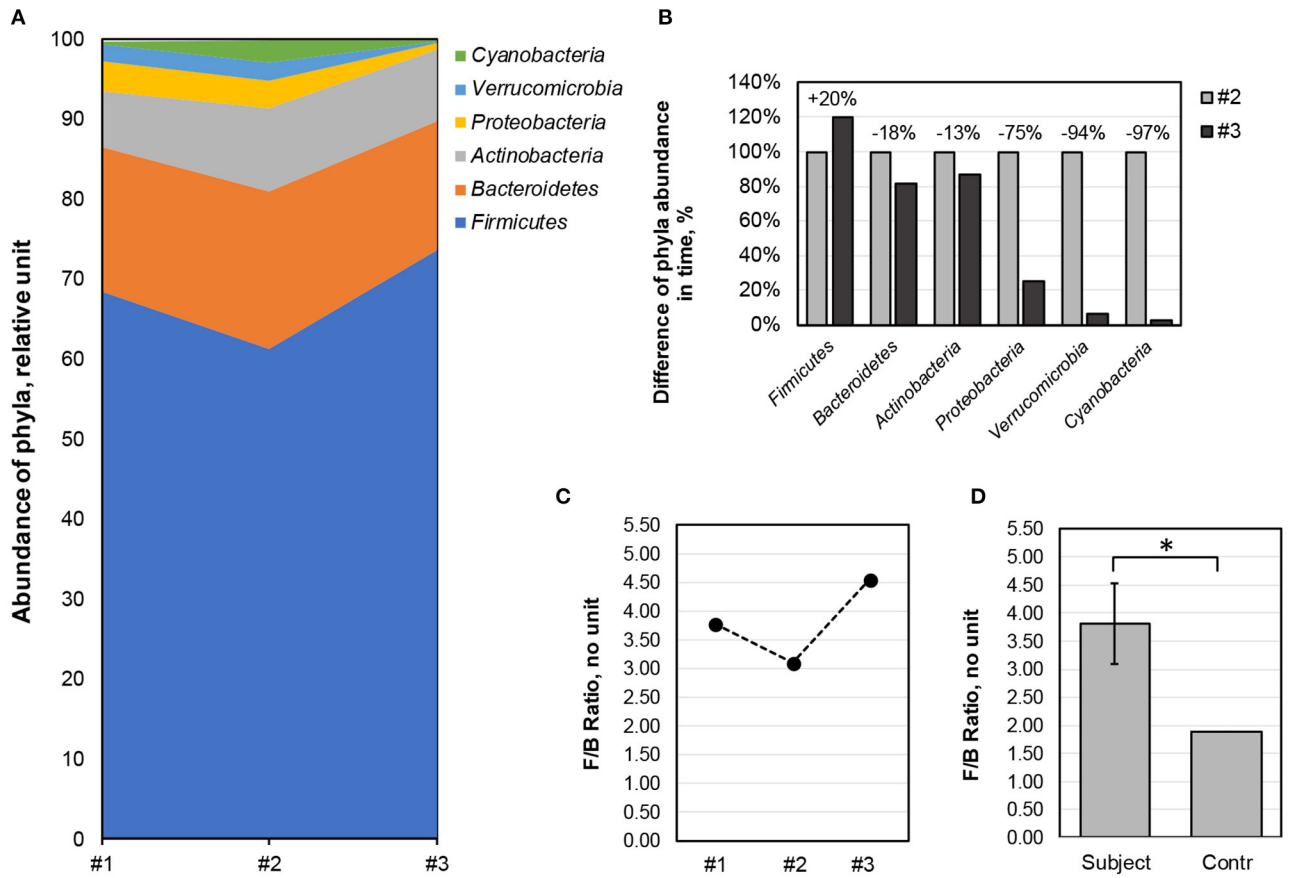

FIGURE 3 | Dynamic changes in bacterial phyla composition over the study period. **(A)** Changes in the abundance of the six most numerous phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Cyanobacteria*) in three serial samples (*x*-axis). *y*-axis—abundance of phyla, in relative units. **(B)** Proportional changes in the abundance of the six major phyla upon fiber consumption. The abundance of each phylum in sample #2 was equated to 100%, and the change in sample #3 relative to sample #2 is indicated above the columns. *y*-axis—relative abundance (%) of phylum in samples #2 and #3; *x*-axis—phyla. **(C)** Changes in *F/B* ratio over the 31-week study period, measured at three serial time points (*x*-axis). *y*-axis—*F/B* ratio. **(D)** The young athlete showed a high *F/B* ratio as compared with the control group (single mean *t*-test, *p*-value < 0.05, denoted by *). The bar graph represents the arithmetic mean of the *F/B* ratio in study samples and the error bars represent the standard deviation. *y*-axis—*F/B* ratio. #1, time point week 1 (baseline); #2, time point week 27 (high training and competition period); #3, time point week 31 (after 30 days of dietary supplement intake).

Firmicutes (30, 31) and that *F/B* ratio correlated significantly with cardiorespiratory fitness (32).

Opposite Dynamics of Butyrate-Producing Bacteria in Periods of Competition and Upon Dietary Fiber Intake

The relative abundance of 77 genera, of which 29 were shared across all samples, was significantly different between samples (*p*-values < 0.05, Wilcoxon rank sum test, **Figure 4**). At the genus level, *Prevotella* [11.7 $\pm$ 2.1 (mean abundance across three time points $\pm$ SD)], *Faecalibacter* (5.1 $\pm$ 1.8), *Blautia* (5.4 $\pm$ 1.2), *Ruminococcus* (3.8 $\pm$ 2.8), and *Bifidobacterium* (5.0 $\pm$ 3.8) were the most abundant genera (**Figure 4**). The predominance of *Prevotella* compared with the families of *Bacteroides* and *Ruminococcus* indicated that the subject had *Prevotella*-predominant enterotype, e.g., enterotype II (**Figure 4A**). *Prevotella*'s abundance was associated with long-term fiber intake (33).Similar trends were noted also in the current study whereupon dietary fiber intake resulted in enhanced abundance of *Prevotella* that became 41.7% more abundant as compared with the previous time point (**Figure 4A**). It is known that extreme dietary changes can lead to wide-ranging shifts in the gut bacterial community (34). Herein, the relative abundance of the acetate-producing bacteria (e.g., *Blautia*, *Bifidobacterium*, *Sutterella* groups) and the lactate-producing bacteria (e.g., *Bifidobacterium*, *Streptococcus*, *Lactococcus* groups) increased during high training and competition period (sample #1 vs. #2), but showed decreasing patterns (except *Blautia*) by the end of the dietary supplement intake period when acetate- and lactate-consuming and butyrate-producing genera including *Faecalibacterium* increased significantly (sample #2 vs. #3, *p*-value < 0.05, Wilcoxon rank sum test, **Figures 4B,C**). Interestingly, the propionate-producing genera (35) showed differential patterns.

Bacteroides and *Acidaminococcus* showed increasing trends in contrast to *Phascolarctobacterium* and *Veillonella* that decreased upon competition, with only the levels of *Veillonella* slightly rescued upon fiber consumption (**Figures 4B,C**).

The abundance of the shared largest 29 genera was better correlated in samples #1 and #2 (Spearman's correlation coefficient $r_s = 0.87$) as compared with that in samples #2 and #3 (rs = 0.76, **Figure 4D**). However, the strongest correlation in abundance of these major genera was observed between baseline and endpoint (samples #1 and #3, $r_s = 0.92$). This result was somewhat surprising. Similar to other studies (36), we also noted strong patterns of individuality of the response to exercise and diet, with a possible explanation that these activities supported the original (primary, "keystone") bacterial community dynamics of the subject.

Taken together, high exercise along with dietary fiber intake resulted in dynamic shifts in genera composition especially in the balance of lactate- and acetate/butyrate-producing bacteria.

Selective Effects of the Dietary Fiber Supplement on Individual Species of the Gut Microbiota

Next, we compared the mean relative abundance of 32 individual species to identify those that were the most affected by the dietary switch. Among the studied species, the six most abundant bacterial species (*Prevotella copri*, *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, *Bifidobacterium adolescentis*, *Coprococcus eutactus*, *Collinsella aerofaciens*) accounted for 92.5% on average of the abundance of the top species. For most of these analyzed species, a notable variation was associated both with the intense exercise loads and the dietary fiber consumption (**Figure 5**). Interestingly, *A. muciniphila* (*Verrucomicrobia*) that produces both propionate and acetate (37, 38) showed decreased proportions upon fiber consumption and was replaced by the abundance of the butyrate producer *C. aerofaciens* (**Figure 5A**). Another major shift upon fiber consumption was noted in the abundance of *C. eutactus* with known beneficial effects on butyrate production (39). However, the abundance of 12 species attributed with a protective role on the intestinal mucosa reduced significantly after dietary fiber intake (p-value < 0.05, Wilcoxon rank sum test), from on average 50.4 to 33.5% of abundance of the detected species (**Figure 5B**). Herein, two of the five species which were among the most significantly affected were the *Bacteroidetes* spp. Also, the species of *Akkermansia*, *Bacteroides*, *Bifidobacterium*, and *Ruminococcus* with protective functions on the intestinal mucosa showed a significant decrease upon fiber intake, whereas, *F. prausnitzii*, one of the major manufacturers of butyrate, showed increased abundance upon dietary fiber (**Figure 5B**). *Veillonella dispar* species were specifically monitored because of their potential impact on performance enhancement as a lactic acid-utilizing community (6). Although, changes in *V. dispar* abundance during periods of high exercise load and dietary fiber intake were detected, the statistical significance of these findings could not be determined (**Figure 5C**). It is noteworthy that the number of *V. dispar* in the microbiome of the young athlete was significantly higher than of the control group (**Figures 5C,D**, single mean t-test, p-value < 0.05). The observed changes in the abundance of bacteria producing SCFAs associated with energy consumption of the skeletal muscle (40) supported the initial work hypothesis that dietary fiber intake could facilitate athletic endurance by favorable shifts in microbial composition.

DISCUSSION

This work investigated the effects of dietary fiber supplement on the microbiome of the young rower at high-intensity exercising loads. Several consistent patterns in the gut microbiota were observed. First, the shifts induced by high exercise and dietary fibers were restricted to a limited number of phyla and genera, but were remarkable at the species level contributing to energy production. Second, the magnitude of change in microbial alpha diversity upon fiber consumption was drastic, constituting a 20.3% drop in diversity, by substantially enhancing the *Firmicutes*/*Bacteroidetes* ratio. Third, as suggested previously (41, 42) but now confirmed by this longitudinal case study, microbial response to dietary fiber consumption included the keystone species of the individual.

Our data showed that fiber consumption at high exercise loads led to decreased alpha diversity of the gut microbiota (**Figure 2**). Recent findings suggest a dynamic positive relationship between gut microbiota diversity and physical activity as professional athletes exhibit more diverse composition [ref (43)]. Paradoxically, in our study, there was a significant drop in alpha diversity upon dietary fiber consumption, most conceivably due to the rise in select advantageous bacterial species, such as those involved in butyrate production (**Figure 5**). Overall, these data support the view (44) that complex fibers of the dietary mix are highly selective for specific bacteria. It has become clear that animals can get by and often have high fitness with low-diversity microbiota (45, 46). Also, high gut microbial diversity has been linked to longer colonic transit time and systemic circulation of potentially harmful protein degradation products (47). Therefore, interpreting the health of the athlete's gut based on alpha diversity values of the microbiome implies a personal approach.

We observed a strong association between exercise loads, fiber intake, and *F*/*B* ratio (**Figure 3**), in good agreement with previous findings (48, 49). The proportional composition of the phyla clearly differentiated the subject from the matched control cohort with the abundance of *Firmicutes* and *Prevotella*. The *Prevotella* enterotype is supported by the diet of the subject in good support with earlier findings (50, 51). The dietary fiber intake has a limited influence on the communal stability of the latter two as compared with the baseline (**Figure 3**). However, dietary fiber intake resulted in enhanced abundance of *Prevotella* and *Roseburia* that became 41.7 and 4.2% more abundant, respectively, as compared with the time point of high competition (**Figure 4**). A recent *in vitro* study elucidating the mechanism of action of select dietary fibers on gut microbiota found that beta-glucan from oats induced the growth of *Prevotella* and *Roseburia* with a concomitant increase in SCFA propionate production (52).

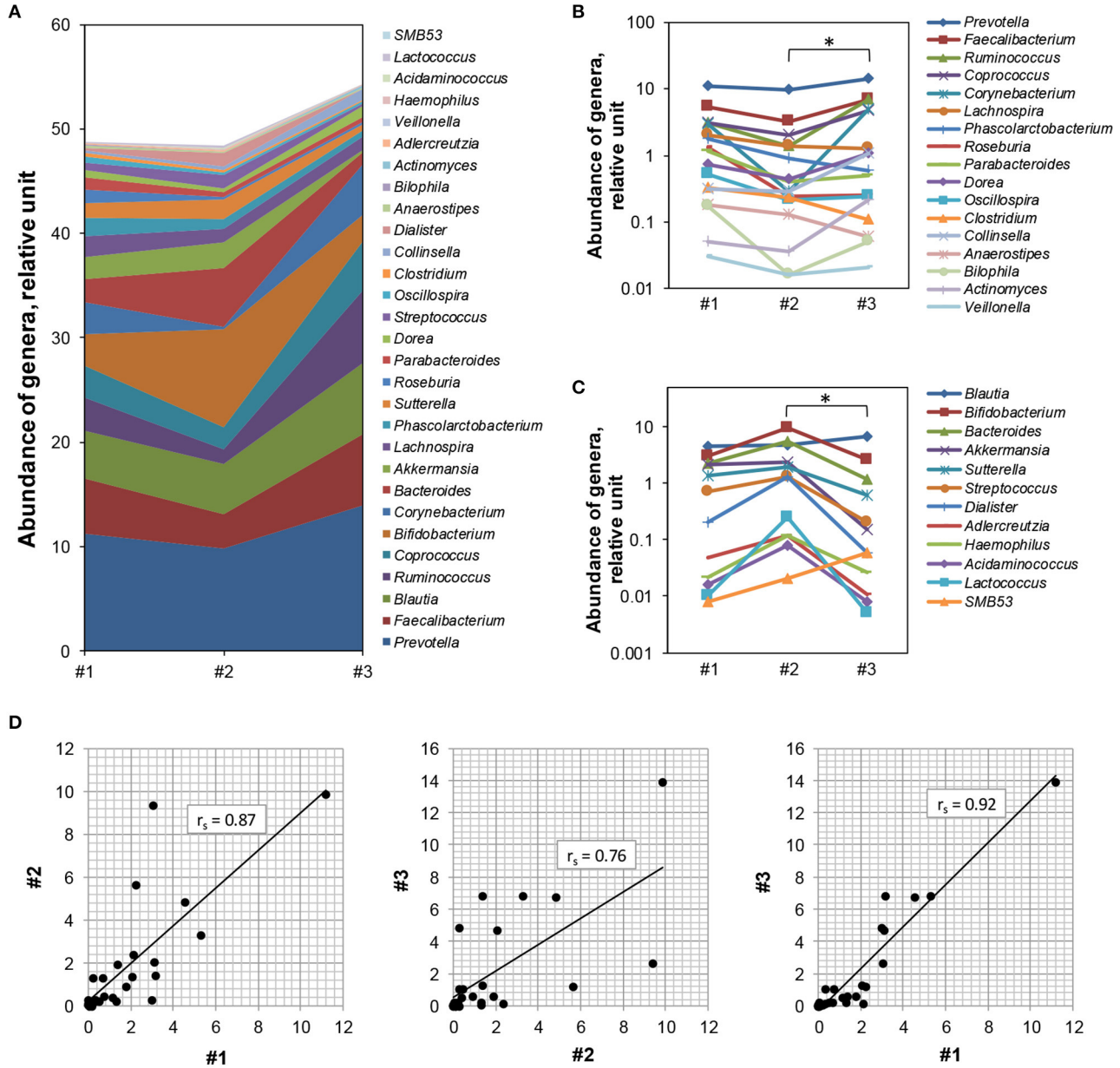

FIGURE 4 | Dynamic changes in bacterial genera composition during the study period. **(A)** The bacterial genera ($n = 29$) detected in all three samples at different time points (*x*-axis) are presented. *y*-axis—abundance of genera, in relative units. **(B,C)** Those species ($n = 17$) predominantly producing acetate had higher abundance in sample #2 compared with sample #1 but were significantly lower in sample #3 (Wilcoxon rank sum test, *p*-value < 0.05, denoted by *) **(B)**, whereas, butyrate-producing species were higher in sample #3 and low in samples #1 and #2 (Wilcoxon rank sum test, *p*-value < 0.05, denoted by *) **(C)**. *x*-axis—serial time points; *y*-axis—abundance of genera, in relative units. The number of bacterial genera is plotted in logarithmic scale. **(D)** Correlations between the differential abundance of large bacterial genera ($n = 29$) in the microbiome composition. The difference was high before vs. after the intake of fiber [sample #2 vs. #3, middle graph, Spearman's correlation coefficient $r_s = 0.76$ ($p < 0.0001$)], as compared with that at the beginning and the end of the intense training period [sample #1 vs. #2, left graph, Spearman's correlation coefficient $r_s = 0.87$ ($p < 0.0001$)], and also to that at the beginning and the end of the study [samples #1 and #3, Spearman's correlation coefficient $r_s = 0.92$ ($p < 0.0001$)]. *x*- and *y*-axes—abundance of genera, in relative units. #1, time point week 1 (baseline); #2, time point week 27 (high training and competition period); #3, time point week 31 (after 30 days of dietary supplement intake).

This study also showed that non-digestible sugars like inulin and oligosaccharides increase SCFA levels (52). Thus, our data allow concluding that non-digestible carbohydrates of the dietary fiber supplement promoted the growth of beneficial microorganisms for the performance of the athlete. In addition, there was less *Actinobacteria* (**Figure 3**), in harmony with previous studies (53). Interestingly, *P*/*A* was found to be <1 for all samples (0.5, 0.3, and 0.1), suggesting that decreased abundance of the *Proteobacteria*

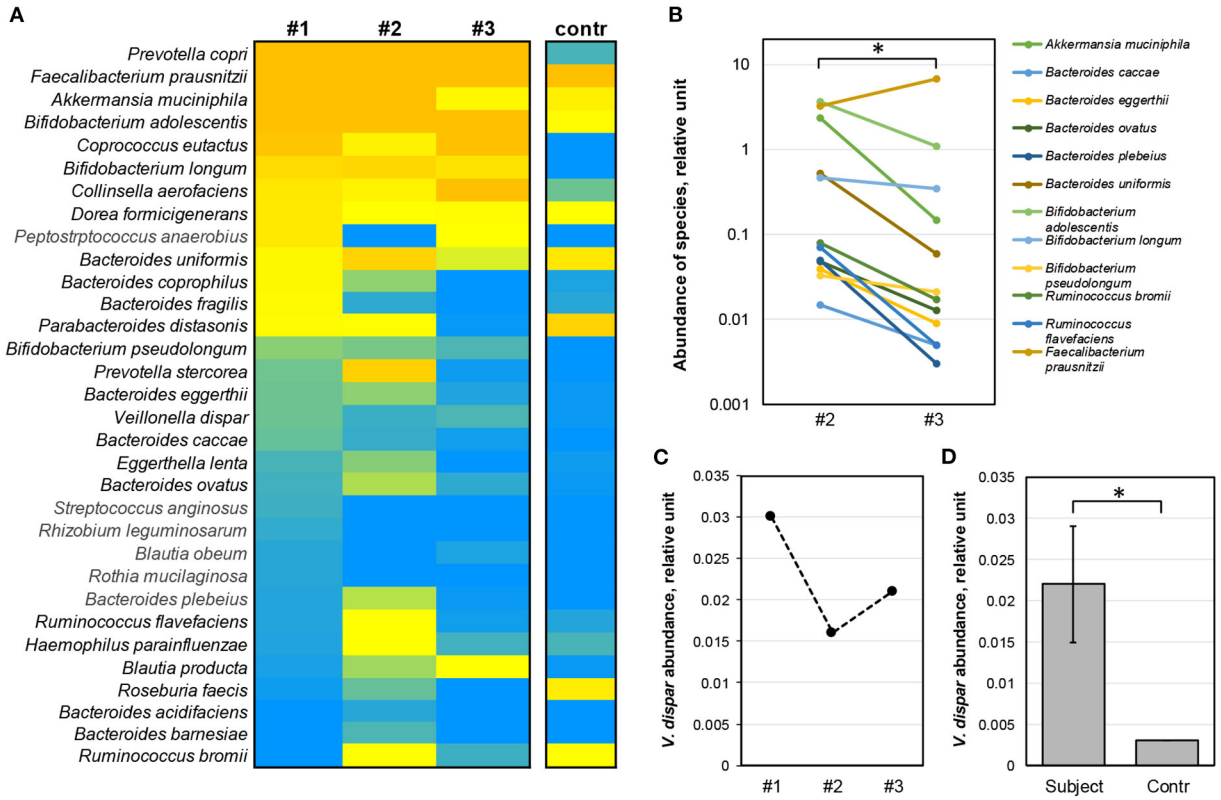

FIGURE 5 | Changes in composition of the 32 most abundant bacterial species during the study period. **(A)** Dynamic changes in the abundance of observed species in different samples. Relative abundance of specific species (*rows*) in samples (*columns*) are shown in color scale with orange depicting abundance values >1 RUs, yellow >0.07, green >0.03, and blue = 0. **(B)** Abundance of species with a protective role on the intestinal mucosa ($n = 12$) showed a significant reduction after monthly fiber intake (sample #2 vs. sample #3, Wilcoxon rank sum test, p-value < 0.05, denoted by *); y-axis—number of bacterial species, in relative units. **(C)** Differential changes in the level of abundance of *Veillonella dispar* (in relative units) before the competition season (#1), after the end of the intensive competition period (#2) and after the fiber intake (#3). x-axis—serial time points; y-axis—abundance, in RU. **(D)** High abundance of *V. dispar* in the microbiome under study (*Subject*) was observed as compared with the control group (p-value < 0.05, single t-test, denoted by *). Bar graph expressed as arithmetic mean of abundance of *V. dispar* in samples and error bars depicted by standard deviation. y-axis—abundance of *V. dispar*, in RU. #1, time point week 1 (baseline); #2, time point week 27 (high training and competition period); #3, time point week 31 (after 30 days of dietary supplement intake).

was linked to high exercise loads [see also (54)]. Of note, *Proteobacteria* are a major group behind the gut's metagenome functional variability (55).

Furthermore, we observed opposite dynamics of lactate- and acetate/butyrate-producing bacteria in periods of competition and upon dietary fiber intake, supporting the mechanism where during exercise the gut supplies lactate (56) and acetate (57) as fuel energy source (58). Though, generally fiber tends to increase SCFA-producing bacteria such as *Bacteroidetes* and *Actinobacteria* and decrease *Firmicutes* (59) as also observed in our study, whether it is a direct cause or because of changes in training and competition routine (outdoor vs. indoors rowing) along with dietary fiber intake that might have changed the metabolism needs further evaluation.

Our data show that high-endurance exercise and a prebiotic fiber-supplemented diet resulted in significant shifts across the key genera. We found that seven genera, namely *Prevotella*, *Parabacteroides* (Bacteroidetes), *Faecalibacterium*, *Ruminococcus*, *Coprococcus*, *Lachnospira* (Firmicutes), and *Corynebacterium* (Actinobacteria), were reduced upon high exercise loads, but the levels of these were restored upon dietary fiber consumption. These findings suggested that these seven genera affected primarily the levels of acetate and propionate available to the host. Both of these SCFAs are the known substrates for energy production, as well as in skeletal muscle (60, 61). In contrast, six genera, namely *Streptococcus* and *Dialister* (Firmicutes), *Bacteroides* (*Bacteroidetes*), *Bifidobacterium* (*Actinobacteria*), *Akkermansia* (*Verrucomicrobia*), and *Sutterella* (*Proteobacteria*), were specifically stimulated at high exercise loads, but inhibited by dietary fiber intake. These findings suggested higher butyrate production upon dietary fiber consumption with also potentially ameliorative effects on gut mucosal inflammation and oxidative status (62, 63). Elite athletes' dietary plans are based on the consumption of certain micronutrients, but the health of the gut microbiota is rarely considered (27). Here, we show that despite

the individual features of the microbiota composition of the athlete, exercise-driven prevalence of acetate- and propionate-producing species was flexibly switched to butyrate producers after dietary fiber intake.

Baseline bacterial composition has repeatedly been observed to be a key factor of changes in the gut microbiota following dietary interventions (17, 36). What was noticeable upon dietary fiber consumption by the athlete was the increase in taxa, such as *F. prausnitzii*, with known beneficial effects on muscle function (15). However, in contrast to the published studies, we found that while having a positive effect on bacterial families associated with athletic excellence, fiber intake had detrimental contracting effects on the overall microbial community. This was surprising although in concordance with the notions that the host might lack keystone species (64) or lack strains to utilize specific dietary fiber (65). It is expected that the microbiome reverts to its original state after short-term dietary interventions (33, 34), although, positive impacts on the gut microbiota could be maintained for at least a year (12).

Strengths

The major strength of the study was that it was conducted in "real-life" scenario as the temporal dynamics of the athlete's microbiota was explored by combining high-endurance exercise specifically with the athlete-designed dietary fiber supplement. The benefits of this study may lead to new insights into the cumulative effects a particular physiological interference has on the gut microbiome, that is on the role of the host's enterotype (with defined keystone species) has on the covariation of microbial communities upon dietary shifts and at high exercise loads. Finally, this is an individual athlete's study, and as such, it does not allow to draw solid and supportive conclusions. The value of the study lies in the aspect that overall variability in the physiological response of athletes to training and nutrition has not yet been adequately explored.

Limitations

Firstly, it was not possible to fully control dietary intake, although, the participant was instructed to maintain normal habits. Secondly, the study did not examine the causal relationship between exercise performance and the gut microbiota of the athlete, although, a high number of intestinal bacteria of the *Veillonella* family that would be of advantage to the athlete were observed. Also, recording of metrics inflammation and immunosuppression could have helped to examine the microbial gut stress levels of the athlete. Finally, given that microbiome sequencing had a limited capacity to resolve taxa to the species level, therefore, the study focused on the proportionally largest reservoir of multiple species' diversity and functionality.

CONCLUSIONS

Testing the microbiome of young athletes is necessary to obtain information on the dynamics and composition of this activity during the training process and at competition. At high levels of endurance exercise, athletes may have serious problems getting the amount of energy they need, so taking supplements to increase or to recover gut microbiota diversity at times of physical exertion is highly recommended. Our observations suggest that the dietary fiber-supplemented diet produces pronounced changes in the gut microbiota of the subject with high fractions of *Bacteriodetes* (*Prevotella*). This fact solely could be used to stratify athletes by their baseline gut bacterial composition before assigning such a fiber-supplemented diet. We evidenced that high dietary fiber intake at high exercise loads might produce profound changes beneficial to human health. Establishing the causal role of the GI microbiota and the underlying mechanisms would remain essential for the development of improved next-generation personal nutritional strategies. Only this type of in-depth understanding will allow for the selection of dietary fibers (or) mixtures thereof, to systematically target specific features of the GI microbiome (i.e., specific taxa, diversity, metabolites) with the goal of alleviating the immunometabolic features (frequently dysbiotic) that are characteristic of athletes' gut.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MJ, UT, TT, and KP collected the samples, carried out the analysis, wrote the paper, and reviewed and accepted the final version of the manuscript. KP (corresponding) is responsible for the integrity of the work as a whole. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was supported by funding grants of Protobios (5.1-4/20/170 and PRG573) from the Estonian Ministry of Education and Estonian Research Council, respectively, and H2020 PANBioRA (EU760921) projects from the European Union.

ACKNOWLEDGMENTS

We would like to thank the athlete's coach and the team of Tallinna Sõudeklubi (www.soudeklubi.ee) for their contributions to the athlete's performance. The authors gratefully acknowledge Helle Sadam, Arno Pihlak, Nadezda Pupina, and Annika Rähni of Protobios for their kind support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 16 July 2021
doi: 10.3389/fphys.2021.696018

Importance of Nutrient Availability and Metabolism for Skeletal Muscle Regeneration

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Skeletal muscle is fundamentally important for quality of life. Deterioration of skeletal muscle, such as that observed with advancing age, chronic disease, and dystrophies, is associated with metabolic and functional decline. Muscle stem/progenitor cells promote the maintenance of skeletal muscle composition (balance of muscle mass, fat, and fibrotic tissues) and are essential for the regenerative response to skeletal muscle damage. It is increasing recognized that nutrient and metabolic determinants of stem/progenitor cell function exist and are potential therapeutic targets to improve regenerative outcomes and muscle health. This review will focus on current understanding as well as key gaps in knowledge and challenges around identifying and understanding nutrient and metabolic determinants of skeletal muscle regeneration.

Keywords: skeletal muscle, regeneration, tissue recovery, metabolism, nutrients

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *16 April 2021*
Accepted: *17 June 2021*
Published: *16 July 2021*

Citation:
Blum J, Epstein R, Watts S and Thalacker-Mercer A (2021) Importance of Nutrient Availability and Metabolism for Skeletal Muscle Regeneration. Front. Physiol. 12:696018. doi: 10.3389/fphys.2021.696018

INTRODUCTION

Maintenance of skeletal muscle mass and function are essential for quality of life. Skeletal muscle is the largest organ system in the body, accounting for 30–40% of whole body mass of a healthy adult (Janssen et al., 1985). Each skeletal muscle is composed of an intricately connected network of nerves, blood vessels, and bundles of myofibers that contain myofibrils with the contractile units (sarcomeres), all covered and supported by layers of connective tissue. The contractile property of skeletal muscle allows for voluntary contractions in the body that facilitate physical locomotion, posture maintenance, breathing, urinary bladder control, mastication, swallowing, and blinking. Additionally, muscle is a major metabolic organ that sustains body temperature and plays a central role in whole body nutrient homeostasis.

Skeletal muscle repair and regeneration of damaged tissue following trauma is essential to regain tissue homeostasis. Impairments in muscle regeneration are associated with pathological tissue remodeling, which includes the gain of fat and fibrotic tissues—adverse changes that impact the organ and organismal function and metabolism. Unfortunately, existing therapies to improve regenerative outcomes are limited, and the demographic of individuals at risk for impaired muscle regeneration and poor muscle health is projected to increase in the future (**Table 1**). In 2016, 15% of the population was over the age of 65, by 2030 it is estimated that this will rise to 21% (Ortman et al., 2014; Medina, 2018). The current prevalence of obesity is 42% and the prevalence of overweight and obesity is 71.6% (Hales et al., 2020). According to one report that used multiple prediction models,

the prevalence of obesity in 2030 is projected to range from 42 to 51% (Finkelstein et al., 2012). Further, an estimated 85% of adults will have overweight or obesity by 2030 (Wang et al., 2008). According to the National Diabetes Statistics Report, 13% of the U.S. population has diabetes (Centers for Disease Control and Prevention, 2020). According to this report, 90–95% of reported cases were type 2 diabetes. From 2015 to 2030, the proportion of adults with type 2 diabetes is projected to increase by 54% (Krohe et al., 2016). The current prevalence of chronic kidney disease is 13.2% and is projected to increase to 16.7% by 2030 (Hoerger et al., 2015).

Coordination of the regenerative process is multifaceted, and it is increasingly recognized that nutrient availability and cell metabolism are important determinants of muscle stem and progenitor cell function and ultimately tissue regeneration. The primary focus of this review is to discuss current knowledge in nutrient and metabolic determinants of skeletal muscle regeneration. To provide context for understanding nutrient and metabolic determinants of muscle regeneration and their importance, we will provide an overview of the regenerative process and the requirement for muscle regeneration. Further, we will briefly discuss impairments in muscle regeneration, which set the stage for the identification of therapies to improve regenerative outcomes. Finally, this review will shed light on key gaps in knowledge that limit the use of nutrient and metabolic targets as therapy to improve regenerative outcomes.

OVERVIEW OF SKELETAL MUSCLE REGENERATION

Skeletal muscle regeneration is an obligatory process to repair damaged tissue in response to contusion, laceration, burn, and mechanical overload, enabling muscle to return to a homeostatic state. Regeneration is dependent on a well-orchestrated myogenic program that includes the activation of muscle-specific stem cells (MuSCs) and expansion of MuSCs and their committed progeny, the muscle progenitor cells (MPCs) (**Figure 1A**). MuSCs and MPCs will collectively be referred to as MPCs throughout, except where cell specificity is necessary. Following population expansion, MPCs undergo terminal differentiation, fuse to each other or into damaged myofibers, and undergo maturation. MuSCs generally reside in a quiescent state until activated by trauma-associated cues. Quiescent and proliferating cells are characterized by their expression of the transcription factor paired box 7 (Pax7) (Seale et al., 2000). Pax7 expression declines during terminal differentiation (Zammit, 2006). Myoblast determination protein 1 (MyoD) is an early regulator of MuSC commitment to the muscle lineage (Megeney et al., 1996). Myogenin (MyoG) is a transcription factor expressed in differentiating MPCs (Wright et al., 1989). Later stages of differentiation are characterized by the expression of embryonic myosin heavy chain (eMHC), which persists during MPC fusion, and is eventually replaced by adult myosin isoforms (Schiaffino et al., 2015).

Studies in rodents identified that MPC depletion severely impairs the muscle regeneration process, following an acute injury (Sambasivan et al., 2011; Fry et al., 2015). Additionally, MPCs are essential to prevent tissue fibrosis. Following synergist ablation surgery in mice, MPC depletion caused an increase in extracellular matrix deposition and expansion of the fibroblast cell population (Fry et al., 2014). Follow-up studies revealed that MPCs secrete exosomes containing microRNAs (specifically miR206), that can attenuate collagen biosynthesis and secretion from nearby fibroblasts (Fry et al., 2017). Thus, in response to an acute injury, robust MPC activation is required to restore muscle mass and prevent fibrosis.

The myogenic/regenerative process can be mirrored *in vitro*. For example, freshly isolated MPCs are used as model of activation. Growth media is used to promote the proliferation of MPCs. Serum withdraw (i.e., differentiation media) or allowing proliferating MPCs to become confluent, initiates differentiation of MPCs. Duration of MPC differentiation is associated with myotube formation. For example, the first day in differentiation media little to no fusion of cells is observed. However, with extended duration in differentiation media, there is an increase in the number of fused cells representing myotube formation. *In vitro* systems have been used extensively for examining nutrient and metabolic determinants of myogenesis, as detailed in the following sections.

IMPAIRED MUSCLE REGENERATION AND EXISTING THERAPIES

Impaired skeletal muscle regeneration is commonly observed with advancing age, and in individuals with metabolic conditions including obesity, type two diabetes, and chronic kidney disease (Chakravarthy et al., 2008; Day et al., 2010; Hu et al., 2010; Chakkalakal et al., 2012; D'Souza et al., 2013; Sousa-Victor et al., 2014; Blau et al., 2015; Avin et al., 2016; Fu et al., 2016; Liu et al., 2018; O'Sullivan et al., 2018; Teng and Huang, 2019). This impaired capacity for muscle regeneration leads to pathological tissue remodeling. Specifically, older animals or animals with obesity and insulin resistance show increased fibrotic tissue deposition and intramuscular fat deposition after injury (Brack et al., 2007; Hu et al., 2010; Lee et al., 2013). Adipose and fibrotic tissue do not perform the same essential functions as skeletal muscle (i.e., locomotion, nutrient homeostasis) and replacement of skeletal muscle with adipose or fibrotic tissue is associated with functional impairment and metabolic disease.

Impaired muscle regeneration, as observed in aging and chronic disease, is driven by a combination of changes in intracellular factors and cell-extrinsic factors. Key intracellular factors shown to be altered in conditions of impaired muscle regeneration include oxidative stress, inflammatory signaling, signal transduction, and altered metabolism (Aragno et al., 2004; Fulle et al., 2005; Price et al., 2014; Avin et al., 2016; Zhang et al., 2016; Liu et al., 2018; Pala et al., 2018). Cell-extrinsic factors include those localized to the MuSC niche (Gopinath and Rando, 2008) or circulating factors that derive from the diet or other tissues and act on MuSCs/MPCs through the bloodstream (Conboy et al., 2005), which will be discussed in the following sections.

TABLE 1 | The prevalence of conditions known to impact muscle regeneration is going to increase.

	Recent estimate of prevalence (%)	Projected prevalence for 2030 (%)
Advanced age (>65 years) (Medina, 2018)	15	21
Obesity (Finkelstein et al., 2012; Hales et al., 2020)	42	42–51
Type 2 diabetes (Krohe et al., 2016; Centers for Disease Control and Prevention, 2020)	13	15.3
Chronic kidney disease (Hoerger et al., 2015)	13.2	16.7

While many therapies for improving muscle function after injury, in individuals with impairment have been proposed, as reviewed elsewhere (Baoge et al., 2012; Judson and Rossi, 2020), the overall range of options is rudimentary and there are no clinically available options. Clinical trials are planned or are already completed to test the role of cell based (MPCs and other muscle-related/resident cells) therapies to augment regeneration following a rotator cuff injury, improve muscle outcomes after hip replacement, and counteract fecal and urinary incontinence (Qazi et al., 2019). However, despite some promising results, these therapies are not yet clinically available. Identifying novel regulators of MPC function may provide new strategies to augment proposed therapies.

OVERVIEW OF CENTRAL METABOLISM

All cells, including muscle stem cells, require conversion of nutrients to energy and biosynthetic intermediates to support maintenance and cell division. Glycolysis, the breakdown of glucose into pyruvate, and the TCA cycle, a coordinated set of enzymatic reactions that convert acetyl-coA to carbon dioxide, are key energy generating pathways within cells (**Figure 2**). Acetyl-coA can be derived from pyruvate, lactate, acetate, or catabolism of fatty acids and certain amino acids (leucine, lysine, phenylalanine, tryptophan, and tyrosine). Additionally, glutamine, and other amino acids (asparagine, aspartate, valine, methionine, threonine, proline, arginine, histidine) feed the TCA

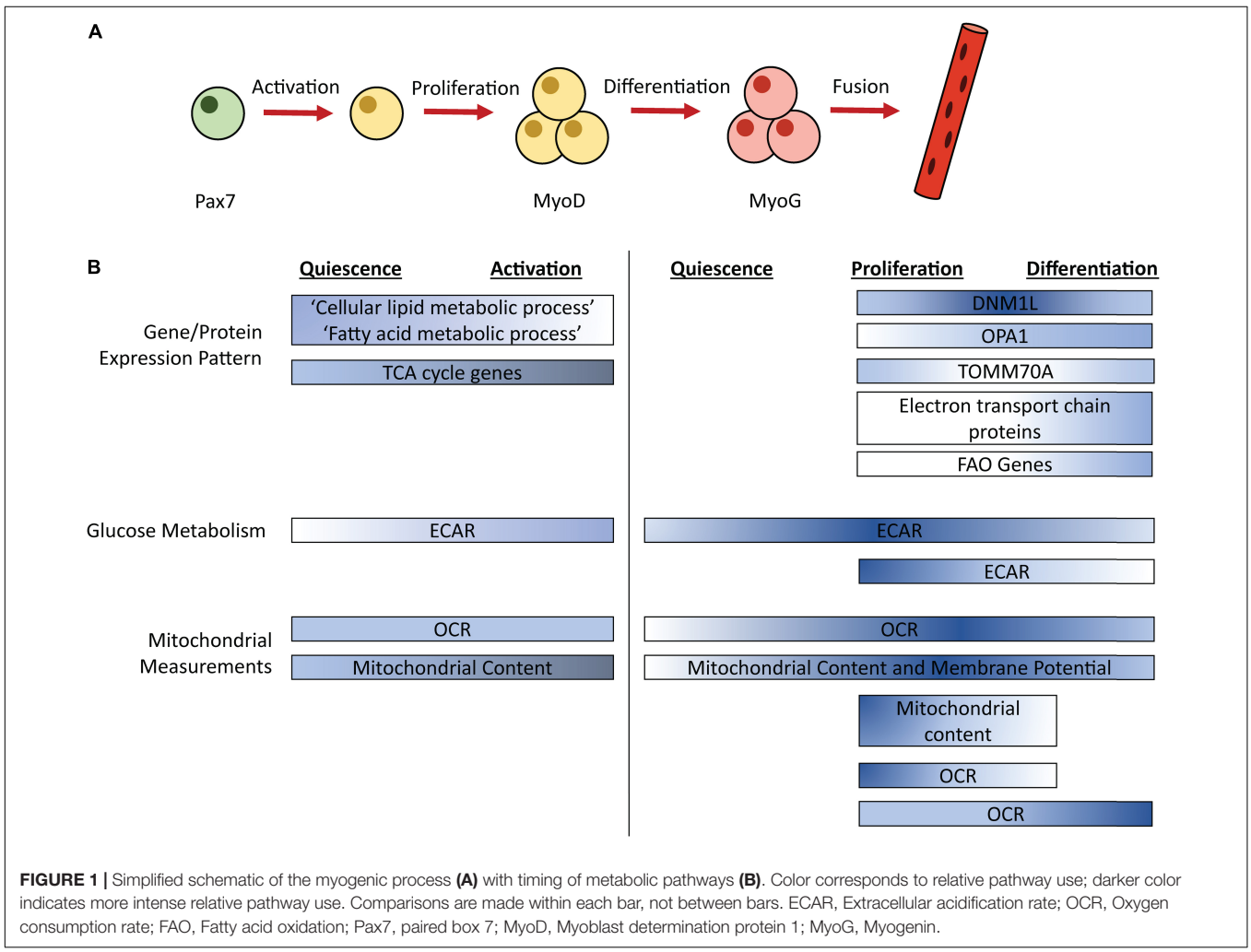

FIGURE 1 | Simplified schematic of the myogenic process **(A)** with timing of metabolic pathways **(B)**. Color corresponds to relative pathway use; darker color indicates more intense relative pathway use. Comparisons are made within each bar, not between bars. ECAR, Extracellular acidification rate; OCR, Oxygen consumption rate; FAO, Fatty acid oxidation; Pax7, paired box 7; MyoD, Myoblast determination protein 1; MyoG, Myogenin.

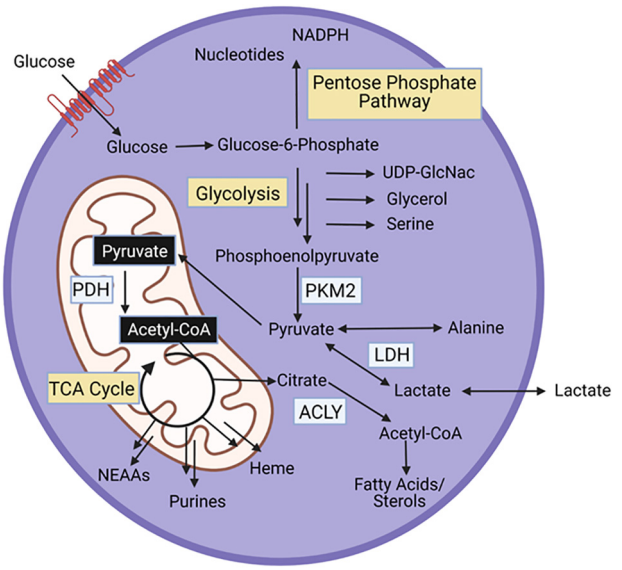

FIGURE 2 | Energy generating pathways and key protein in MPCs. PKM2, Pyruvate kinase M2; PDH, pyruvate dehydrogenase; ACLY, ATP Citrate Lyase.

cycle at entry points other than acetyl-coA. Both glycolysis and the TCA cycle generate NADH. The electron transport chain in the mitochondria, uses the energy gained from oxidizing NADH to NAD+ to power the generation of ATP. In the cytosol, lactate dehydrogenase uses NADH to reduce pyruvate to lactate, generating NAD+, which enables further glycolysis.

In addition to energy generation, glycolysis and the TCA cycle support synthesis of biosynthetic intermediates (**Figure 2**). Glucose-6-phosphate, the first intermediate of glycolysis is a substrate for the pentose phosphate pathway, a series of reactions that produce nucleotides, and NADPH, an essential cofactor for fatty acid synthesis and oxidative stress management. Fructose-6-phosphate, the next glycolytic intermediate is a precursor for the hexosamine pathway, which generates UDP-GlcNAc, a substrate that glycosylates proteins. Glycolytic intermediates provide a glycerol backbone for triacylglyceride synthesis, and also feed the serine biosynthetic pathway, which generates serine and glycine. Pyruvate, the final glycolytic intermediate can be transaminated to alanine. Intermediates of the TCA cycle are substrates for synthesis of fatty acids and sterols, nutritionally, non-essential amino acids (NEAAs), purines, and heme. Importantly, as intermediates in the TCA cycle are used for biosynthetic pathways, these intermediates can be replenished through anaplerotic reactions.

In the following sections we will review changes in relative occurrence of glycolytic and oxidative metabolism during myogenesis. Important to note, while these processes can be compared across the myogenic stages, the glycolytic and oxidative pathways are not mutually exclusive. Next, we will review knowledge about essentiality and function of glycolytic and oxidative metabolism, which will be combined since they are inherently linked. Following, we will briefly discuss alteration in metabolism in MPCs from populations with impaired regeneration, and then review metabolic proteins that contribute to coordination of cellular metabolism. Lastly, we will discuss historical and recent advances in understanding the role of circulating nutrients in MPC function.

TIMING OF GLYCOLYSIS

Studies have examined changes in energy generating pathways that occur throughout the myogenic process (**Figure 1B**). Extracellular acidification rate (ECAR), a proxy measurement for glycolysis, is increased in freshly isolated MPCs (a model of activation) and proliferating MPCs compared to quiescent MPCs (Ryall et al., 2015; Pala et al., 2018). In proliferating human MPCs, mRNA levels of the glucose transporters GLUT1 and GLUT4 did not differ between an early and later timepoint of proliferation (Riddle et al., 2018). ECAR returns to levels observed in quiescent MPCs during MPC differentiation, in primary mouse MPCs isolated from injured mice (Pala et al., 2018). Similarly, in cultured primary mouse MPCs, ECAR levels are lower in differentiating, compared to proliferating, MPCs (Yucel et al., 2019). Thus, maximum ECAR occurs during MPC proliferation.

TIMING OF MITOCHONDRIAL METABOLISM

Similar to glycolytic metabolism, oxidative metabolism is dynamic during myogenesis. Quiescent MPCs show enrichment of expression of genes in the "cellular lipid metabolic process" and "fatty acid metabolic process" compared to activated MPCs, suggesting that lipid catabolism may be particularly important for quiescent cells (Ryall et al., 2015). Oxygen consumption rate (OCR), a measurement of mitochondrial respiration, is unaltered between quiescent and freshly isolated MPCs (Ryall et al., 2015). Interestingly, despite no change in OCR during the transition from quiescence to activation, activation is associated with an increase in mitochondrial content and expression of genes that coordinate TCA cycle activity, which may indicate increases in mitochondrial capacity precede increases in oxidative metabolism (Ryall et al., 2015). This was confirmed by a second study that showed the transition from quiescence to activation is associated with increased mitochondrial content, both in MPCs at the site of injury and in MPCs in distant muscles that also were activated by the injury (Rodgers et al., 2014). In proliferating MPCs, OCR is upregulated compared to quiescent MPCs and mitochondrial content and membrane potential remain elevated (Pala et al., 2018). Taken together, evidence suggests an increase in mitochondrial content during MPC activation that leads to increased OCR during MPC proliferation.

Oxidative metabolism is also dynamic during differentiation and myotube formation. OCR, mitochondrial content, and mitochondrial membrane potential remain elevated during MPC differentiation (5 days post-injury) compared to quiescent levels in MPCs isolated from injured mice (Pala et al., 2018). In immortalized MPCs, OCR is higher in MPCs that are

differentiated for 6 days, a time when myotube formation is prevalent, compared to proliferating MPCs. Conversely, cultured primary mouse MPCs showed decreased OCR and reduced mitochondrial content in MPCs that had differentiated for 24 h compared to proliferating MPCs (Das et al., 2017; Yucel et al., 2019). Thus, it is possible that early differentiation is associated with a dip in OCR and mitochondrial content, which is recovered and perhaps magnified at later stages of differentiation.

In conjunction with elevated OCR, MPC differentiation is associated with increased fatty acid oxidation (FAO). In cultured primary mouse MPCs, only differentiated MPCs (not proliferating) show increased OCR in response to palmitate treatment, suggesting that differentiating MPCs may use more FAO compared to proliferating MPCs (Sin et al., 2016). Though interestingly, in MPCs isolated from injured mice, transcript levels of genes involved in mitochondrial and peroxisomal FAO are elevated in both proliferating and differentiating MPCs compared to quiescent MPCs (Pala et al., 2018). Thus, increases in mRNA levels of genes involved in FAO may precede increased reliance on FAO pathways. Consistent with an increase in energy demand, ATP levels were increased in both proliferating and differentiating MPCs compared to quiescent MPCs (Pala et al., 2018).

Mitochondrial morphology is also dynamic during the myogenic process. In immortalized MPCs, dynamin 1 like (DNM1L), a protein involved in mitochondrial fission, shows peak expression at 1 day differentiation compared to proliferating MPCs or MPCs differentiated for 3 or 6 days (Sin et al., 2016). OPA1, a mediator of mitochondrial fusion shows increasing expression during the differentiation process (Sin et al., 2016). Levels of translocase of outer mitochondrial membrane 70 (TOMM70A), a protein that imports mitochondrial precursor proteins, show a slight dip at the transition from proliferation to early differentiation, recovering to proliferation levels by 3–6 days in differentiation (Sin et al., 2016). Further, complexes of the electron transport chain showed increased expression after 3 days of differentiation (Sin et al., 2016). Overall, proteins involved in mitochondrial fusion and the electron transport chain increase from proliferation to differentiation.

ESSENTIALITY OF GLYCOLYTIC AND MITOCHONDRIAL METABOLISM DURING THE MYOGENIC PROCESS

In addition to understanding the timing of metabolic pathway enrichment, studies have addressed the essentiality of glycolytic and mitochondrial metabolism and further elucidated the specific functions that these pathways serve (e.g., energy generation, biomass production, *de novo* synthesis of metabolic intermediates and amino acids).

Glucose is essential for MPC proliferation. In the absence of glucose, EdU incorporation, a marker of cell proliferation, is strongly repressed (Chen et al., 2019). Additionally, in the absence of glucose, ATP levels are reduced, suggesting that glucose carbons are essential for energy generation in proliferating MPCs (Chen et al., 2019). Interestingly, in one study, glucose deprivation in proliferating MPCs increased basal OCR, but dramatically reduced maximal (stress-induced) respiration (Yucel et al., 2019). This could indicate that loss of glucose increases basal OCR to compensate for loss of glycolytic ATP production. Additionally, since maximal respiration was reduced in the absence of glucose, glucose might also contribute substantially to mitochondrial metabolism under stressed conditions. In support of this claim, knockout of pyruvate dehydrogenase (PDH), the enzyme that converts pyruvate to acetyl-CoA, and thus links glycolysis to the TCA cycle, blunts MPC proliferation and decreases mitochondrial membrane potential and mitochondrial mass (Hori et al., 2019). Interestingly, the ratio of phosphorylated (Ser293)/total PDH increases during MPC proliferation (Yucel et al., 2019). Phosphorylation at Ser293 inhibits PDH activity, thus this finding indicates suppression of PDH activity during MPC proliferation. Pairing findings from these studies could suggest that PDH activity is low, but essential during MPC proliferation. PDH may be important for metabolism of pyruvate from glucose or other sources (e.g., lactate, alanine), or may have other unidentified cellular roles that are important for MPC proliferation. Together, this suggests that one reason for the essentiality of glucose during MPC proliferation is metabolism of glucose into acetyl-CoA, which is a powerful generator of NADH to fuel ATP production and continued mitochondrial metabolism. In addition to supporting mitochondrial metabolism, acetyl-coA is a substrate for histone acetylation. One tracing study, using ^{13}C labeled glucose, identified that glucose contributes to histone acetylation in proliferating MPCs (Yucel et al., 2019). Another study demonstrated, using ^{13}C glucose, that glucose contributes minimally to the synthesis of the non-essential amino acids, serine and glycine, in proliferating MPCs; however, the contribution of glucose to serine or glycine is not sufficient to maintain proliferation in the absence of extracellular serine and glycine (Gheller et al., 2020). The necessity of glucose for other biosynthetic pathways has received limited attention. Similar to other proliferating cells, it is likely that glucose also feeds biosynthetic pathways such as the pentose phosphate pathway, and that flux of glucose through these pathways is essential for MPC proliferation, though this has never been explicitly demonstrated.

In addition to proliferation, studies have also addressed the essentiality and specific function of metabolic pathways during differentiation. Knockout of PDH blunts MPC differentiation, highlighting the importance of mitochondrial glucose metabolism during differentiation (Hori et al., 2019). Glucose availability also regulates levels of the myogenic protein MyoD (Chen et al., 2019). In differentiating MPCs, glucose deprivation decreases basal and maximum OCR, suggesting glucose is a major substrate for mitochondrial respiration (Yucel et al., 2019). ^{13}C labeled glucose is used for histone acetylation in differentiating MPCs, though the amount of ^{13}C labeled glucose incorporated into histones is lower in differentiating MPCs compared to proliferating MPCs (Yucel et al., 2019).

Impairing mitochondrial metabolism impacts myogenesis. In immortalized mouse MPCs, an inhibitor of mitochondrial protein synthesis significantly impaired myotube formation,

despite no change in ATP levels, suggesting a requirement for mitochondrial protein synthesis that is independent of energy demand (Hamai et al., 1997). In this model, mRNA levels of the early myogenic marker myogenin (*MyoG)* was not impacted suggesting mitochondrial protein synthesis is essential for later stages of differentiation and fusion (Hamai et al., 1997). In contrast, in immortalized quail MPCs, inhibiting mitochondrial protein synthesis reduced myogenin expression and MPC proliferation, but, similarly, reduced fusion (Rochard et al., 2000). Additionally, an ATP synthase inhibitor and mitochondrial uncoupling agent reduced myogenin levels in quail myoblasts, suggesting that mitochondrial function is essential for MPC differentiation (Rochard et al., 2000). Therefore, during differentiation, glucose is likely essential to maintain mitochondrial metabolism, and mitochondrial metabolism (from all substrates) is essential for induction of myogenic proteins and successful MPC fusion.

METABOLISM IN CONDITIONS OF IMPAIRED MPC FUNCTION

Alterations in metabolic processes have been documented in conditions of impaired muscle regeneration, most extensively in aging models. Studies demonstrate that OCR is reduced in old rodent MPCs compared to young counterparts (Zhang et al., 2016; Pala et al., 2018). This is accompanied by changes in transcript profile including decreased expression of genes in the TCA cycle, oxidative phosphorylation, and lipid metabolism (Pala et al., 2018). One study documented a corresponding increase in ECAR in MPCs from old mice (Pala et al., 2018). Interestingly, research in human primary MPCs (hMPCs) did not fully corroborate this result. While old male hMPCs had lower OCR compared to young male hMPCs, there was extensive interindividual variation among MPCs from young and old females with no age effect on OCR observed (Riddle et al., 2018). This result urges caution when directly extrapolating from mouse studies to humans and a need to consider sex differences and heterogeneity of the human population.

METABOLIC PROTEINS IN SKELETAL MUSCLE REGENERATION

An emerging area of research is linking activity of specific metabolic proteins to control of MPC metabolism, myogenesis, and muscle regeneration. Numerous metabolic proteins that directly interconvert substrates are essential for MPC function. As described above, PDH knockout impairs several steps of the myogenic process. In mice, PDH knockout impairs regeneration after a muscle injury (Hori et al., 2019; Yucel et al., 2019). In addition to PDH, ATP citrate lyase (ACLY), the enzyme that converts cytosolic citrate to acetyl-coA regulates myogenesis. ACLY is essential for differentiation, but not proliferation, in MPCs. Further, ACLY overexpression improves muscle regeneration after injury (Das et al., 2017). Lactate dehydrogenase (LDHA), the protein that converts pyruvate to lactate is implicated in maintenance of quiescence in MPCs. Injecting muscles with a plasmid that causes LDHA overexpression, 5 days after inducing a muscle injury, caused an increase in quiescent cells per muscle fiber, suggesting LDHA plays a central role in the return of activated MPCs to the quiescent state (Theret et al., 2017). Pyruvate kinase M2, the enzyme that converts phosphoenolpyruvate to pyruvate, the final step in glycolysis, is also a regulator of myogenesis (Ryall, 2013). MPCs from mice with a whole-body PKM2 deletion show impaired proliferation *in vitro* (Lunt et al., 2015). Further, giving supplemental PKM2 to MPCs increases proliferation (Kodani et al., 2018). It is unknown whether PKM2 impacts regeneration after injury.

Proteins that deposit or remove post-translational modifications also control the regenerative process. The sirtuin family of enzymes regulate protein activity by removing acyl moiety post-translational modifications (i.e., acetyl, succinyl, malonyl groups) from lysine residues of proteins. There are seven members of the sirtuin family that differ in subcellular compartment and specificity for different acyl moieties (Carafa et al., 2016). The sirtuin family of enzymes have been shown to regulate broad cellular functions including metabolism, inflammation and DNA damage repair (Bosch-Presegue and Vaquero, 2014; Vachharajani et al., 2016). Roles for a few sirtuin enzymes have been described in MPCs. For example, the deacetylase enzyme sirtuin 1 (SIRT1) promotes MPC proliferation (Rathbone et al., 2009) and is essential for recovery from muscle injury (Ryall et al., 2015). Another member of the sirtuin family, SIRT3, regulates MPC differentiation (Khalek et al., 2014). Roles for other sirtuin enzymes, such as sirtuin 5 (SIRT5), a desuccinylase and demalonylase enzyme, have been described in cancer cell proliferation (Park et al., 2016; Xiangyun et al., 2016; Chang et al., 2018), and thus are putative regulators of muscle regeneration. Succinylation of lysine residues impacts proteins involved in FAO, branched chain amino acid catabolism, ketone body synthesis, the pentose phosphate pathway, and the TCA cycle (Rardin et al., 2013; Zhang et al., 2015; Zhou et al., 2016). Additionally, over half of glycolytic enzymes are modified by malonylation, another modification removed by SIRT5 (Nishida et al., 2015). SIRT5 has been identified as a mediator of proliferation in lung tumors, hepatocellular carcinoma, melanoma, and mouse embryonic fibroblasts (Zhou et al., 2016; Xiangyun et al., 2017; Chang et al., 2018). Overexpression of SIRT5 in HEK cells increases ECAR and SIRT5 knockout reduces lactate production in hepatocytes (de Moura et al., 2014; Nishida et al., 2015). Interestingly the opposite is observed in MDA-MB-231 cells, SIRT5 reduction increased lactate levels (Polletta et al., 2015). Together these findings suggest SIRT5 is a key regulator of glucose flux. SIRT5 may also be important for oxidative phosphorylation, as SIRT5 overexpression increased basal OCR in HEK293 cells and SIRT5 reduction reduced mitochondrial membrane potential in MPCs and MDA-MB-231 cells (de Moura et al., 2014; Polletta et al., 2015). Given the role of SIRT5 in cell proliferation and metabolism, and link of metabolism to MPC function, it is possible SIRT5 is an important mediator of MPC function. In addition to the sirtuin enzymes, AMP activated protein kinase (AMPK) is a cellular energy sensor that regulates metabolism

through phosphorylating other proteins (Fu et al., 2015; Theret et al., 2017). AMPK knockout MPCs have impaired activation and proliferation, and loss of AMPK impairs regeneration after injury (Fu et al., 2015; Theret et al., 2017).

Lastly, some proteins with non-metabolic canonical functions are regulators of MPC metabolism and muscle regeneration. STAT3, an intracellular signaling protein best recognized for its role in promoting inflammation, also increases OCR in MPCs (Sala et al., 2019). MPC specific loss of STAT3 impairs proliferation and blunts regeneration after injury (Zhu et al., 2016). Ying Yang 1 (YY1) is a transcription factor with numerous targets, many involved in cell growth (Deng et al., 2010). Recently YY1 was shown to be a hypoxia inducible factor 1 (HIF1α) binding partner in MPCs that coordinates metabolic capacity (Chen et al., 2019); YY1 deletion decreases both ECAR and OCR, causing a 70% reduction in ATP production (Chen et al., 2019). YY1 is essential for MPC activation and proliferation, and deletion impairs muscle regeneration after injury (Chen et al., 2019).

Expanding access to and use of proteomics has enabled and will continue to accelerate identification of metabolic proteins that are important for MPC function. Further, immunoprecipitation coupled to proteomics should continue to be leveraged to identify protein-protein binding partners, which enables the identification of "moonlighting" effects of proteins and provides crucial information about coordination of different metabolic processes or coordination of metabolism with other cellular processes. Immunoprecipitation for post-translational modifications, coupled with proteomics, can also clarify the role of these modifications in myogenesis by identifying protein targets. In addition to generating fundamental knowledge about MPC biology, continued research investigating metabolic proteins has the potential to identify novel therapeutic targets to boost regeneration in populations with impairment.

NUTRIENT AVAILABILITY AND DIETARY FACTORS IMPACT SKELETAL MUSCLE REGENERATION AND RECOVERY

Nutrient availability and requirements for myogenesis is also a growing area of research. Identification and characterization of nutrients that are essential for MPC function has the potential to improve skeletal muscle regeneration in individuals/populations that have impairments. Nutrient availability and dietary compounds will be discussed below.

Heterochronic parabiosis studies cemented the importance of circulating factors (i.e., hormones, nutrients, cytokines in the serum) for controlling muscle regeneration (Conboy et al., 2005). In heterochronic parabiosis, the circulatory system of a young and old mice are surgically joined. When challenged to recover from a muscle injury, old mice joined to young mice (heterochronic) showed improved regeneration compared to old mice joined to another old mouse (isochronic) (Conboy et al., 2005). Follow-up studies showed that, compared to isochronic parabiosis, young mice in heterochronic parabiosis showed lower BrdU incorporation, a marker of cell proliferation, and increased fibrotic tissue infiltration (Andrew et al., 2007). The opposite was observed in old mice, in which heterochronic parabiosis improved cell proliferation and reduced fibrosis. Interestingly, replacing a portion of old plasma with saline solution improves muscle regeneration in mice (Mehdipour et al., 2020). This suggests that factors which antagonize muscle regeneration may accumulate with age, and dilution of these factors is beneficial. Additionally, therapeutic plasma exchange (TPE), an FDA approved procedure in which a portion of a person's plasma is replaced with saline, resulted in a plasma sample that increased proliferation of cultured MPCs compared to plasma from the same individual before TPE (Mehdipour et al., 2020).

Follow-up research has identified specific factors in circulation, such as nutrients, that are essential for muscle tissue regeneration. For example, our lab demonstrated that the NEAA L-serine and the closely related NEAA L-glycine are required from an extracellular source for human MPC proliferation in cell culture models (Gheller et al., 2020). Additionally, a diet deficient in L-serine and glycine impaired recovery from myotoxin-induced muscle injury by reducing the number of MPCs and inducing intramuscular fat infiltration in aged animals (Gheller et al., 2020). Of note, several studies have identified a decline in endogenous serine levels in humans (Pitkanen et al., 2003; Houtkooper et al., 2011; Fazelzadeh et al., 2016; Gheller et al., 2020) and a decline in endogenous glycine in mice (Houtkooper et al., 2011) with advancing age. Thus, supplementation may be required to support optimal tissue regeneration. In another study, using genome-scale metabolic modeling, Shcherbina et al. (2020) predicted reduced retinoic acid signaling in aged MPCs. They identified a reduction in retinoic acid (Vitamin A) receptor mRNA levels in MPCs from aged mice compared to MPCs from young mice. Intriguingly, in both the young and aged MPCs, treatment with all-trans-retinoic acid promoted quiescence and reduced proliferation *in vitro*. However, while all-trans-retinoic acid increased Pax7 levels in young MPCs, no difference in Pax7 levels were observed in old MPCs (Shcherbina et al., 2020). Vitamin D has been shown to improve functional recovery at 48 h and 7 days post-mechanical load induced muscle injury in young human males (Owens et al., 2015). Further, this group identified a positive relationship between circulating 25(OH)D and peak torque recovery. These studies suggest a potential benefit of dietary supplementation with nutrients in the timeframe post-skeletal muscle trauma to improve tissue regeneration and functional recovery, particularly for individuals who may have reduced nutrient availability.

The relationship between nutrient availability and MPC function highlights an opportunity for habitual diet to impact MPC function through changing circulating nutrient availability or levels of nutrient linked hormones (e.g., insulin). Yet, few studies have investigated the impact of dietary patterns or whole foods on muscle regeneration and recovery. Caloric restriction is one of the best-known interventions to extend life-expectancy across species. Young mice (5 months) fed a reduced calorie diet had an increased MPC number and an increased number of MPCs per myofiber. The MPCs from the calorie restricted mice demonstrated increased OCR and reduced ECAR compared to mice consuming the control diet

(Cerletti et al., 2012). Additionally, the engraftment capacity of MPCs from calorie restricted mice or engraftment capacity into a calorie restricted mouse was significantly improved compared to control mice (Cerletti et al., 2012). Conversely, muscle regeneration was impaired in mice fed a high fat diet that induced obesity (Fu et al., 2016). In terms of whole foods, a recent study from our laboratory demonstrated blueberries alter the serum environment to provide benefits to MPCs, which may optimize muscle regeneration. Our lab developed a novel paradigm to culture isolated human MPCs in human serum. Using this model, we cultured MPCs from young and old donors in serum from before and after a 6-week dietary intervention of consuming a daily freeze-dried blueberry supplement. In young cultures, the dietary intervention serum boosted MPC proliferation (Blum et al., 2020). While results for MPC function are promising, a current limitation with using dietary patterns and whole foods is identification and understanding of specific biological components that promote regeneration.

Further investigation into circulating factors that alter MPC function will likely greatly accelerate diagnosis and treatment for impaired tissue regeneration. Metabolism is a composite of human biological information, including genetic and environmental factors (e.g., diet, physical activity, microbiome, etc.); therefore, the serum metabolome offers comprehensive knowledge of an individual (Hill et al., 2019). From a diagnostic perspective, studying the serum metabolome will identify biomarkers of individuals at risk for, or already experiencing, impaired tissue regeneration. For example, circulating metabolites (e.g., lactate, aspartate/asparagine, glutamate/glutamine, and fatty acids) were identified as biomarkers that explain the variation in physical recovery in response to hip replacement surgery (Parker et al., 2020). From a treatment perspective, identifying nutrients that are essential for MPC function, and commonly altered in groups with impairment (e.g., serine/glycine are essential for MPC proliferation and decrease in older adults/individuals with metabolic conditions) can lead to rational individual nutrient supplementation or dietary pattern recommendations after muscle trauma.

DISCUSSION

Skeletal muscle health is fundamentally important for overall human health. One component of skeletal muscle health is preservation of the regenerative process of muscle post-injury. However, with advancing age and chronic disease the regenerative response is disrupted. Additionally, even in healthy young individuals, the nutrient intakes and metabolic pathways that are important for optimizing tissue regeneration are largely unknown.

Fundamental Challenges to Identify Nutrient and Metabolic Requirements for Muscle Regeneration in Humans

One of the challenges in identifying and understanding nutrient and metabolic requirements in human health and disease is heterogeneity that exists among individuals. This diversity is induced by non-modifiable variables such as age, sex, and genetics; potentially non-modifiable variables such as obesity and chronic disease; and modifiable variables such as nutritional status, exercise, and medications (Lavin et al., 2019). As such, it is difficult to assess nutritional requirements in large cohorts of humans with similar conditions including trauma. Therefore, novel methods to determine nutrient and metabolic requirements are necessary. Identifying outcome variability in response to nutrient dose, such as those described for exercise induced muscle hypertrophy (Petrella et al., 1985; Thalacker-Mercer et al., 2013; Stec et al., 2017), will help identify responders and non-responders and target therapies to those most likely to benefit. Further, high-throughput technologies that enable the identification of multiple nutrients and metabolic targets should be leveraged to study MPC biology.

Current Research Gaps

Nutrients that are essential for tissue regeneration and homeostasis need to be defined. This gap in knowledge is critical to address for (i) a fundamental, detailed understanding of MPC biology and the regenerative process; and (ii) for the development of potentially more readily available/feasible therapies that can be implemented through the diet or with supplementation to improve regenerative outcomes post-injury. Identifying metabolites that have a meaningful impact on cell function is challenging due to the intrinsic variability in human metabolism that is governed by genetic and environmental factors in the absence of disease (Hill et al., 2019). Novel and emerging methods to study individual variation in metabolism may lead to major advances in this area. Continued research into the relationship between nutrients or dietary intervention and recovery from muscle injury may lead to therapies that can be used independently, or in conjunction with cell-based therapies, to maximize recovery for patients suffering from muscle damage.

CONCLUSION

Skeletal muscle regeneration and homeostasis are important for maintaining human health; however, with advancing age, metabolic disease, and pathological conditions skeletal muscle regeneration and homeostasis are perturbed. Existing therapies to augment muscle regeneration may be inadequate for the growing population of individuals with impairment (**Table 1**). Additionally, nutritional and pharmaceutical approaches to improve skeletal muscle regeneration are lacking. Nutrient based therapies are necessary, not only for the improvement of skeletal muscle heath, but also for the prevention of skeletal muscle deterioration. Thus, research is needed to improve upon existing and develop better therapies for the growing population with impaired skeletal muscle regeneration.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This material is based upon the work supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE-1650441 (to JB). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. Funding for AT-M received through NIH U01AR071133I. This work was supported through the NIH Nutrition Obesity Research Center at UAB P30 DK056336 to SW.

ACKNOWLEDGMENTS

Images were created with BioRender.com.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 20 July 2021
doi: 10.3389/fphys.2021.702826

Branched-Chain Amino Acids: Catabolism in Skeletal Muscle and Implications for Muscle and Whole-Body Metabolism

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Branched-chain amino acids (BCAAs) are critical for skeletal muscle and whole-body anabolism and energy homeostasis. They also serve as signaling molecules, for example, being able to activate mammalian/mechanistic target of rapamycin complex 1 (mTORC1). This has implication for macronutrient metabolism. However, elevated circulating levels of BCAAs and of their ketoacids as well as impaired catabolism of these amino acids (AAs) are implicated in the development of insulin resistance and its sequelae, including type 2 diabetes, cardiovascular disease, and of some cancers, although other studies indicate supplements of these AAs may help in the management of some chronic diseases. Here, we first reviewed the catabolism of these AAs especially in skeletal muscle as this tissue contributes the most to whole body disposal of the BCAA. We then reviewed emerging mechanisms of control of enzymes involved in regulating BCAA catabolism. Such mechanisms include regulation of their abundance by microRNA and by post translational modifications such as phosphorylation, acetylation, and ubiquitination. We also reviewed implications of impaired metabolism of BCAA for muscle and whole-body metabolism. We comment on outstanding questions in the regulation of catabolism of these AAs, including regulation of the abundance and post-transcriptional/post-translational modification of enzymes that regulate BCAA catabolism, as well the impact of circadian rhythm, age and mTORC1 on these enzymes. Answers to such questions may facilitate emergence of treatment/management options that can help patients suffering from chronic diseases linked to impaired metabolism of the BCAAs.

Keywords: branched-chain amino acids, skeletal muscle, catabolism, mTORC1, protein synthesis

OPEN ACCESS

Edited by:
Enrique Jaimovich, University of Chile, Chile

Reviewed by:
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Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *30 April 2021*
Accepted: *24 June 2021*
Published: *20 July 2021*

Citation:
Mann G, Mora S, Madu G and Adegoke OAJ (2021) Branched-Chain Amino Acids: Catabolism in Skeletal Muscle and Implications for Muscle and Whole-Body Metabolism. Front. Physiol. 12:702826. doi: 10.3389/fphys.2021.702826

INTRODUCTION

Branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) are a special class of amino acids (AA). In addition to being used as substrates for protein synthesis, they can stimulate skeletal muscle protein synthesis (Yoshizawa, 2004; Norton and Layman, 2006; Kamei et al., 2020) and suppress proteolysis (Béchet et al., 2005; Kamei et al., 2020). They also promote glucose transport (Nishitani et al., 2005; Yoon, 2016; Crossland et al., 2020) and have been linked to the regulation

of body weight (She et al., 2007a; Siddik and Shin, 2019). In addition, 3-hydroxy-3-methylglutaryl-CoA, a product of leucine catabolism, can be used as a substrate in cholesterol synthesis (Mathias et al., 1981; Zhang et al., 2007) and therefore is important in membrane integrity and cellular communication. In spite of these roles, sustained elevations of the BCAAs in the plasma and skeletal muscle are associated with insulin resistance (Newgard et al., 2009) and type 2 diabetes mellitus (T2DM) (Flores-Guerrero et al., 2018).

The anabolic effect of the BCAAs, especially of leucine, are mediated in part through the activation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) (**Figure 1**) (Gran and Cameron-Smith, 2011). mTORC1 is a serine/threonine kinase complex that is critical in promoting and maintaining muscle mass (Adegoke et al., 2012). Activation of mTORC1 is triggered by a number of factors, including nutrition [especially BCAAs and other AAs (Bar-Peled and Sabatini, 2014; Moberg et al., 2016), glucose (Kwon et al., 2004), and fatty acids (Yasuda et al., 2014)], growth factors [insulin, insulin-like growth factor 1 (IGF-1)] (Yoon, 2017), energy (Bond, 2016), oxygen status (Cam et al., 2010), statins (Henriksbo et al., 2020) and/or resistance exercise (Adegoke et al., 2012). Full activation of mTORC1 in response to nutrition requires two components. First, insulin/IGF1 induces the activation of the insulin receptor substrate 1 (IRS-1)/phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway, leading to GTP-loading of the mTOR activator Rheb (Hemmings and Restuccia, 2012; Hassan et al., 2013). Second, full activation of mTORC1 requires mTORC1 sensing of the BCAAs via many mediators, the best understood of which is the sestrins/gator/RAG/ragulator pathway (Chantranupong et al., 2014). Activation of these components ultimately leads to the transfer of mTORC1 to the lysosomal membrane where activated Rheb is localized. Once activated, mTORC1 phosphorylates many downstream targets, of which ribosomal protein S6 kinase (S6K1) and eukaryotic mRNA translation initiation factor 4E-binding protein 1 (4E-BP1) are the most studied. Activation of mTORC1 and subsequent phosphorylation of downstream targets stimulates protein synthesis, leading to increases in skeletal muscle fiber size and mass (Bodine et al., 2001b).

mTORC1 also inhibits skeletal muscle proteolysis. Activation of either the ubiquitin proteasome pathway (UPP) and/or autophagy/lysosomal pathways leads to skeletal muscle protein breakdown (Bodine et al., 2001a). Upon activation by the BCAAs, mTORC1 can inhibit signaling events involved in protein breakdown through multiple mechanisms, one of which is the suppression of autophagy via the phosphorylation and inhibition of a number of autophagy regulators, including Unc-51 like autophagy activating kinase (ULK-1) (Hosokawa et al., 2009), transcription factor EB (TFEB) (Martina et al., 2012), Beclin-1-regulated autophagy (AMBRA1) (Nazio et al., 2013) and autophagy-related protein 13 (ATG13) (Hosokawa et al., 2009). Leucine can also suppress proteolysis by suppressing the UPP (Nakashima et al., 2005).

Here, we review the catabolism of BCAAs in skeletal muscle (with reference to other tissues where relevant), and the impact of the BCAAs and their metabolites on skeletal muscle, whole-body metabolism, energy production and disease. Lastly, we comment on outstanding questions that need to be investigated, including mechanisms of regulation of the abundance of enzymes involved in BCAA catabolism, the effects of post translational modifications on the activities of these enzymes in skeletal muscle, effect of age on BCAA catabolism, and the role of mTORC1 in the regulation of BCAA catabolism.

Abbreviations: 3-HIB, 3-hydroxyusobutyrate; 4E-BP1, eukaryotic mRNA translation initiation factor 4E-binding protein 1; α-KG, α-ketoglutarate; AA, amino acids; AKT, protein kinase B; AMBRA1, autophagy and beclin 1 regulator 1; ASD, autism spectrum disorder; APN, adiponectin; ATG13, autophagy-related protein 13; BCAA, branched-chain amino acids; BCAT, branched-chain aminotransferase; BCAT1, cytosolic BCAT; BCAT2, mitochondrial BCAT; BCKDH, branched-chain α-keto acid dehydrogenase complex; BT2, 3-chlorobenzo[b]thiophene-2-carboxylic acid; CHREBP-β, carbohydrate response element binding protein; CRF, chronic renal failure; DLD, dihydrolipoamide dehydrogenase; E1, heterodimeric branched-chain α-keto acid decarboxylase; E2, dihyrolipoyl transacyclase homodimeric; E3, dihydrolipoyl dehydrogenase; GLN, glutamine; GR, glucocorticoid receptor; HEK293, human embryonic kidney 293 cells; HFD, high-fat diet; HMB, beta-hydroxy-beta-methylbutyrate; IGF1, insulin like growth factor 1; IMP, inosine monophosphate; IRS1, insulin receptor substrate 1; KIC, 2-ketoisocaproate/4-methyl-2-oxopentanoic acid; KLF15, kruppel like factor 15; KMV, α-keto-β-methylvaleric acid/3-methyl-2-oxopentanoate; KO, knockout; KIV, 2-keto-isovalerate/3-methyl-2-oxobutanoic acid; MIR, myocardial ischemia reperfusion; miRNA, microRNAs; mKO, muscle knockout; MSUD, maple syrup urine disease; mTORC1, mammalian target of rapamycin complex 1; mPK, mitochondrial protein kinase; PDAC, pancreatic ductal adenocarcinoma; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-*1alpha*; PHK, protein histidine kinase; PI3K, phosphatidylinositol-3 kinase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PP2CM, mitochondrial protein phosphatase 2 cm; PPARα, peroxisome proliferator activated receptor alpha; PPM1K, protein phosphatase mg^{2+}/mn^{2+} dependent 1K; ROS, reactive oxygen species; S6K1, ribosomal protein S6 kinase; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TFEB, transcription factor EB; T3, thyroid hormone (3,5,3′-triiodothyronine); TNFα, tumor necrosis factor alpha; TRIM21, tripartite motif containing-21; UBE3B, ubiquitin E3 ligase; ULK-1, unc-51 like autophagy activating kinase; UPP, ubiquitin proteasome pathway.

BCAA CATABOLISM

The first two steps of BCAA catabolism are shared amongst the three BCAAs (**Figure 2**). These are transamination catalyzed by branched-chain aminotransferase (BCAT) and oxidative decarboxylation catalyzed by the branched-chain α-keto acid dehydrogenase complex (BCKDH). The reversible transamination reactions yield branched-chain α-keto acids [BCKAs; 2-keto-isocaproate/4-methyl-2-oxopentanoic acid (KIC), α-keto-β-methylvaleric acid/3-methyl-2-oxopentanoate, (KMV), and 2-keto-isovalerate/3-methyl-2-oxobutanoic acid (KIV), respectively, from leucine, isoleucine, and valine]. The BCKAs are then irreversibly oxidatively decarboxylated by BCKDH to produce the corresponding acyl CoA derivates (isovaleryl-CoA from KIC, 2-methylbutyryl-CoA from KMV, and isobutyryl-CoA from KIV). The BCKDH reaction is the rate-limiting step in BCAA catabolism and is therefore tightly regulated (Harper et al., 1984; Harris et al., 1990). Beyond this step, the acyl-CoA derivatives are metabolized along separate pathways. Ultimately, leucine catabolism produces acetoacetate and acetyl-CoA, isoleucine yields propionyl-CoA and acetyl-CoA, and valine yields propionyl-CoA (**Figure 2**). Because high circulating concentrations of BCAAs, which may arise from

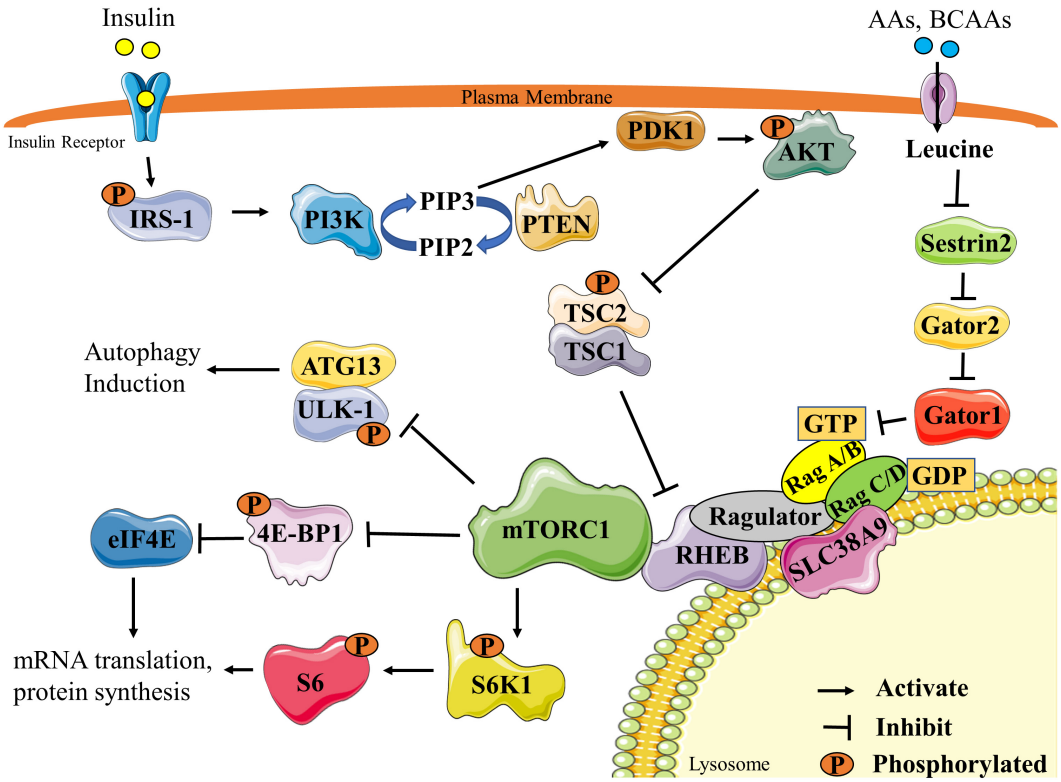

FIGURE 1 | Schematic representation of mTORC1 signaling pathway. Stimulation by insulin ultimately leads to the activation of the PI3K pathway and PIP3 synthesis. PIP3 activates PDK1, which can then phosphorylate AKT. Activated AKT phosphorylates and inactivates the TSC2/1 complex, allowing RHEB to remain GTP loaded to activate mTORC1. Activated mTORC1 activates translation machinery and protein synthesis by phosphorylating two main downstream targets, S6K1 and 4E-BP1. mTORC1 also inhibits autophagy by phosphorylating the ULK1/ATG13 complex. mTORC1 can also be activated through AA or BCAA stimulation of the sestrins/gator/RAG/ragulator pathway. Leucine, the most commonly studied BCAA, inhibits sestrin-2 leading to eventual GTP loading and RHEB activation of mTORC1. Re-drawn and modified from Fan et al. (2017) and Wolfson and Sabatini (2017). 4E-BP1, eukaryotic mRNA translation initiation factor 4E-binding protein 1; AA, amino acids; ATG13, autophagy-related protein 13; BCAA, branched-chain amino acids; eIF4E, eukaryotic mRNA translation initiation factor 4E; IRS1, insulin receptor substrate 1; mTORC1, mammalian/mechanistic target of rapamycin complex1; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphatidylinositol-3 kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; RHEB, Ras homolog enriched in brain; S6K1, ribosomal protein S6 kinase; S6, ribosomal protein S6; SLC38A9, solute carrier family 38 member 9; TSC1/2, Tuberous sclerosis proteins 1 and 2; ULK-1 unc-51 like autophagy activating kinase.

impaired catabolism of the AAs, have been linked to insulin resistance, T2DM (Chen and Yang, 2015), and cardiovascular diseases (White and Newgard, 2019), an understanding of mechanisms of regulation of tissue catabolism of BCAAs is required for a better understanding of these diseases and how to manage/prevent them.

Regarding quantitative tissue contribution to BCAA metabolism, the largest contributors of isoleucine disposal into protein synthesis are the liver (27%), skeletal muscle (24%), pancreas (24%), followed by the kidneys (9%), brown adipose tissue (6%) and other tissues (10%) (Neinast et al., 2018). The pancreas has the highest fractional synthesis rate of protein compared to other tissues (Neinast et al., 2018), which could explain why it is a major contributor to whole body disposal of isoleucine as well as the other BCAAs into proteins. On the other hand, skeletal muscle is the largest contributor to whole-body BCAA oxidation (59%), followed by brown adipose tissue (19%), liver (8%), kidneys (5%), heart (4%), and other tissues (5%). The relative predominance of skeletal muscle in BCAA catabolism is related at least in part to the fact that BCAA transamination, the first step of BCAA catabolism, occurs largely (65%) in the skeletal muscle (Suryawan et al., 1998). In addition to its contribution to BCAA oxidation, muscle metabolism of BCAAs is vital in whole body AA metabolism. As depicted in **Figure 3**, BCAA-derived ammonia, via glutamate dehydrogenase and glutamine synthetase reactions, is ultimately funneled into glutamine, an AA with roles in many vital body processes. The muscle AMP deaminase reaction (**Figure 3**), especially during exercise, is also a source of ammonia that can be used to make glutamine. In addition to the ATP that can be generated from the complete oxidation of BCAA to CO_2, it is also evident from **Figure 3** that muscle BCAA catabolism contributes to energy production via the NADH generated from the glutamate dehydrogenase reacction and the anaplerotic supply of α-ketoglutarate into the TCA cycle. The significance of skeletal muscle in whole body BCAA catabolism is emphasized by the fact that even though

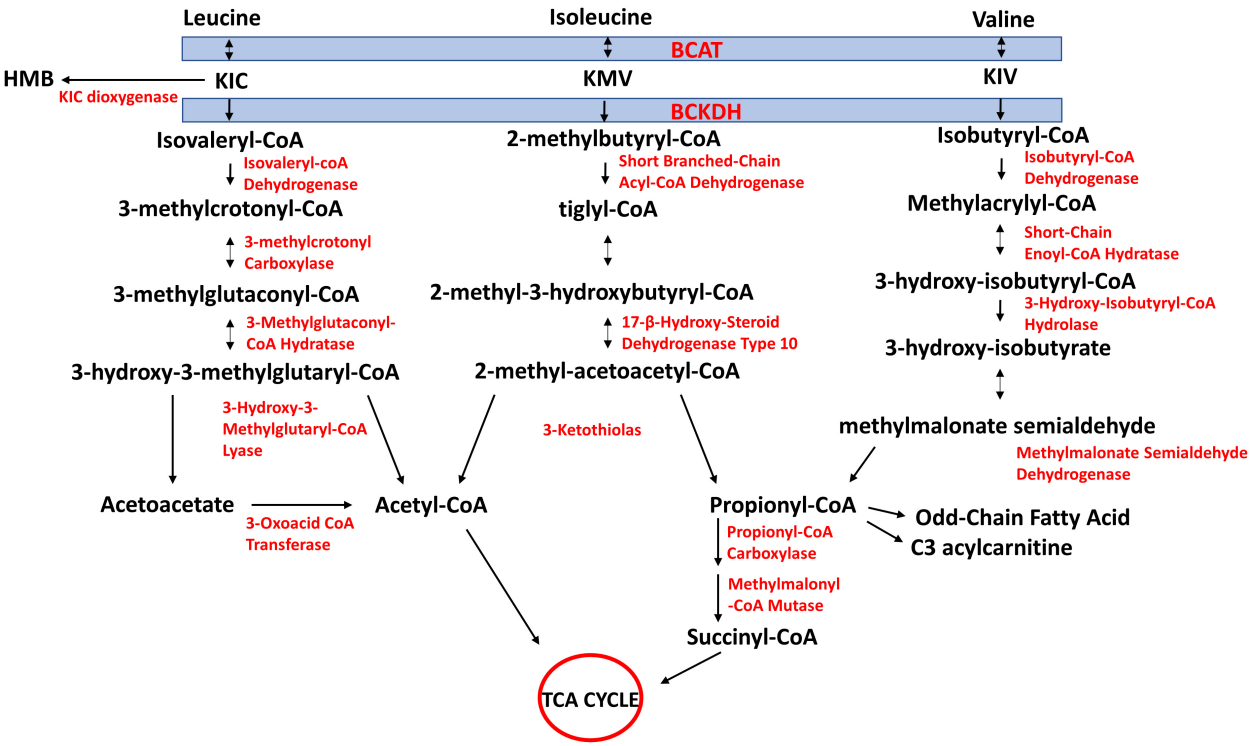

FIGURE 2 | Schematic of branched-chain amino acid (BCAA) catabolism. BCAAs share the same first two steps in their catabolism. They undergo reversible transamination, catalyzed by mitochondrial or cytosolic isoforms of branched-chain aminotransferase (BCAT). Branched-chain ketoacids (BCKAs) produced from this reaction are irreversibly decarboxylated to yield respective CoA compounds, which divide into their respective metabolic pathways. KIC, 2-keto-isocaproate/4-methyl-2-oxopentanoic acid; KMV, α-keto-β-methylvaleric acid/3-methyl-2-oxopentanoate; KIV, 2-keto-isovalerate/3-methyl-2-oxobutanoic acid. Re-drawn and modified from Adeva-Andany et al. (2017).

insulin infusion or inhibition of BCKDH kinase, the enzyme that inhibits BCKDH (see below), increases whole body BCAA oxidation in healthy animals, this is largely driven by the increase in muscle BCAA oxidation. The significance of muscle is even more evident in insulin resistance state: in high fat diet (HFD) fed mice or db/db mice, although BCAA oxidation is decreased by up to 60% in tissues like liver and adipose tissue, BCAA oxidation in skeletal muscle either remained unchanged or increased ∼50% (Neinast et al., 2018). Therefore, our focus is the regulation of BCAA catabolism in skeletal muscle, although appropriate references will be made to other tissues.

Branched-Chain Aminotransferases (BCAT)

Branched-chain aminotransferases catalyzes the reversible transamination of BCAAs into their respective BCKAs (**Figure 2**). In one direction, when BCAAs are transaminated to their respective BCKAs, α-ketoglutarate (α-KG) receives the amino group producing glutamate. In the opposite direction, when the BCAAs are produced from their respective BCKAs, glutamate donates the amino group and is converted to α-KG. In the transamination reaction, BCAT requires a coenzyme form of vitamin B6, pyridoxal 5′-phosphate (PLP), that serves as the amino group carrier. The transamination reaction is accompanied by the interconversion between the PLP- and the pyridoxamine 5′-phosphate (PMP)-bound forms of the enzyme. PMP transfers the amino group to α-KG to produce glutamate, restoring BCAT-PLP conformation (**Figure 4**) (Goto et al., 2005).

There are two isoenzymes of BCAT, cytosolic and mitochondrial forms. The *BCAT1* gene encodes the cytosolic BCAT (BCAT1), while the *BCAT2* gene encodes the mitochondrial BCAT (BCAT2) (Bledsoe et al., 1997). The mitochondrial isoform is much more widespread, being found in skeletal muscle, kidney, cortex, heart, subcutaneous adipose tissue, stomach, colon, ileum, and liver. BCAT1 is restricted to the brain, ovary and placenta (Bledsoe et al., 1997). Both isoforms are not present in the same tissue (Sweatt et al., 2004). BCAT2 is most abundant in skeletal muscle, followed by the kidney, and is least abundant in the liver (Suryawan et al., 1998). Substrate preferences for BCAT proteins is isoleucine, leucine and then valine (Wallin et al., 1990; Hall et al., 1993; Davoodi et al., 1998). Due to the low levels of BCAT2 in the liver, BCAAs are often not metabolized in this tissue but BCKAs arising from the transamination of BCAAs in other tissues can travel to the liver where they can serve as substrates for BCKDH (Kainulainen et al., 2013). Here (see section "BCAT2 and Its Regulation in Skeletal Muscle" below), we will focus on BCAT2, the mitochondrial isoform whose expression is high in skeletal

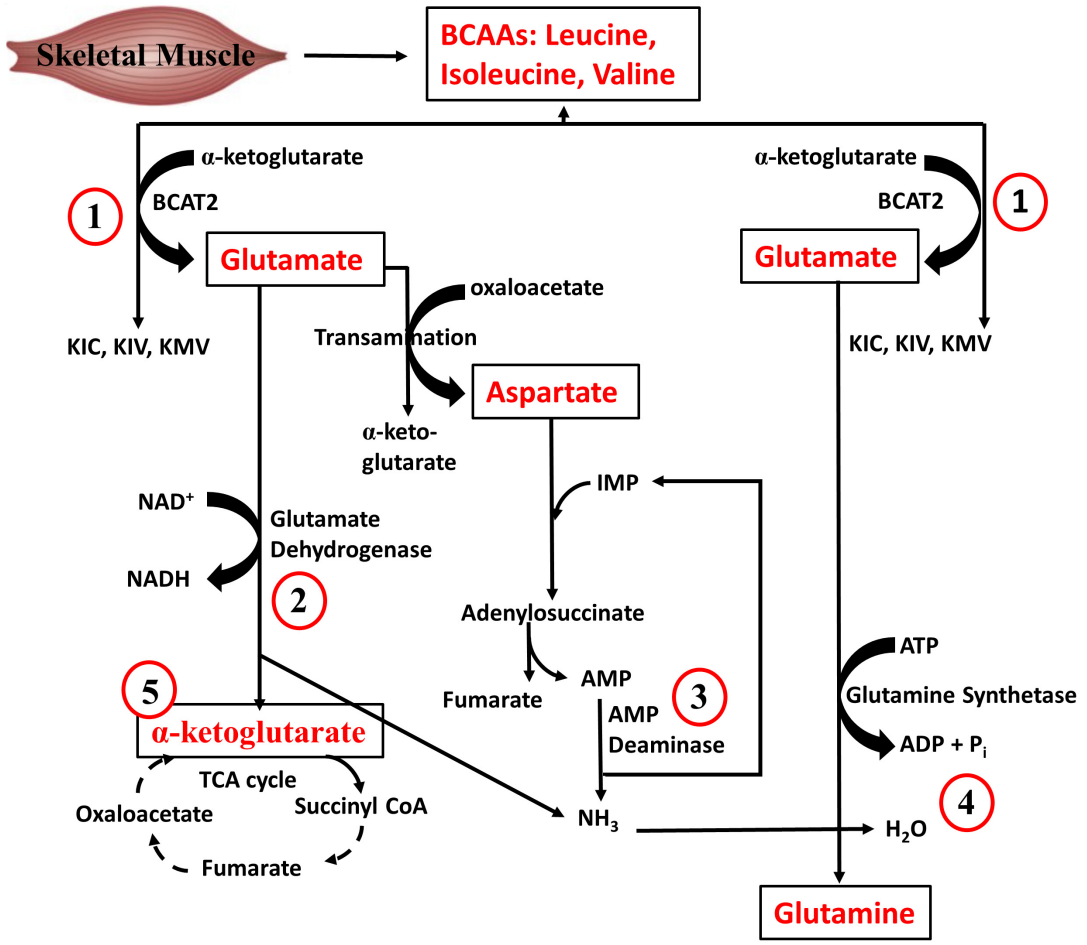

FIGURE 3 | Branched-chain amino acid metabolism in skeletal muscle. (1) BCAAs are transaminated with α-ketoglutarate by BCAA transaminase to generate glutamate. (2) Glutamate deamination yields α-ketoglutarate and ammonia. (3) During exercise, AMP is generated from ATP degradation in the skeletal muscle. The muscle AMP deaminase reaction also forms ammonia. (4) Glutamine is formed from ammonia and glutamate, a reaction catalyzed by glutamine synthetase. (5) The α-ketoglutarate formed by glutamate dehydrogenase can anaplerotically enter the TCA cycle. Re-drawn and modified from Groper and Smith (2013).

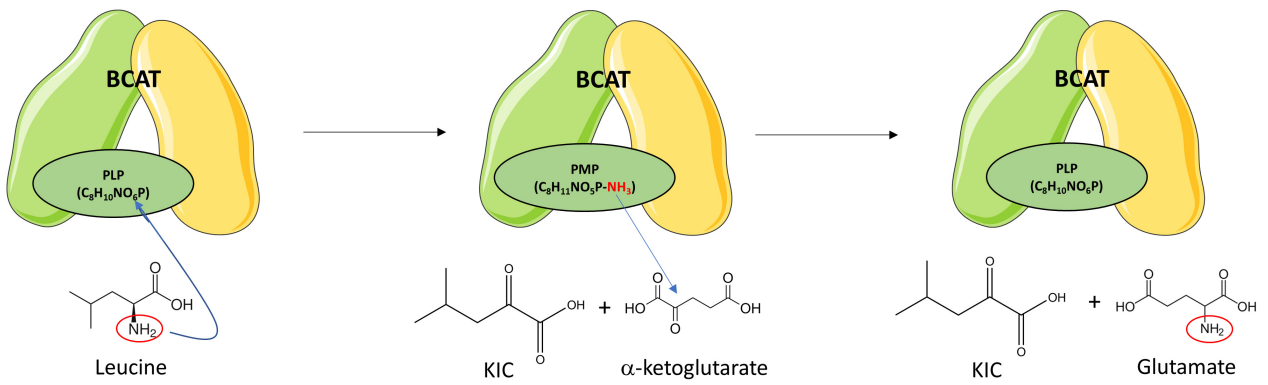

FIGURE 4 | Branched-chain amino transferase reaction. BCAT catalyzes the reversible transamination of branched-chain amino acids (BCAAs) into their respective branched-chain ketoacids (BCKAs). Leucine is transaminated to ketoisocaproic acid (KIC), isoleucine to keto-beta-methylvaleric acid (KMV), and valine to ketoisovaleric acid (KIV). The α-amino group of the BCAA is transferred to BCAT-PLP yielding BCAT-pyridoxamine phosphate (PMP) and the respective BCKA. The α-amino group is then transferred from the BCAT-PMP to α-ketoglutarate producing glutamate and restoring BCAT-pyridoxal phosphate (PLP). Leucine transamination is shown as representative of transamination reactions of the other BCAAs. Re-drawn and modified from Conway and Hutson (2016).

TABLE 1 | Relative distribution of BCAT activity and BCAA transamination in human tissues.

Tissue	BCAT activity (mU/g)	Distribution of BCAA transamination
Skeletal muscle	124 ± 14	65.4%
Kidney	880 ± 48	3.8%
Stomach	241 ± 11	7.7%
Liver	248 ± 32	3.8%
Brain	510 ± 49	15.4%
Heart	387 ± 23	N/A
Pancreas	N/A	N/A
Colon	254 ± 23	N/A
Adipose tissue	84 ± 4	N/A

Table re-drawn and modified from Suryawan et al. (1998). BCAT, branched chain aminotransferase; BCAA, branched-chain amino acids; g, grams of tissue; mU, milliunits; N/A, not applicable.

muscle, the tissue where over half the whole body activity of BCAT2 is found (**Table 1**) (Suryawan et al., 1998).

Branched-Chain Ketoacid Dehydrogenase (BCKDH)

Branched-chain ketoacid dehydrogenase catalyzes the irreversible oxidative decarboxylation of BCKAs (**Figures 2**, **5**), producing the respective branched-chain acyl-CoA derivates (isovaleryl-CoA from KIC, 2-methylbutyryl-CoA from KMV and isobutyryl-CoA from KIV) along with CO_2 and NADH. The BCKDH complex consists of three subunits, heterodimeric branched-chain α-keto acid decarboxylase (E1), dihyrolipoyl transacyclase (E2) and homodimeric dihydrolipoyl dehydrogenase (E3). The E1 subunit is a tetramer comprised of two α and two β subunits (α2β2) and is organized in a tetrahedral arrangement of the two α and two β subunits. The α subunits of E1 are encoded by *BCKDHA* gene and the β subunits by *BCKDHB* gene. Between the α and β subunits are two thiamine-binding pockets, which allow E1 to bind thiamine pyrophosphate (TPP) (Indo et al., 1987) (**Figure 5**).

The E2 subunit is encoded by *dihydrolipoamide branched-chain transacyclase* gene (DBT) and is the subunit to which E1 and E3 subunits are attached at the center of the complex. The E2 subunit has three domains: the core domain, the binding domain and the lipoyl domain. The core domain has the active site of the enzyme while the binding domains are responsible for the attachment of the E1 and E3 subunits (Conway and Hutson, 2016) through ionic interactions (Ananieva and Conway, 2020). The lipoyl domain is for substrate channeling within the complex (Chuang et al., 2006). The E3 subunit is encoded by *dihydrolipoamide dehydrogenase* (DLD) gene and is a homodimeric flavoprotein that contains a bound flavin adenine dinucleotide molecule (Litwer et al., 1989).

BCKDH reaction occurs in multiple steps. Firstly, the E1 subunit catalyzes the decarboxylation of the BCKA, releasing CO_2 and the corresponding branched-chain acyl intermediate. This branched-chain acyl group is then transferred by the lipoyl domain of the E2 subunit to the core of the complex where it is attached to coenzyme A by the catalytic domain of E2,

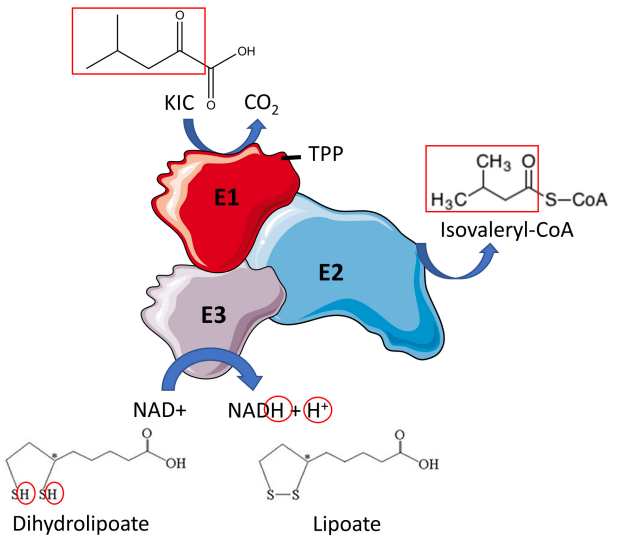

FIGURE 5 | Branched-chain ketoacid dehydrogenase reaction. Oxidative decarboxylation of the branched-chain ketoacid (BCKA) (shown here for ketoisocaproic acid) is initiated when the E1 subunit of BCKDH binds thiamine pyrophosphate (TPP). An acyl group is formed (both red boxes) which is simultaneously transferred to E2, where it is attached to coenzyme A (CoA). Lipoate is oxidized in the process, producing dihydrolipoate. E3 reduces dihydrolipoate producing lipoate and NADH + H^+. Then the metabolites of the BCKAs are metabolized in their respective pathways. Re-drawn and modified from Conway and Hutson (2016).

producing branched-chain acyl-CoA ester, and lipoate is reduced to dihydrolipoic acid in the process. E3 then reoxidizes the dihydrolipoic acid using NAD+ and produces NADH and lipoic acid (Adeva-Andany et al., 2017). Valine-derived KIV is the preferred substrate for BCKDH in cultured fibroblasts (Yoshida et al., 1986), but no data is available in skeletal muscle. In rat tissues, the BCKDH activity is highest in the liver, intermediate activity in the heart and kidney and is lowest in skeletal muscle, adipose tissue, and brain (Harper et al., 1984). In rats, 83% of BCKD's oxidative capacity is in the liver, with 3% in skeletal muscle, 10% in the kidney, 1% in the brain, and 2% in the stomach and intestine. The distribution of BCKD oxidative capacity differs greatly in humans, as 54% is in skeletal muscle, 13% in the liver, 8% in the kidney, 20% in the brain, and 4% in the stomach and intestine (**Table 2**) (Suryawan et al., 1998).

Apart from the BCKDH reaction, BCKAs can have alternative fates. They can be reduced at the α-carbon, forming branched chain α-hydroxy ketoacids in a number of metabolic disorders including maple syrup urine disease (MSUD), propionic acidemia and in ketoacidosis (Liebich and Först, 1984; Anderson et al., 2017). These are present in adult urine at low levels and are directly degraded by α-hydroxyacid oxidases, probably in the liver (Jones et al., 2000). Also, cytosolic dioxygenase, present predominantly in the liver, with a small abundance in the skeletal muscle (Nissen et al., 1996), can convert a small percentage of α-KIC to beta-hydroxy-beta-methylbutyrate (HMB) (Van Koevering and Nissen, 1992) (**Figure 2**). HMB can increase mTORC1 signaling (Kimura et al., 2014; Suryawan et al., 2020)

and muscle protein synthesis (Wilkinson et al., 2013) and suppress proteolysis (Kimura et al., 2014), thus promoting muscle anabolism.

BCAA Metabolism Downstream of BCKD

Leucine-derived isovaleryl-CoA is ultimately metabolized, via multiple steps, to acetyl CoA and acetoacetate (**Figure 2**). Acetoacetate can yield two molecules of acetyl CoA. The three acetyl-CoA derived from leucine can enter the TCA cycle or alternate pathways. Similarly, isoleucine-derived 2-methylbutyryl-CoA ultimately yields acetyl-CoA and succinyl-CoA, while the catabolism of valine-derived isobutyryl-CoA yields propionyl-CoA (Adeva-Andany et al., 2017). Whether the catabolism of an AA ultimately leads to the production of only acetyl-CoA/acetoacetate and/or intermediates such as propionyl-/succinyl-CoA that can be converted, via gluconeogenesis, into glucose is the basis of classification of AAs as glucogenic, ketogenic, or both. Because it is generally accepted that acetyl-CoA derived carbon cannot end up in glucose, leucine is the only BCAA that is considered strictly ketogenic; valine is considered glucogenic while isoleucine is both (**Figure 2**). However, there is still some debate on the fate of acetyl-CoA carbon vis-à-vis gluconeogenesis (Green, 2020; Tetrick and Odle, 2020).

BCAT2 AND ITS REGULATION IN SKELETAL MUSCLE

Importance of BCAT2 in Muscle

BCAT2 (whole body) knockout (KO) mice are leaner and smaller in size. Knockout mice exhibit reduced endurance in response to exercise (∼70%), and increased muscle S6K1 phosphorylation (∼2-fold) (She et al., 2007a, 2010). These mice also display increased expression of genes associated with protein degradation, apoptosis, and necrosis (Lynch et al., 2015). They have increased plasma BCAA levels (fivefold), which is more remarkable in female animals even when they consumed BCAA-free diets (She et al., 2007a). Blood levels of KMV and KIV are reduced 40–50% in KO animals (She et al., 2007a,b). The increased levels of BCAAs could activate protein synthesis through the activation of mTORC1 (Gran and Cameron-Smith, 2011). Also, the transamination of leucine to KIC is required to inhibit protein degradation in skeletal muscle (Tischler et al., 1982), so a lack of BCAT2 could suppress muscle KIC (which was not measured in the study), which could explain the increase in muscle protein degradation (She et al., 2007a).

BCAT2 KO mice have greater muscle glycogen (∼45%) in a re-fed state after starvation (She et al., 2010). They also have reduced exercise capacity and higher intramuscular lactate/pyruvate ratio (∼1.5-fold), along with reduced intramuscular TCA cycle intermediates (malate and citrate), indicating a potential energy crisis. These observations in BCAT2 knock out mice outline the importance of BCAT2 in skeletal muscle and whole-body metabolism. This data was generated from study of whole-body knockout of BCAT2. It would be interesting to see the effects of muscle specific deletion of BCAT2 on skeletal muscle and whole-body metabolism. Interestingly, myoblasts depleted of BCAT2 are impaired in their ability to form myotubes (Dhanani et al., 2019).

TABLE 2 | Relative distribution of BCKD activity and BCKD oxidative capacity in human tissues.

Tissue	Distribution of BCKD activity (mU/g)	Distribution of BCKD oxidative Capacity
Skeletal muscle	1.3 ± 0.3	54%
Kidney	15.9 ± 2.6	8%
Stomach + intestine	2.5 ± 0.1	4%
Liver	4.2 ± 0.41	13%
Brain	6.4 ± 0.7	20%
Heart	3.3 ± 0.5	N/A
Pancreas	N/A	N/A
Colon	2.3 ± 0.2	N/A
Adipose tissue	1.1 ± 0.1	N/A

Table re-drawn and modified from Suryawan et al. (1998). BCKD, branched-chain ketoacid dehydrogenase; BCAA, branched-chain amino acids; g, grams of tissue; mU, milliunits; N/A, not applicable.

Physiological Regulation of Muscle BCAT2

Exercise

Plasma BCAA levels during exercise (∼67% VO_{2max} for 1 h) and 30 min after exercise do not change in young individuals (19–22 years old) (Poortmans et al., 1974). Refsum et al. (1979) demonstrated 40% decrease in plasma BCAA levels (∼40%) after prolonged exercise (4–6 h of cross-country skiing), in trained individuals aged 23–45 years. In another study, there are significant increases in plasma leucine (10%) and isoleucine (35%) levels 4 h post low-intensity exercise (30% $VO2_{max}$), measured in the leg of individuals aged 24–32 years (Ahlborg et al., 1974). Differences in the change in BCAA levels across studies could be attributed to differences in workload intensity and duration. Whole-body leucine oxidation is higher after exercise compared to rest, and leucine oxidation is higher in a fasted state compared to a post absorptive state in human subjects who had exercised. There is no difference in leucine oxidation between starvation and post absorptive state amongst non-exercised controls (Knapik et al., 1991). In the post-absorptive state, there was a greater KIC to leucine ratio after exercise compared to rest (Knapik et al., 1991) suggesting BCAT2 activity could be upregulated. Fielding et al. (1986) support this as intramuscular KIC levels increased during exercise, suggesting an increase in leucine transamination.

Most studies attribute changes in BCAA metabolism after exercise with changes in BCKDH activity (Wagenmakers et al., 1989; Shimomura et al., 1995; Fujii et al., 1998), but there is evidence that BCAT2 too can be regulated by exercise. Roberson et al. demonstrated that BCAT2 protein expression is higher after endurance training compared to resistance training but normalized to similar levels after 3 days of repetitive exercise training (Roberson et al., 2018). They did not measure if

this correlates with increased BCAA metabolism, but it is consistent with the literature as endurance exercise increases BCAA oxidation (Wagenmakers et al., 1989) and leucine oxidation (Mazzulla et al., 2017) while resistance exercise does not (Tarnopolsky et al., 1991). In another study, mice underwent bilateral synergist ablation (mechanical overload), removing the gastrocnemius and soleus to induce hypertrophy in the plantaris muscle for up to 14 days. BCAT2 mRNA expression was downregulated (0.48-fold) days 3–7 of mechanical overload in the plantaris. The authors suggested that reduced BCAT2 levels increase BCAA levels (Chaillou et al., 2013), which could in turn activate mTORC1, leading to muscle hypertrophy.

Diabetes/Insulin Resistance

Skeletal muscle BCAT2 mRNA level is reduced by 25% in type 2 diabetes patients compared to body mass index-matched controls (Hernández-Alvarez et al., 2017). However, muscle BCAT2 protein level is increased (∼50%), but without a change in BCAT2 mRNA expression in db/db mice, a T2DM mouse model (Hernández-Alvarez et al., 2017). On the other hand, in another study, db/db mice exhibit increased plasma levels of valine (∼50%), suggesting reduced BCAT2 activity (Neinast et al., 2018). In ob/ob mice, an obese mouse model, there is no change in gastrocnemius muscle BCAT2 mRNA or protein levels compared to control, irrespective of whether the animals were fasted or fed (Hernández-Alvarez et al., 2017). In another study, gastrocnemius muscle BCAT2 protein level is not different between lean and obese Zucker rats, irrespective of their nutritional status (She et al., 2007b). Finally, in rats fed fructose for 45 days to produce a non-obese insulin resistance state, there were reductions in muscle BCAT2 activity (15%), even though activities of the enzyme in liver and adipose tissue were not affected (David et al., 2019).

Interestingly, supraphysiological supplementation of metformin (2 mM), a commonly prescribed drug for T2DM, for 12 h increases BCAT2 mRNA expression levels (30%) but significantly reduces it after 24 h in C2C12 myotubes. Protein levels of BCAT2 were also decreased (25%) after 24 h of treatment (Rivera et al., 2020b). Thus, elevation in circulating BCAAs in obesity/insulin resistance states cannot be totally explained by changes in the expression of BCAT2. Although not consistent across all reports, the studies reviewed suggest a reduction in BCAT2 level in diabetes/insulin resistance. The discordance may relate to study models and/or length of study. For example, it is conceivable that in studies with prolonged duration, elevated BCAA levels may induce BCAT2 expression in an effort to reduce the levels of these AAs via increased transamination. Furthermore, increased BCAT2 abundance can regulate BCKDH function (Islam et al., 2010).

Similarly, BCAAs are increased fivefold in animals with type 1 diabetes mellitus (T1DM) (Hutson and Harper, 1981; Aftring et al., 1988; Rodríguez et al., 1997), reviewed in Holeček (2020). This could be due to the significant reduction in BCAT2 mRNA levels in skeletal muscle and liver of rabbits with T1DM induced by alloxan (Gürke et al., 2015). Although insulin is a potent activator of overall muscle and whole body BCAA oxidation (Neinast et al., 2018), much of the work on BCAA metabolism has focussed on insulin resistance/T2DM. Thus, more studies are required to assess the effect of T1DM on BCAT2 and BCAA catabolism, and whether changes in BCAA metabolism in T1DM are linked to the pathologies or complications of the disease.

Nutritional Regulation

Valine supplementation in C2C12 muscle cells has no effect on BCAT2 mRNA or protein expression, whether or not insulin resistance was present (Rivera et al., 2020a). In adipocytes, leucine supplementation on day 4 of differentiation increases BCAT2 mRNA levels (twofold), but on day 10 of differentiation, it reduces BCAT2 protein levels (Kitsy et al., 2014). As mentioned, dexamethasone had no effect on BCAT2 mRNA levels, but dexamethasone and 5 mM of leucine (but not 10 mM) supplementation increases BCAT2 mRNA (100%) in C2C12 myoblasts (Wang et al., 2016).

Compared to a diet that contains 17% protein, a diet with 30% protein has no effect on rat muscle BCAT2 mRNA (Cheon and Lim, 2015). On the other hand, a HFD has a more robust effect on BCAT2 expression (Liu et al., 2017). Rats on a HFD for 24 and 32 weeks display increased BCAT2 protein expression in skeletal muscle (twofold for 24 weeks, threefold for 32 weeks), and leucine supplementation with a HFD further increased BCAT2 expression (25%) (Liu et al., 2017). This correlates with a significant reduction in serum isoleucine and valine levels, but no change in leucine levels. Additionally, a HFD increases serum KIV (50%) and KIC (25%) levels after 32 weeks on an HFD, further suggesting the increase in BCAT2 activity. Interestingly, supplementation of leucine in a HFD at a later time (when hyperglycemia had already developed) attenuated the reduction in insulin sensitivity seen in the HFD group by mechanisms including increase in muscle mitochondrial biogenesis (Liu et al., 2017). This is an interesting finding, as obesity/T2DM exhibit increased BCAAs and reduced BCAA catabolism (Lian et al., 2015). While the finding of beneficial effects of leucine supplementation at a later stage of HFD consumption may suggest that timing of the leucine administration is an important factor to consider, it should also be noted that, unlike what is commonly seen in insulin resistance/T2DM, BCAA levels were not increased in the HFD-fed animals studied by Liu et al. (2017).

Mechanisms of Effects of Physiological/Disease States on BCAT2

The effects of physiological and disease states on BCAT2 and BCAA metabolism may be mediated by one or more of the known regulators of metabolism. For example, mice overexpressing peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) in skeletal muscle have increased BCAA catabolism (Hara et al., 1987; Hall et al., 1993) and BCAT2 protein expression (1.25-fold) (Hara et al., 1987), and overexpression of PGC-1α in C2C12 muscle cells significantly elevates BCAT2 mRNA (40%) and protein expression (Hara et al., 1987). Increases in BCAA catabolism in the PGC1α transgenic mice coincides with increased TCA cycle intermediates. The increases in BCAT2 mRNA and protein expression in these transgenic mice could be due to the increased number of mitochondria, as BCAT2 is a mitochondrial enzyme. PGC-1α may also activate BCAT2 via

its effects on glucocorticoid receptor (GR), as the GR, working through Kruppel like factor 15 (KLF15), increases BCAT2 expression in skeletal muscle (Tischler et al., 1982; Sahlin, 1985). KLF15 KO mice have reduced (∼30%) skeletal muscle BCAT2 mRNA expression (Islam et al., 2010), while its overexpression increases BCAT2 protein levels in primary skeletal myocytes (Flores-Guerrero et al., 2018). In another study, dexamethasone does not increase BCAT2 mRNA levels in C2C12 myoblasts (Tulp et al., 1979), so more work is required to understand the link between glucocorticoids and BCAT2. Moreover, the supposed link between regulators of mitochondrial abundance and BCAT2 is not always observed. For example, KO of nuclear receptor Rev-erb-α, an activator of mitochondrial biogenesis and function, increases, rather than decreases, muscle BCAT2 mRNA levels (30%) (Marchesini et al., 2003). Similarly, 5-aminolevulinic acid, a compound that activates PGC-1α and mitochondrial content in quadriceps, reduces BCAT2 mRNA expression (80%). Thus, there might be more to BCAT2 regulation than merely PGC-1α expression and oxidative capacity (Espinal et al., 1986).

The effects of diverse physiological states on BCAA metabolism may also be mediated by small metabolites. For example, reduced levels of BCAAs in liver cirrhosis patients occur in parallel with hyperammonemia (Lackey et al., 2013). Ammonia infusions results in decreases in BCAA levels in skeletal muscle. This decline could be because of the requirement to use BCAAs in transamination reactions with α-KG to produce glutamate to facilitate ammonia detoxification in skeletal muscle (Henriksson et al., 1986). The decline in BCAAs to detoxify high ammonia levels is also present in urea cycle disorders (Perng et al., 2014). Similarly, in chronic renal insufficiency BCAA levels are reduced and the authors suggest that these AAs are metabolized to produce glutamine to counter the increase in ammonia (Holeček, 2018), implying that an increase in BCAT2 activity in individuals with liver cirrhosis, urea cycle disorders and chronic renal insufficiency can mitigate the increases in ammonia levels.

Post-transcriptional/Translational Modifications of BCAT2

Except for the relatively well studied effects of branched-chain ketoacid dehydrogenase kinase (BDK) and PP2Cm on the E1α subunit of BCKDH (please see section "BCKDH Regulation in Muscle"), not much is known about posttranslational modifications of enzymes that catabolize BCAAs. In addition to regulation of its mRNA and protein levels, BCAT2 is subject to other forms of regulation, in particular post translational modifications. Overexpression of the mutant form of the proto-oncogene GTPase KRAS ($KRAS^{G12V}$) in pancreatic ductal adenocarcinoma (PDAC) samples increases, whereas knockdown of KRAS decreases, BCAT2 protein, with a minimal effect on BCAT2 mRNA level. The increased stability of BCAT2 under this condition is due to reduced interaction of BCAT2 with tripartite motif containing-21 (TRIM21), a member of the tripartite motif family that functions as a RING finger E3 ubiquitin protein ligase. This results in reduced ubiquitylation but increased stability of BCAT2. The ubiquitylation and degradation of BCAT2 by TRIM21 requires the phosphorylation of BCAT2 by spleen tyrosine kinase (SYK) on Tyr228 (Li J. T. et al., 2020). In addition, BCAT2 is acetylated on Lys44, a modification that does not affect the activity of the enzyme but rather promotes its ubiquitin-dependent degradation. cAMP-responsive element-binding (CREB)-binding protein (CBP) and sirtuin4, respectively, acetylates and deacetylates BCAT2. Interestingly, BCAA depletion promotes acetylation and ubiquitin dependent degradation of BCAT2 in pancreatic cancer cells (Lei et al., 2020). It will be interesting to see whether these novel mechanisms of regulation of BCAT2 are also observed in skeletal muscle.

In a *Escherichia coli*, BCAT2 is negatively regulated by H_2O_2. Two cysteine residues (Cys315 and Cys318) form a disulfide bond under oxidizing conditions, reducing the activity of BCAT2 (Conway et al., 2003). Supplementation of dithiothreitol, an antioxidant compound, completely reversed the oxidation and restored activity of BCAT2 (Davoodi et al., 1998; Conway, 2020). This demonstrates that there is redox-linked regulation of BCAT2 activity but this is yet to demonstrated in mammalian skeletal muscle.

BCAT2 is also regulated by microRNA. MicroRNAs (miRNA) are non-coding short RNA molecules that regulate gene expression (Ambros, 2004). *BCAT2* gene is a translational target of microRNA (miR)-182, which is important in axon outgrowth and dendrite maturation (Wang et al., 2017). BCAT2 expression is downregulated by miR-182 (Wang et al., 2017). miR-330-5p is another negative regulator of BCAT2, as there is an inverse relationship between the two during ovine preadipocyte differentiation (Shi et al., 2018). miR-125a-3p is involved in AA and glucose metabolism, and can inhibit BCAA metabolism by negatively regulating KLF-15, an upstream regulator of BCAT2 in skeletal muscle of fish (Li H. et al., 2020).

Much of what is known about BCAT2 relates to the regulation of its abundance. The recent discovery of the miRNAs that can regulate BCAT2 abundance, and of the ubiquitin protein ligase that regulates acetylation/phosphorylation-dependent ubiquitination and degradation of this protein represents novel mechanisms of regulation of BCAT2 and BCAA oxidation (Lei et al., 2020; Li J. T. et al., 2020). First, there is a need to identify mammalian skeletal muscle expression of these enzymes and how they are regulated. In conditions where BCAT2 expression changes, it will be interesting to assess whether these recently discovered enzymes mediate the change in BCAT2. One would predict that conditions that increase BCAT2 abundance and/or BCAA oxidation, for example increased supply of the BCAA, would lead to reduced phosphorylation, acetylation, and ubiquitination of the enzyme. Secondly, because post translational modifications like ubiquitination (Adegoke et al., 2020) and oxidation (Davoodi et al., 1998; Conway, 2020) can affect enzyme functions/localization, an intriguing question would be whether BCAT2 activity too can be so regulated, a discovery that may explain studies in which, for example, changes in BCAT2 expression do not correspond with changes in BCAA oxidation (She et al., 2007b; Hernández-Alvarez et al., 2017). Because these posttranslational modifications happen relatively quickly and are reversible, they may be used by skeletal muscle

for short-term regulation within a time frame during which changes in gene or protein expression might not be apparent.

BCKDH REGULATION IN SKELETAL MUSCLE

BCKDH activity is regulated by a kinase and a phosphatase (Nobukuni et al., 1989). BCKDH kinase (BDK), encoded by the *BCKDK* gene, phosphorylates Ser293 and Ser303 of the E1 subunit of the BCKDH complex and inactivates it (Shimomura et al., 1990; Popov et al., 1991, 1992; Wynn et al., 2000). Conversely, protein phosphatase 2Cm (PP2Cm), encoded by protein phosphatase Mg^{2+}/Mn^{2+} dependent 1K (PPM1K), is responsible for the reactivation of the complex by dephosphorylation (**Figure 6**) (Damuni et al., 1984; Damuni and Reed, 1987). There is also evidence that BCAT2 can physically interact with the E1 subunit of BCKDH, which allows for substrate channeling, increasing the rate of decarboxylation of the complex as a whole (Islam et al., 2007).

The abundance and activity of the BCKDH complex is responsive to nutritional, hormonal, and metabolic conditions. The effects of these conditions are relayed to the BCKDH complex at least in part through phosphorylation/dephosphorylation of the complex. In addition, BCKDH levels are allosterically regulated by branched-chain acyl-CoA esters and NADH, end products of the BCKDH reaction in kidney (Boyer and Odessey, 1991) and liver (Parker and Randle, 1978). Whether these allosterically regulate BCKDH specifically in muscle remains to be seen.

BDK Structure and Regulation

BDK Structure

BDK is a mitochondrial protein kinase (mPK) that contains a nucleotide-binding domain and a four-helix bundle domain. It is similar in structure to protein histidine kinases (PHKs) (**Figure 6**) (Popov et al., 1992; Machius et al., 2001). However, unlike PHKs, phosphotransfer carried out by BDK on BCKDH E1α subunit is not mediated by histidine, but by potassium and magnesium (**Figure 7**) (Machius et al., 2001). In solution, the enzyme can exist as a homodimer (Machius et al., 2001) or tetramer (Wynn et al., 2000; Machius et al., 2001). As a homodimer, it can undergo auto-phosphorylation (Wynn et al., 2000). Changes in the ATP-binding domain attenuates BDK catalytic activity, suggesting that it is of the ATPase kinase superfamily (protein kinases with intrinsic ATPase activity), rather than the PHK family (Wynn et al., 2000). Crystal structure of BDK shows that an allosteric inhibitor such as (S)-α-chloro-phenylpropionic acid [(S)-CPP] binds to a site in the N-terminal domain to cause a movement of the helix-bundle and a conformational change in BDK to promote full activation and dephosphorylation of BCKDH (**Figure 7**) (Popov et al., 1991; Obayashi et al., 2001; Shimomura et al., 2006; Tso et al., 2013).

BDK Regulation

BDK mRNA expression is highest in skeletal muscle, intermediate in the brain and kidney and lowest in the liver and small intestine (Suryawan et al., 1998). Although BDK concentration is lowest in the small intestine, BCKDH complex activity in the small intestine is also low (Suryawan et al., 1998). BDK exists in the mitochondrial matrix of a cell in two forms: a free form and bound form (Popov et al., 1991; Obayashi et al., 2001; Shimomura et al., 2006). A small number of BDK binds BCKDH complex before BDK can be catalytically active and promote the phosphorylation and inactivation of the BCKDH complex. The ketoacids of leucine and valine, KIC and KIV, respectively, induce conformational change and annul the attachment of BDK to the BCKDH complex (Harris et al., 1990). BDK inhibitors such as clofibric acid and thiamine diphosphate (ThDP) also promote the dissociation of BDK from BCKDH complex (Murakami et al., 2005). The inhibition of ThDP is influenced by the physiological concentrations of potassium (Akita et al., 2009).

There is a high ratio of BDK to the E2 subunit of BCKDH in skeletal muscle compared to other tissues, hence the low activity of BCKDH in muscle (Suryawan et al., 1998). The fully lipoylated E2 transacyclase interacts with BDK to promote the phosphorylation of the E1 subunit of

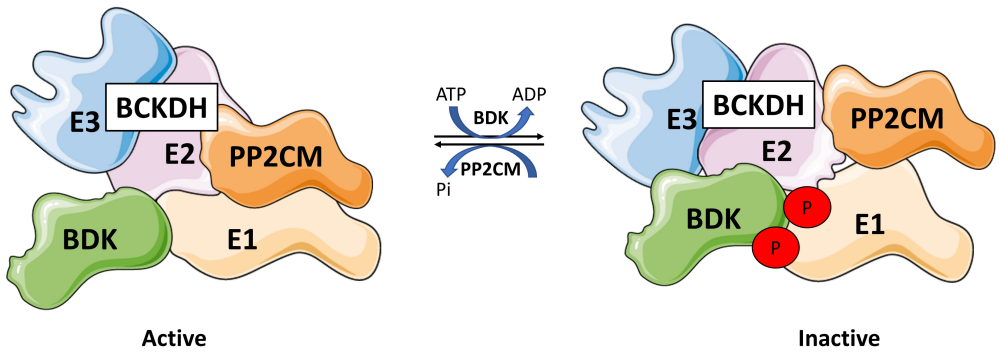

FIGURE 6 | Branched-chain ketoacid dehydrogenase regulation. BCKDH is regulated by a kinase, branched-chain ketoacid dehydrogenase kinase (BDK), and a phosphatase, protein phosphatase 2Cm (PP2Cm) that interact with E1 subunit. BDK phosphorylates BCKDH at Ser293 and 303 inhibiting BCKDH, while PP2Cm dephosphorylates BCDKH, reactivating the complex. Drawn using information from Damuni et al. (1984) and Popov et al. (1991).

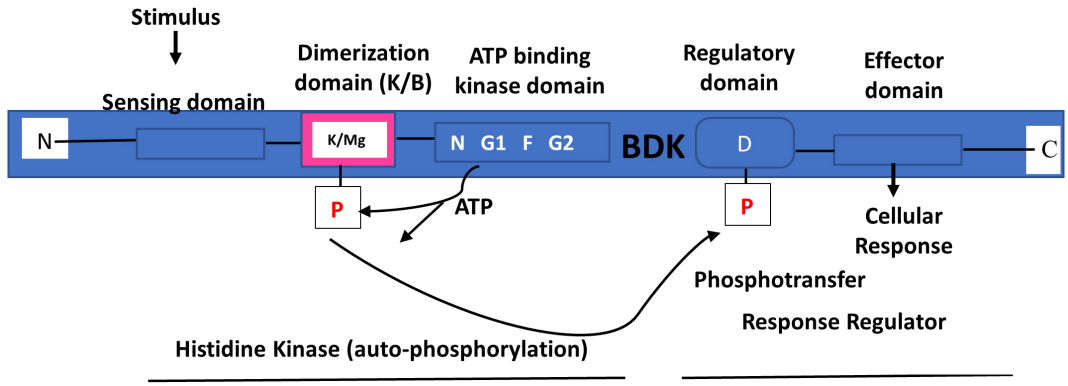

FIGURE 7 | Branched-chain ketoacid dehydrogenase kinase structure and the two-component signal transduction pathway. BDK contains ATP kinase domain with conserved N, G1, F, and G2 motifs. The phosphorylation of BCKDH by BDK is mediated by magnesium (Mg) and potassium (K). Drawn using information from Machius et al. (2001).

BCKDH (Wynn et al., 2000). Maximal decarboxylation activity of BCKDH is determined by the full lipoylation of the E2 subunit of BCKDH (Chuang et al., 2000). Consequently, increased BDK activity decreases the decarboxylation activity and the concentration of the subunits of BCKDH (Chuang et al., 2000).

Mice with muscle-specific deletion of BDK (BDK-mKO) have low skeletal muscle BCAA concentrations, with reduced leucine (∼50%), isoleucine (∼75%), and valine (50%). These mice also exhibit decreased phosphorylation of S6K1 on Thr389 (50%) and of 4E-BP1 (∼25%) when fed a low protein diet (Ishikawa et al., 2017). They have increased BCKDH activity in the heart (20%) and kidney (∼10-fold) (activity in muscle was not measured). They also show a decrease in myofibrillar protein content (∼20%) suggesting that increased BCAA catabolism negatively regulates protein synthesis in skeletal muscle (Ishikawa et al., 2017).

Nutritional Regulation of BDK

The activity state of rat hepatic BCKDH complex is regulated by its substrates KIC, KIV, and KMV via their effect on BDK. KIC inhibits BDK and thus activates BCKDH complex in rat liver and heart (Paxton and Harris, 1984; Popov et al., 1991). KIV and KMV also inhibit BDK but with reduced efficiency (Paxton and Harris, 1984; Shimomura et al., 2001). In rat liver, BCAA starvation and low protein diets (8% protein) increase BDK mRNA levels to inactivate the BCKDH complex by more than 90% (Espinal et al., 1986; Popov et al., 1995; Kobayashi et al., 1999; Harris et al., 2001). There is a decreased E1α/E2 ratio with low protein diet (5% leucine) that promotes BCKDH complex sensitivity to phosphorylation-induced inhibition by BDK (Zhao et al., 1994). On the other hand, high glucose levels suppress the activity of the E1α component of BCKDH and the whole complex in the islet of the pancreas (MacDonald et al., 1991). Rats re-fed a high fructose diet (60% fructose) for 4 h after an overnight fast exhibit 75% higher BDK mRNA expression in the liver (White et al., 2018). Because BDK also activates liver lipogenesis by phosphorylating ATP-citrate lyase at Ser454 residue (White et al., 2018), others have studied the effect of HFD on the activity of the enzyme. Rats on a HFD for 24 weeks have reduced (−25%) muscle BDK protein levels but calorie restriction and leucine treatment attenuate this decrease. However, there is no change in BCKDH E1α protein levels. Rats on a HFD for 32 weeks have increased (∼50%) muscle BDK protein levels, consistent with a ∼25% reduction in BCKDH E1α protein levels (Liu et al., 2017). These data correlate with a significant increase in serum KIC (50%) and KIV (25%) after 32 weeks of a HFD, further emphasizing the reduction of BCAA catabolism in a HFD model (Liu et al., 2017).

Hormonal Regulation of BDK

There is evidence for hormonal regulation of BDK. Hyperthyroidism, induced by thyroid hormone (T3, 3,5,3′-triiodothyronine) treatment, reduces BCKDH activity in the liver (∼70%), and does so through a 3× increase in BDK activity (Kobayashi et al., 2000). This effect is not seen in skeletal muscle, as BCKDH activity is already low (Kobayashi et al., 2000). Furthermore, as mentioned in Section "Nutritional Regulation of BDK," protein starvation increases BDK expression, and it also increases serum T3 (Edozien et al., 1978; Tulp et al., 1979), supporting the role of T3 in regulating BDK. Glucocorticoids on the other hand suppresses the expression of BDK. Liver BDK mRNA levels are reduced after dexamethasone treatment in protein starved rats (Huang and Chuang, 1999). Dexamethasone treatment also decreased BDK mRNA levels, increasing BCKDH activity in rat hepatoma H4IIE cells (Huang and Chuang, 1999). Glucocorticoids stimulate gluconeogenesis and since valine and isoleucine are glucogenic, the effect of glucocorticoids may be through the activity of BCKDH, as they downregulate BDK to activate BCKDH to potentially provide more substrate for gluconeogenesis (Harris et al., 2001). One study demonstrated that the glucocorticoid methylprednisolone upregulates BCKDH activity in skeletal muscle, similar to dexamethasone treatment in liver cells, although the effect on BDK was not measured (Block et al., 1987).

Diurnal rhythm and sex hormones may also regulate BDK. BCKDH activity is subject to diurnal rhythm, with activity being low at the beginning of the dark cycle, which also corresponds to increased BDK activity (Kobayashi et al., 1997; Obayashi et al., 2004). This rhythm is observed in females, but

not in male rats, which is indicative of regulation of BDK activity by the sex hormone, estrogen (Kobayashi et al., 1999; Obayashi et al., 2004). Indeed, the rhythm is lost in gonadectomized animals (Kobayashi et al., 1999; Harris et al., 2001). Finally, insulin effects a one-fold increase in BDK mRNA expression and a two-fold increase in BDK protein levels in Clone 9 rat cells (Nellis et al., 2002). These data are at odds with other reports that show that insulin increases whole body (Castellino et al., 1987) and skeletal muscle (Neinast et al., 2018) BCAA oxidation. Additionally, as discussed in Section "Diabetes/Insulin Resistance," T1DM increases BCAA levels, and Aftring et al. (1988) showed that insulin treatment attenuated the increases in plasma and intramuscular BCAAs in diabetic rats. Given that protein metabolism is influenced by sex (Chevalier et al., 2005; Murton and Greenhaff, 2009; Comitato et al., 2015), it is perhaps not surprising that BDK, and by implication, BCAA catabolism can be regulated by sex hormones. Nevertheless, this variable has not been rigorously studied. For example, it would be interesting to examine if alterations to BDK (and related enzymes involved in BCAA catabolism) in insulin resistant/diabetic individuals are sex-dependent, and if such differences are somewhat linked to differences between the sexes in measures of protein metabolism and abnormalities associated with insulin resistance and its sequelae like T2DM and cardiovascular disease. Diurnal regulation of BDK and BCAA catabolism has been even less studied. Because disruption of circadian rhythm is implicated in a number of diseases, including cardiovascular disease, obesity, and some cancers (Scheer et al., 2009; Baron and Reid, 2014; Rijo-Ferreira and Takahashi, 2019), that BDK regulation is under circadian rhythm regulation is of particular interest. Along this line, KLF15 is a transcriptional factor that regulates rhythmicity of AAs (including BCAAs) and nitrogen metabolism (Jeyaraj et al., 2012). Interestingly, a recent study in an animal model of spinal muscular atrophy (SMA) links disruption of circadian rhythm in the regulation of skeletal muscle BCAA catabolism to severity of phenotypes in these animals (Walter et al., 2018). Additional studies are needed to examine whether other enzymes involved in BCAA catabolic pathway, in addition to BDK, are also subject to circadian rhythm regulation, mechanisms of such regulation, and whether such regulation is altered in insulin resistance and its sequelae.

Transcriptional/Post-translational Regulation of BDK

There is a carbohydrate response element-binding protein-β (ChREBP-β) binding site 2.5 kb upstream of the BDK transcriptional start site (Jeong et al., 2011). Furthermore, adenovirus ChREBP-β treatment for 7 days upregulates BDK transcripts (∼50%) in rat liver (White et al., 2018). As discussed in Section "Nutritional Regulation of BDK," BDK is implicated in lipogenesis by activating ATP-citrate lyase. Since ChREBP-β activation contributes to fatty liver and dyslipidemia (Iizuka, 2017), regulation of BDK by this transcription factor further emphasizes the connection between the BCAA catabolic pathway and fatty acid metabolism.

BDK is also regulated post-translationally. Phosphorylation of BDK by Src on Tyr246 enhances BDK activity and stability which promotes metastasis in human colorectal cancer, by enhancing migration, invasion and epithelial to mesenchymal cell transition of colorectal cancer cells (Tian et al., 2020). BDK promotes colorectal and hepatocellular cancers through an alternative pathway that does not involve catabolism of BCAAs. In this context, BDK overexpression increases MEK and ERK activation, which has been linked to carcinogenesis (Lu et al., 2012; Cheng et al., 2016; Xue et al., 2017; Zhai et al., 2020). BDK inhibition by phenyl butyrate reverses this effect (Xue et al., 2017), suggesting that BDK could be a suitable target in the prevention of these cancers. In hepatocellular cancer, Aminopeptidase N is thought to regulate phosphorylation of Ser31 of BDK, which then increases BDK-mediated phosphorylation and activation of ERK1/2, a modification that is associated with increased hepatocellular carcinoma metastasis. However, the specific kinase that phosphorylates BDK on this site has not been elucidated (Zhai et al., 2020). This further emphasizes the importance of BDK in cancer progression and the link between BDK and ERK signaling, a link that has not been investigated in skeletal muscle. Combined with the already discussed effect of BDK on ATP-citrate lyase, this effect of BDK on ERK1/2 signaling demonstrate roles for BDK that appear independent of BCAA catabolism. It also raises the intriguing question of whether signaling pathways that regulate BCAA-independent functions of BDK can regulate this enzyme and whether such a regulation would have a 'spilling' effect on BCAA catabolism.

Ubiquitination of BDK is promoted by a ubiquitin E3 protein ligase (UBE3B) and this ubiquitination is disrupted in individuals with Kaufman oculocerebrofacial syndrome. In skeletal muscle of UBE3B KO mice, there is an accumulation of BDK that leads to a decrease in BCKDH complex activity, but this does not correlate with changes in BCAA levels, indicating that BCKAs could be driven to other pathways (Cheon et al., 2019). As discussed above, BDK phosphorylates ATP-citrate lyase, which is crucial for lipogenesis, an important process for neuronal development (Ziegler et al., 2017), therefore BDK accumulation may disrupt the regulation of lipogenesis in developing neurons. The focus of BDK transcriptional/translational regulation of BDK has mostly been in liver so more work on transcriptional/translational modifications of BDK in skeletal muscle is required.

PP2Cm Structure and Regulation

PP2Cm Structure

PP2Cm is a BCKDH phosphatase encoded by the *PPM1K* gene and is highly conserved in vertebrates (Lu et al., 2009; Dolatabad et al., 2019). The phosphatase activity of PP2Cm is dependent on Mn^{2+} bound in the active site (Wynn et al., 2012). PP2Cm is highly expressed in the liver, brain, heart, kidney and diaphragm, but low in skeletal muscle (Zhou et al., 2012). The enzyme binds and dephosphorylates BCKDH complex at Ser293 of the E1α (Lu et al., 2009). Mutation of the Ser293 residue, but not Ser303, affected the interaction of BCKDH with PP2Cm (Lu et al., 2009), suggesting that Ser293 is the critical residue in BCKDH activation. This interaction is disrupted in the presence of BDK suggesting that PP2Cm and BDK compete for interaction with the BCKDH complex (Zhou et al., 2012). Whole body knockout of PP2Cm abrogates the dephosphorylation and increase the

hyperphosphorylation of the E1α subunit of BCKDH complex in mice liver and heart (Lu et al., 2009; Wynn et al., 2012; Zhou et al., 2012). Mice lacking PP2Cm show 3–4-fold increases in circulating BCAAs and 5–10-fold increases in liver BCKAs (Abell, 2019), which is associated with numerous diseases, including insulin resistance, obesity, T2DM (Perng et al., 2014; Giesbertz et al., 2015). In liver, PP2Cm also can dephosphorylate ATP-citrate lyase to prevent lipogenesis (White et al., 2018).

PP2Cm Regulation

PP2Cm is regulated transcriptionally in response to nutrients (Zhou et al., 2012). Food deprived mice show a decrease in PP2Cm mRNA levels (Lu et al., 2009). BCAAs and BCKAs promote the interaction of PP2Cm with the BCKDH complex (Lu et al., 2009). In HepG2 cells, removal of BCAAs decreases PP2Cm expression (40%) while BCAA replenishment in medium rescues PP2Cm mRNA levels (Zhou et al., 2012). The effect appears to be at the transcriptional level, as PP2Cm promoter activity is decreased by 50% when BCAAs are removed from the medium (starvation) (Zhou et al., 2012). Expression of PP2Cm (mRNA and protein) in rat heart is down-regulated in pressure overload model of heart stress (Lu et al., 2007), which is not what you would expect, as catabolic pathways are induced during stress (Church et al., 2019). Similar to BDK discussed in Section "Transcriptional/Post Translational Regulation of BDK," PP2Cm is also regulated by ChREBP-β in the liver, but in a negative manner. This results in an upregulation of lipogenesis (White et al., 2018). Also, rats referred a high fructose diet (60% fructose) for 4 h after an overnight fast exhibit lower PP2Cm mRNA expression (25%) (White et al., 2018). High fructose diets induce insulin resistance (Basciano et al., 2005), and reduced PP2Cm is consistent with reduced BCKDH activity seen in insulin resistant states like diabetes, as discussed later in Section "Type 2 Diabetes/Obesity." The fact that substrate availability and insulin resistant regulate PP2Cm expression are consistent with what one might expect. Unlike BCAT2, BCKDH-E1α subunit, and BDK, for which there is at least a few evidence of posttranslational modifications, we did not see evidence that PP2Cm is so regulated. Furthermore, mechanisms that regulate the abundance of the enzyme are yet to be identified.

MicroRNAs

Like BCAT2, PP2Cm too is regulated by miRNA. PPM1K mRNA expression is suppressed by miR-204/211 in mouse cell (NIH 3T3) and miR-22 in human cells (HepG2 and HeLa) (Pan et al., 2015). Overexpression of these miRNAs decreases the 3-UTR′ activity of endogenous mRNA of PPM1K (Otulakowski and Robinson, 1987). Finally, BCKDH mRNA expression is controlled by human miR-29b and miR-222. miR-29b interacts with the mRNA of the E2 subunit of BCKDH complex to prevent its translation in human HEK293 cells (Menkes et al., 1954). Studies on the regulation of BCAA catabolism by miRNA are only at the initial stage: we are unaware of data on muscle miRNA that controls PP2Cm expression. Such regulation presents an attractive option to control the metabolism of these AAs since the expression of miRNA can be upregulated or downregulated to control the abundance of their targets (Peng and Wang, 2018).

Nutritional and Physiological Regulation of BCAA Metabolism and Related Enzymes

Fatty Acid Oxidation

Clofibric acid, a drug that promotes fatty acid oxidation, increases gastrocnemius muscle BCKDH activity by ∼50% (Paul and Adibi, 1980). Peroxisome proliferator-activator receptor-α (PPARα) might mediate the effect of free fatty acids (FFA)/FFA oxidation on BCAA oxidation (Kobayashi et al., 2002). Circulating long-chain FFA released due to starvation and exercise bind to PPARα to activate fatty acid oxidation in skeletal muscle and liver (Minnich et al., 2001). Interestingly, PPARα that is released in response to exercise inhibits hepatic BDK expression and promotes BCKDH activity in skeletal muscle (Brabin et al., 2001; Shimomura et al., 2004b).

Exercise

Endurance exercise promotes BCAA catabolism and activation of BCKDH complex in human (Wagenmakers et al., 1989) and rat (Shimomura et al., 1995; Fujii et al., 1998) skeletal muscles via an increase in fatty acid oxidation. Manipulation of the BDK-BCKDH interaction is a potential short-term regulatory mechanism for the activity of BCKDH complex (Xu et al., 2001). Exercise training increases human skeletal muscle BDK protein content by 30% and decreases BCKDH complex activity (Howarth et al., 2007). This could be due to the significant increase in NADH levels during maximal exercise and submaximal isometric contractions (Sahlin, 1985; Henriksson et al., 1986), as NADH inhibits BCKDH activity (Brosnan and Brosnan, 2006). However, most studies show the opposite. Endurance training reduces the number of BDK bound to BCKDH complex in rat liver and skeletal muscle, with a greater reduction in liver (∼71%) compared to skeletal muscle (∼30%) (Fujii et al., 1998; Kobayashi et al., 1999; Xu et al., 2001; Shimomura et al., 2004a). This reflects the low activity state of BCKDH complex in the muscle (Xu et al., 2001). The reduction in BDK levels in response to exercise could partly explain increased BCKDH activity after exercise but increases in KIC concentration too could mediate the effect of exercise on BDK and BCKDH activity because of the inhibitory effect of KIC on BDK. This is in line with the observation of an increase in KIC concentration in response to electrically stimulated muscle contraction (Shimomura et al., 1993). BCAA catabolism may be increased due to the reduction in glutamine levels post exercise. Glutamine synthesis is an ATP-dependent condensation of glutamate and ammonia. Glutamate and glutamine levels and glutamine synthesis are reduced post-exercise while glutamine uptake is unchanged in rat skeletal muscle (Dos Santos et al., 2009). Additionally, plasma ammonia levels are increased post exercise (Chen et al., 2020). Thus, it is possible that increased BCAT activity is needed to produce glutamate for ammonia detoxification (Holecek, 2013). BCAA catabolism also feeds into the TCA cycle to ultimately produce ATP. Therefore, BCAA catabolism could be enhanced to replenish the glutamate and ATP pool for glutamine synthesis/ammonia detoxification post exercise. During exercise, there is a net breakdown of proteins

to produce AAs for oxidation and gluconeogenesis (Lynis Dohm et al., 1987). This could also partly explain the increase in BCAA catabolism during exercise.

Adiponectin

Adiponectin (APN) is an adipokine that regulates glucose metabolism and fatty acid breakdown (Turer and Scherer, 2012). There is evidence that it can regulate BCAA catabolism. APN whole-body KO mice fed HFD have reduced (50%) PP2Cm expression and BCKDH activity in liver and adipose tissue. In addition, these mice exhibit ∼50% increases in liver, adipose tissue and skeletal muscle BDK mRNA expression, as well as elevated plasma BCAA (40%) and BCKA (∼30%) levels (Lian et al., 2015). APN treatment reverses these effects. The effect of APN on BCKDH activity is mediated by PP2Cm, as PP2Cm deficiency partially inhibits APN-induced activation of BCKDH. This effect of APN on BCKDH was also abolished when AMPK was inhibited. Supplementation of AMPK activator, AICAR, decreased liver, adipose tissue, and skeletal muscle BDK protein abundance, and increased PP2Cm protein abundance in adipose tissue and liver. AICAR supplementation also increased BCKDH activity in skeletal muscle (Lian et al., 2015). Another study supports this as APN treatment corrects increases in BCAA concentrations from a HFD in skeletal muscle (Liu et al., 2013). APN is reduced (∼25%) in obesity and diabetes (Weyer et al., 2001), conditions that are also associated with reduced liver, adipose tissue and skeletal muscle PP2Cm and BCKDH activity (Lian et al., 2015; Biswas et al., 2019). Adiponectin regulation of BCAA metabolism adds to the connection between BCAA catabolism, fatty acid oxidation and adipose tissue regulation of whole body insulin sensitivity via regulation of BCAA catabolism.

EFFECT OF DIFFERENT DISEASES ON BCAA CATABOLISM IN SKELETAL MUSCLE

Skeletal muscle is a major site for the initial step of BCAA catabolism, leading to the release of alanine and glutamine in the blood (Holeček, 2018). There are links between BCAA catabolism and disorders such as MSUD, isovaleric acidemia (IVA), methylmalonic acidemia (MMA), propionic acidemia (PA), liver cirrhosis, sepsis, chronic renal failure (CRF), muscle wasting, type 2 diabetics/obesity and neurodegenerative disorders like Alzheimer's disease (AD) (Burrage et al., 2014; Manoli and Venditti, 2016; Siddik and Shin, 2019). In this section we will discuss how these diseases affect muscle BCAA catabolism. While other diseases too may affect BCAA catabolism, we have focussed on those for reason of space and because there is sufficient data to support the link between these conditions and BCAA catabolism. In the next section, we discuss the effects of altered muscle BCAA catabolism on whole body metabolism.

Liver Cirrhosis

Reductions in both plasma BCAAs and protein synthesis are seen in liver cirrhosis patients (Holecek et al., 2011). Mechanistically, decreased BCAAs in this condition may be due to an enhancement of BCKDH complex activity (Shimomura et al., 2006). Reduced BCAA concentrations resulting from elevated BCKDH activity suggest that BCAA supplementation may be beneficial for patients. Indeed, 6 months of daily BCAA consumption (12.45 g of BCAAs daily) improved liver disease scores and other indicators of liver cirrhosis severity (Park et al., 2017). Randomized control trials have also found improvements in general health scores (Marchesini et al., 2003), fatigue (Nakaya et al., 2007), and sleep disturbances (Ichikawa et al., 2010) following BCAA supplementation. Further, BCAA supplementation improves muscle strength (Tajiri and Shimizu, 2013), increases protein metabolism and suppresses further worsening of symptoms (Togo et al., 2005). Additionally, patients with liver cirrhosis also develop insulin resistance (Kawaguchi et al., 2011). However, both intravenous (Tabaru et al., 1998) and oral (Korenaga et al., 2008) BCAA supplementation has been found to reduce blood glucose levels. This literature highlights the therapeutic potential of BCAA supplementation in limiting the severity of symptoms and outcomes in liver cirrhosis patients.

Hyperammonemia is another cause of reduced BCAAs in liver cirrhosis patients (Holecek et al., 2011). Following hyperammonemia, skeletal muscle can absorb ammonia from the blood and detoxify it via synthesis of glutamine, a process known as ammonia detoxification (Holecek et al., 2011). In slow twitch soleus muscles, hyperammonemia reduces (∼80%) BCAA (leucine, isoleucine, and valine) release and increases (∼1.3-fold) BCKA (KIV, KIC, and KMV) release. Similarly, in fast twitch EDL, hyperammonemia reduced BCAA release (60%), increased BCKA release (∼160%) and increased leucine oxidation (∼1.7-fold). In addition, AAs glutamine, glutamic acid and alanine are all reduced following hyperammonemia in the soleus and EDL. Interestingly, these alterations in BCAA catabolism had no effect on protein synthesis, protein turnover or myofibrillar proteolysis in skeletal muscle (Holecek et al., 2011). However, studies in patients with liver cirrhosis have found BCAA supplementation to improve muscle strength (Tajiri and Shimizu, 2013), increase protein metabolism and suppress further worsening of liver cirrhosis (Togo et al., 2005). These beneficial effects of BCAA are contrary to what has been described about the link between BCAA and insulin resistance/T2DM and show that the effects of BCAA on health outcomes are context-dependent.

Chronic Renal Failure

Chronic renal failure (CRF) is associated with decreased plasma and skeletal muscle levels of the BCAAs and BCKAs (Indo et al., 1987). In addition, metabolic acidosis, a common condition found in CRF patients, is also linked to increased abundance of the BCKDH complex in muscle (Hara et al., 1987). CRF patients display elevated BCAA oxidation in skeletal muscle, which may serve to provide nitrogen for glutamine production and excretion of acids in the kidney (Mitch et al., 1994). Thus, increased abundance and activity of BCKDH in skeletal muscle is primarily responsible for the decreased levels of BCAAs and BCKAs found in CRF. Interestingly, ingestion of AAs in CRF patients increases uptake of the non-essential AAs, but not of the BCAAs in skeletal muscle. Therefore, restricted protein intake along with BCAA supplementation may be required to

delay renal disease progression and uremic toxicity (Cano, 2009; Holeček, 2018). Since the BCKAs are also reduced in CRF (Indo et al., 1987), ketoanalogues of AAs and of BCAAs are also often used in the treatment of CRF. Twelve to 24 weeks of ketoacid treatment enhanced renal function, exemplified by decreased proteinuria, glomerular sclerosis, and tubulointerstitial fibrosis (Gao et al., 2010; Meisinger et al., 1987; Zhang et al., 2016; Liu et al., 2018). Previous studies have reported similar findings (Maniar et al., 1992), in addition to enhanced globular filtration rate and survival in rats (Barsotti et al., 1988). More recently, a study by Wang et al. found that 24 weeks of ketoacid treatment in rats with CRF increased body weight, mitochondrial electron transport chain activity and decrease oxidative damage in muscle (Wang D. et al., 2018). The mechanisms of beneficial effects of supplementation with ketoacids on muscle require further investigation. While the mechanisms may involve BCKA to BCAA conversion, the beneficial effects may also be independent of BCAA, for example by the actions of the ketoacids on mitochondrial integrity and function.

Maple Syrup Urine Disease

Maple syrup urine disease (MSUD) is a hereditary recessive disorder characterized by neurological and development dysfunction, and by a distinct sweet odor in the urine of infants (Indo et al., 1987). Pathogenesis of MSUD is linked to impairments in BCAA catabolism, most often due to alterations in the BCKDH complex. MSUD is classified as types I, II, or III depending on whether E1, E2, or E3 subunit of BCKDH is mutated (Nobukuni et al., 1991). Individuals with type 1A MSUD present with a missense mutation at the C-terminal aromatic residue of the E1α subunit of the BCKDH assembly. This mutation hinders the catalytic activity of BCKDH (Menkes et al., 1954). Similarly, an 11-bp deletion mutation in the E1β subunit of the BCKDH complex that results in a change to the reading frame and subsequent E1α instability, also causes a decrease in enzymatic function *in vitro* (Nobukuni et al., 1991). These findings suggest the importance of E1β in normal functioning of the BCKDH complex in MSUD.

Evidence of abnormalities in skeletal muscle are also found in MSUD patients. Muscle fibers from MSUD patients show abnormal myofibril physiology (Ferrière et al., 1984). In addition, decreases in fiber cross sectional area (CSA) of the quadriceps (∼30%) and gastrocnemius (35%) are observed in MSUD mice. These mice show intra-muscular build up of AMP and NADH, suggesting mitochondria dysfunction as a result of BCKA accumulation, especially KIC (Sonnet et al., 2016). Accumulation of NADH in this condition is another point of link between BCAA catabolism and fatty acid metabolism, as NADH would affect the TCA cycle and impede β-oxidation of fatty acid. The data also suggest that upregulating BCKDH activity by treatment with, for example BT2, an inhibitor of BDK (Neinast et al., 2018), may attenuate accumulation of BCKAs in MSUD.

Sepsis and Trauma Injury

Previous reports have suggested that sepsis and trauma [which often lead to muscle wasting (Owen et al., 2019)] is associated with increased BCAA oxidation in skeletal muscle (Holecek and Sispera, 2014). Mechanistically BCAAs are an important nitrogen donor for glutamine synthesis (Holecek and Sispera, 2014). Glutamine is a conditionally essential AA (Newsholme and Hardy, 1997) that is required for, amongst others, functioning of the immune system (Exner et al., 2002). Due to the reduction in glutamine pools in sepsis and trauma, the skeletal muscle compensates to increase glutamine production and does so through enhanced BCAA oxidation (Roth et al., 1982 and Holecek and Sispera, 2014). This is consistent with an increase in BCKDH activity in sepsis (Holeček, 2018). Also, increased inflammatory cytokines in sepsis decrease BCAA absorption from the gut and inhibit BCAA transport from the blood to muscles (Hasselgren et al., 1988; Gardiner and Barbul, 1993). Leucine release from the liver of endotoxin-treated animals after addition of KIC supplementation (Holecek et al., 1998) suggests that visceral tissues aminate BCKAs to produce BCAAs in sepsis in response to the reduced transport of BCAAs to the muscle from the blood.

Type 2 Diabetes/Obesity

As discussed in Section "Diabetes/Insulin Resistance," BCAA catabolism in skeletal muscle, liver, and adipose tissue is reduced in T2DM. BCKDHβ (∼50%) and BCAT2 (∼25%) mRNA expressions are reduced in skeletal muscle of type 2 diabetic subjects (Hernández-Alvarez et al., 2017). However, db/db mice (a model of diabetes) display no changes in BCKDHα or BCKDHβ mRNA levels, but show a decrease (∼50%) in BCKDHα protein expression and an increase in BCAT2 protein levels (∼25%) in skeletal muscle (Hernández-Alvarez et al., 2017). Similar alterations are seen in liver and adipose tissue. Liver BCKDH activity (∼2-fold), and adipose tissue BCKDHα (∼90%) and BCAT2 (∼90%) protein levels are also reduced in db/db mice (Hernández-Alvarez et al., 2017). Reduced abundance of BCKDH is consistent with suppressed (∼40%) BCKDH activity in skeletal muscle of type 2 diabetic mice. Concomitantly, BDK protein expression is ∼2-fold greater, with no change in PP2Cm (Lian et al., 2015). Furthermore, BCAA levels are elevated in the hearts of diabetic mice (twofold). These mice exhibit a defect in BCAA catabolism by way of reduced PP2Cm expression (∼40%) and diminished BCKDH activity in the heart (Lian et al., 2020). Interestingly, these changes appear specific to diabetes, as ob/ob mice show no change in the mRNA or protein expression of BCAT2 or BCKDH in skeletal muscle (Hernández-Alvarez et al., 2017). In another study, ob/ob mice exhibit significant decreases in BCAT2, BDK, PP2Cm, and BCKDH mRNA expression in adipose tissue (Zhou et al., 2019), but to a lesser extent for BDK. Downstream BCAA catabolic enzymes are also downregulated in ob/ob mice adipose tissue. This is also seen in the liver, but the effect is not as drastic, and BDK expression is increased (∼50%). On the other hand, in skeletal muscle of ob/ob mice, there is no change in the mRNA expression of these BCAA catabolic enzymes (Zhou et al., 2019). This differential tissue regulation of these enzymes results in increased BCAAs in adipose tissue, a decrease in liver BCAAs and no change in skeletal muscle. This suggests that obesity primarily targets BCAA catabolism in adipose tissue and liver but not in skeletal muscle. However, glycolysis, a process that converts

glucose into pyruvate may induce BCAA catabolism in skeletal muscle. Glycolysis increases TCA cycle flux and the supply of α-KG for BCAT2. It is also possible that in insulin resistance, impairment in glucose metabolism may be linked to altered BCAA catabolism. Under such a condition, glycolysis is reduced, resulting in reduced pyruvate, and ultimately less TCA cycle flux and α-KG for transamination of BCAAs by BCAT2 (Holeček, 2020). This leads to reduced BCAT2 activity in skeletal muscle, and increased BCAA levels in plasma. Related to this, treatment with a BDK inhibitor BT2, which increases BCAA catabolism, restores the decrease in insulin sensitivity normally associated with elevated BCAA/BCKAs, suggesting that increasing BCAA catabolism may help against obesity-induced insulin resistance (Zhou et al., 2019). She et al. (2007b) support this, as in an animal model of obesity, plasma BCAA levels were ∼50% greater in Zucker rats compared to lean mice. Interestingly, no changes were found for the protein content of either BCAT2 or BCKDH E1α in the gastrocnemius. However, these mice exhibit reduced BCKD E1α protein content in both the liver (∼30%) and adipose tissue (∼60%) (She et al., 2007b). On the other hand, one study showed significant decreases in BCKDH activity (∼40%) in skeletal muscle of ob/ob mice, consistent with a significant increase in BDK protein levels (∼2-fold), but no change in PP2Cm protein levels (Lian et al., 2015). Although the studies reviewed do not all show a consistent pattern of regulation of BCAA catabolism enzymes in muscle, they all point to altered tissue and whole-body metabolism of these AAs in obesity/insulin resistant states, and that correcting such defects ameliorates insulin resistance.

Skeletal Muscle Wasting and Fatigue

Although not a disease, altered BCAA metabolism is seen in skeletal muscle wasting and fatigue. There is an accumulation of muscle inosine monophosphate (IMP) in BCAT2 KO mice. This finding is likely due to the decreased exercise tolerance and increased muscle fatigue in these mice (She et al., 2010). However, BCAT2 KO mice fed a BCAA diet show a 39% increase in protein synthesis. The increased protein synthesis correlates with elevated mTORC1 signaling, indicated by higher 4E-BP1 and S6 phosphorylation. Interestingly, these mice present without any change in muscle weight and structure (She et al., 2007a). These results suggest that disruption of BCAA metabolism (by way of BCAT2 KO) may not only promote muscle fatigue and decreased exercise tolerance (She et al., 2007a, 2010), but may also upregulate pathways involved in both skeletal muscle protein synthesis and breakdown (Lynch et al., 2015).

EFFECTS OF ALTERED/DISRUPTED BCAA CATABOLISM ON WHOLE-BODY METABOLISM

Insulin Resistance/Type 2 Diabetes Mellitus

BCAAs are nutrient signals required for muscle protein synthesis and growth (Yoshizawa, 2004). However, elevated levels of plasma BCAAs are seen in insulin resistant disorders such as obesity (Newgard et al., 2009; Lackey et al., 2013; Mccormack et al., 2013) and T2DM (Andersson-Hall et al., 2018; Vanweert et al., 2020). T2DM patients also exhibit elevated levels of the BCAAs (∼13%) in skeletal muscle (She et al., 2013) and plasma BCKAs are higher in insulin resistant individuals (Newgard et al., 2009; Mccormack et al., 2013; Giesbertz et al., 2015). Increased BCAA levels in skeletal muscle can lead to sustained mTORC1/S6K1 activation and thus impair insulin signaling/sensitivity via attenuation of PI3K/Akt signaling (Yoon, 2016; Crossland et al., 2020). This is evident as leucine (150 μM) treatment in starved L6 myotubes suppresses insulin-stimulated glucose uptake (∼37%), with an increase in S6K1 phosphorylation (∼30%). KIC (200 μM), the ketoacid of leucine, too suppresses insulin-stimulated glucose uptake (∼60%) (Moghei et al., 2016; Mann and Adegoke, 2021), with an increase in S6K1 phosphorylation (∼5-fold) (Moghei et al., 2016). This effect of KIC is attenuated in BCAT2-depleted cells (Mann and Adegoke, 2021), suggesting that KIC is converted back to leucine to suppress insulin-stimulated glucose uptake. In line with this, myotubes depleted of BCKDH have reduced insulin-stimulated glucose uptake (∼33%), but with no change in the Thr389 phosphorylation of S6K1, suggesting an alternative mechanism of insulin resistance (Mann and Adegoke, 2021). For example, increased levels of 3-hydroxyusobutyrate (3-HIB), a metabolite of valine, may contribute to insulin resistance. Knockdown of 3-HIB dehydrogenase, the enzyme that catabolizes 3-HIB, results in 3-HIB accumulation. This leads to increased endothelial fatty acid uptake by the skeletal muscle, contributing to reduced insulin sensitivity (Jang et al., 2016). Interestingly, BCAA restricted Zucker fatty rats show a reduced level of malonyl CoA, a regulator of fatty acid oxidation (Ruderman et al., 1999; McGarrah et al., 2020), potentiating increased insulin sensitivity (Ruderman et al., 1999). Restricting BCAAs in Zucker rats improves skeletal muscle insulin sensitivity, along with enhanced fatty acid oxidation and acyl-glycine export (White et al., 2016). On the other hand, body builders and athletes that consume BCAA supplements are not insulin resistant (Helms et al., 2014; Shou et al., 2019). This suggests that the effects BCAA consumption on insulin sensitivity likely depend on physiological context (energy and substrate requirements) and underlying (subtle) co-morbidities. For example, as discussed above (see section "Exercise"), exercise increases BCAA catabolism (Wagenmakers et al., 1989; Shimomura et al., 1995; Fujii et al., 1998) and improves insulin resistance, which many offset the increase in BCAA consumption by these athletes.

As discussed in Sections "Nutritional Regulation of BDK" and "PP2Cm Structure," BDK and PP2Cm regulate ATP-citrate lyase (White et al., 2018). These findings suggests a link between increased BCAA catabolic flux and fatty acid uptake/metabolism in the development of insulin resistance (Jang et al., 2016). Consistent with this, mice heterozygous for the BCAA catabolic enzyme methylmalonyl-CoA mutase exhibit insulin resistance and reduced fatty acid oxidation (Lerin et al., 2016). This could be because reduced BCAA oxidation results in a reduced availability of BCAA-derived anaplerotic TCA intermediates, which has been

shown to reduce fatty oxidation and increase the accumulation of acylcarnitines that can activate proinflammatory pathways (Adams et al., 2009), contributing to insulin resistance. In line with this, treatment with BT2 (an inhibitor of BDK) and adenoviral expression of PPM1K significantly increase insulin sensitivity via enhanced BCKDH activity and improvement in lipid metabolism (White et al., 2018).

Heart Failure/Cardiovascular Diseases

Pathological cardiac hypertrophy is an indicator of heart failure. In cardiomyocytes, the transcription factor KLF15 inhibits hypertrophic gene expression and protein synthesis (Fisch et al., 2007). In addition, KLF15 is a central regulator of BCAA metabolism in cardiomyocytes (Fan et al., 2018). Ablation of KLF15 in mice reduces mRNA (by 50–75%) and protein (∼50%) expression of known BCAA catabolic genes, BCAT2, BCKDHα, BCKDHβ and PP2Cm in the heart. The BCAA catabolic defect in these mice led to an impairment in cardiac contractility and greater susceptibility to heart failure. On the other hand, stimulation of BCAA catabolism by BT2 increases cardiac BCKDH activity (∼7-fold) and reduces plasma BCKA levels (Sun et al., 2016). Consistent with this observation, stimulation of BCAA catabolism in cardiomyocytes that mitigates accumulation of BCAAs/BCKAs maintains cardiac function and increases the life span of cardiomyocytes (Tso et al., 2013).

Autism and Neurological Disorders

Autism Spectrum Disorder (ASD) is a neurodevelopment abnormality characterized by an impairment in social interaction (Sparks et al., 2002). Previous reports have suggested causative links between impaired BCAA metabolism and ASD. In one study, deleting an AA transporter (solute carrier transporter 7A5) in the brain results in abnormal AA profiles and neurological abnormalities in mice (Tărlungeanu et al., 2016). In autistic patients, homozygous nonsense mutations in BDK results in reduced phosphorylation of the E1α subunit of BCKDH, leading to decrease in plasma BCAA levels (Novarino et al., 2012). Similarly, whole-body BDK knockout in mice increases brain levels of AAs (threonine, phenylalanine, tyrosine, histidine, and methionine) and causes abnormal neurobehavior, along with reduced BCAA levels. Interestingly, BCAA supplementation reverse the increased levels of neural AAs, the neurological defects and normalizes plasma BCAA levels (Novarino et al., 2012).

Cancer

Different cancer types show alterations in BCAA metabolism (Sivanand and Vander Heiden, 2020). A common feature of many tumor cells is an increased requirement for glutamine (Yang et al., 2017; van Geldermalsen et al., 2018; Jiang et al., 2019; Matés et al., 2019), an AA that can be produced via amino group donation from the BCAAs (Holecek and Sispera, 2014). Some tumor cells can use BCAAs as alternative 'fuel' to drive tumor formation (Keenan and Chi, 2015). Overexpression of BCAT1 in mice accelerates tumor growth in hepatocellular carcinoma (Zheng et al., 2016), breast carcinoma (Siddiqui and Williams, 1989), endometrial cancer (Wang P. et al., 2018), and myeloid leukemia (Hattori et al., 2017), while suppression of BCAT1 reduces proliferation in glioblastoma (an aggressive cancer of the brain and spinal cord) (Tönjes et al., 2013).

In hepatocellular carcinoma, BCAT1/2 mRNA is elevated. However, BCKDH and other BCAA catabolic enzymes are decreased (Ericksen et al., 2019). Mechanistically, BCKAs would be unable to undergo further metabolism, leading to re-amination into BCAAs and subsequent increases in mTORC1 activity. Consistent with this, overexpression of BCAT1 in hepatocellular carcinoma leads to chemoresistance following cisplatin treatment (Zheng et al., 2016).

In breast cancer, BCAT1 and BCKDH are upregulated, enhancing BCAA metabolism and leading to increased substrates for the TCA cycle. BCAT1 knockdown resulted in blunted growth of breast cancers (Zhang and Han, 2017). In addition, expression of BCAT1 activates mTORC1 signaling to increase mitochondrial biogenesis and ATP production contributing to growth and colony formation of breast cancer cells (Zhang and Han, 2017). Similarly, tumors from ovarian cancer patients also have upregulated BCAT1. Mechanistically, inhibition of BCAT1 may not only reduce genes associated with tumorigenesis, but may also result in less energy production from the TCA cycle (Wang et al., 2015).

Elevated BCAA levels are linked to promotion of tumor development in patients with PDAC (Lieu et al., 2020). In addition to other possible roles, PDAC also utilizes BCAAs as a carbon source for fatty acid biosynthesis during cell proliferation (Lee et al., 2019). Consistent with this data, increased BCAT2 and BCAA catabolism are seen in PDAC, and knockout of BCAT2 prevented PDAC development (Li J. T. et al., 2020), highlighting the necessity for BCAT2-mediated catabolism in PDAC. However, a previous report did not demonstrate a role for BCAT1/2 in PDAC (Mayers et al., 2017) suggesting the need for more research in this area. In sum, inhibition of BCAT seems to hold promise for treatment of multiple cancers.

In addition to the impact of altered BCAA catabolism on metabolic diseases such as insulin resistance and its sequelae (T2DM, cardiovascular disease), as well as some cancers, the studies reviewed here show that impairments in BCAA catabolism have implications for neurological disorders.

CONCLUDING COMMENTS AND OUTSTANDING QUESTIONS

A major issue in this discussion is whether the altered metabolism of BCAA in muscle and other tissues cause or are a consequence of the associated diseases. Except in relatively rare conditions such as MSUD, where impairments in BCAA catabolism are causative, it is challenging to prove causality between BCAA catabolism and the indicated human metabolic diseases. Studies in rodents, which permit use of specific inhibitors and genetic perturbations, have proven useful. Replicating such studies in humans will be more challenging if not impossible. As mentioned before, data on the beneficial effects of protein and BCAA supplementation in athletes clearly indicate that the effects of these nutrients on metabolic outcomes are context dependent. It may not be possible to correct for all the metabolic confounders because, for example, the effects of such confounders might be

subtle and additive, even if not measurable because they are at initial stages of impairments.

The link between BCAA catabolism and lipid metabolism suggest that dietary interventions that focus on one of these macronutrients at the expense of other are likely to yield an incomplete picture. Related to this, consumption of whole egg promotes greater myofibrillar protein synthesis compared to egg white (which is a complete, high quality protein) in men, post resistance exercise (Van Vliet et al., 2017). In that study, energy intake was not balanced between study groups but the links between BCAA catabolism and lipid oxidation reviewed here would suggest that the difference might not be due to caloric content of the two foods alone. Related to this is how the nature of the fatty acids (mono or polyunsaturated fatty acids, $n-3$ vs. $n-6$) would modulate the effect of protein/AA supplements in athletes.

The anabolic effects of leucine and other BCAAs are least in part linked to their effects on mTORC1. Interestingly, insulin stimulates BCAA catabolism (Neinast et al., 2018). Moreover, insulin resistance is linked to both altered BCAA catabolism (Lerin et al., 2016; Wang et al., 2019) and increased mTORC1 signaling (Moghei et al., 2016; Yoon, 2016). An intriguing question then is whether mTORC1 regulates BCAA catabolism. White adipocytes from adipose tissue-specific raptor knockout (i.e., attenuated mTORC1 signaling) mice are impaired in their ability to respond to PPARγ-induced catabolism of BCAA (Andrade et al., 2021). In addition, leucine-induced increase in heart BCKDH complex activity is impaired in the presence of rapamycin, an inhibitor of mTORC1 (Zhen et al., 2016). This effect of mTORC1 on BCAA catabolism appears counter-intuitive, given the anabolic nature of the complex. We are unaware of similar studies in skeletal muscle but if mTORC1 activates muscle BCAA catabolism, this would be inconsistent with the beneficial effects of rapamycin in ameliorating insulin resistance (Leontieva et al., 2014; Blagosklonny, 2019).

While the three BCAA have anabolic properties, an emerging concept is the observation that each of these AAs has unique metabolic imprints. For example, a recent study shows that many of the metabolic abnormalities attributed to BCAA are mediated by isoleucine and to a lesser extent valine, with leucine having little to no effect (Yu et al., 2021). Although another recent study in C2C12 did not find an effect of valine on insulin resistance (Rivera et al., 2020a), the data from Yu et al. (2021) are consistent with a previous report of greater effect of valine (compared to leucine) in inducing insulin resistance of glucose transport in L6 myotubes (Tavajohi-Fini, 2012) and of the effect of the valine metabolite 3-hydroxyusobutyrte (3-HIB) in inducing insulin resistance in skeletal muscle (Jang et al., 2016). Because normally, humans do not consume these AAs singly in isolation, it will be interesting to see the relevance of this in management of insulin resistance. In addition, because the three BCAA are essential AAs that are required in relatively large amounts, if the data on the impact of each of them on metabolic abnormalities are confirmed in other human studies, this would most certainly have implications for their requirements. For example, how would consuming a diet perpetually low in isoleucine and valine affect tissue (especially skeletal muscle) protein metabolism and muscle mass?

Aging is associated with increased rate of muscle loss and strength (sarcopenia) which can be improved with essential AA ingestion and resistant exercise (Dickinson et al., 2013). Also, for many of the human diseases, including diabetes, cardiovascular disease, insulin resistance and cancer, age is the most important risk factor (Hall, 2015; Mullard, 2018). How BCAA catabolism, specifically the activities of BCAT2 and BCKDH, change with age is an area that needs to be explored. Finally, as mentioned before, not much is known about the post translational regulation of the BCAA catabolic enzymes in skeletal muscle.

There is growing literature suggesting that diseases such as T2DM, insulin resistance, renal failure and MSUD develop in response to altered whole-body and skeletal muscle BCAA metabolism. Future studies addressing gaps in our understanding of the links between altered metabolism of the BCAAs and muscle and whole-body physiology are warranted. From such studies, we may identify metabolic targets that can be explored and used in the prevention/treatment of many human diseases, especially those that lead to defects in muscle growth and functions.

AUTHOR CONTRIBUTIONS

GaM, SM, and GlM drafted the initial version of the manuscript. GaM, SM, GlM, and OA reviewed and edited the manuscript. All authors approved the final version of the manuscript.

FUNDING

This work was supported by funds from the Natural Sciences and Engineering Research Council of Canada (grant #RGPIN-2021-03603) and from the Faculty of Health, York University.

ACKNOWLEDGMENTS

Figures 1, **3–5** were generated using images assembled from Servier Medical Art (https://smart.servier.com/), used under Creative Commons Attribution 3.0 Unported License.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 27 August 2021
doi: 10.3389/fnut.2021.728477

Dietary Betaine Addition Alters Carcass Traits, Meat Quality, and Nitrogen Metabolism of Bama Mini-Pigs

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Betaine is widely used as feed additives in animal husbandry as it can cause many benefits such as improving antioxidant ability, growth performance, and carcass traits. However, there are limited studies about the effects of betaine on the Bama mini-pigs. The present study was conducted to evaluate the effects of dietary betaine on carcass traits, meat quality, and nitrogen metabolism of pigs. Twenty-six pregnant Bama mini-pigs and then 104 weaned piglets were assigned for experimental treatments. The plasma and muscle samples were collected at 65-, 95-, and 125-d-old pigs, respectively. The results showed that betaine addition in the sow-offspring diets increased the lean meat rate in the 65-d-old pigs, whereas carcass weight, carcass yield, and loin-eye area were increased in the 95-d-old pigs, and carcass weight and backfat thickness in the 125-d-old pigs. Dietary betaine addition in the sow-offspring diets increased the contents of plasma Asp of 65-d-old, Met of 95- and 125-d-old, and Sar of 125-d-old pigs. Moreover, betaine addition increased the contents of Met, His, Ile, and Phe in *Longissimus thoracis et lumborum*, whereas those contents were decreased in *biceps femoris* and *psoas major* muscles at different stages. Betaine addition in the sow and piglets' diets regulated the muscle fiber-type and myogenic regulatory gene expressions. In summary, betaine addition in the sow and sow-offspring diets could improve the carcass traits and meat quality by altering the plasma biochemical parameters, amino acid composition, and gene expressions of skeletal muscle.

Keywords: Bama mini-pig, betaine, carcass traits, meat quality, nitrogen metabolism

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *21 June 2021*
Accepted: *05 August 2021*
Published: *27 August 2021*

Citation:
Cheng Y, Song M, Zhu Q, Azad MAK, Gao Q and Kong X (2021) Dietary Betaine Addition Alters Carcass Traits, Meat Quality, and Nitrogen Metabolism of Bama Mini-Pigs. Front. Nutr. 8:728477. doi: 10.3389/fnut.2021.728477

INTRODUCTION

Pork is the most widely-eaten meat globally, followed by poultry and beef, and the consumption is increasing continuously (1). The demand for pork with better quality, higher nutritional value, and better taste has also increased simultaneously. Therefore, increasing attention has been paid to improve the carcass traits and meat quality (2). In the recent past, antibiotics were widely used to enhance the growth rate, feed intake, and disease resistance, as well as to improve the carcass traits and meat quality (3).

Owing to the ban on in-feed antibiotics in swine production, safer and more effective feed additives with similar effects to antibiotics are indispensable to explore. Betaine (trimethylglycine) is a non-toxic amino acid derivative commonly found in most organisms (4). Furthermore, betaine is widely used as a feed additive in animal husbandry because of its benefits on improving the antioxidant ability, growth performance, and carcass traits (5, 6). Moreover, betaine can involve in the metabolisms of protein, amino acid, and fatty acid of the body (7). Besides the dietary nutrients, betaine can also regulate the growth performance and meat quality of pigs. Maternal nutrients also play a vital role in offspring's traits. During pregnancy, fetal nutrients are derived from the mammal circulation, and the nutrition deficiency of sows may cause the intrauterine growth retardation (IUGR) of pigs (8). The IUGR pigs can hardly adapt to the environment and mainly died after delivery due to the low potential of growth, immune, and digestion (9). Moreover, several studies demonstrated that increasing the maternal diet's energy can promote the development of muscles of the piglets (10, 11). Therefore, changing the maternal nutrients may affect the growth of the offspring. However, there are limited studies about maternal betaine addition on the offspring's carcass traits, meat quality, and nitrogen metabolism.

China is the leading country for swine production, has more than 100 local pig breeds due to the variation in the natural environment and differences in socio-economic conditions (12, 13). Bama mini-pig is one of the local pig breeds in China, is genetically stable and smaller in size. Due to its anatomy and physiological similarities to humans', Bama mini-pigs are a suitable pig breed for medical models, generally used to study metabolic diseases of human beings (14–16). However, Bama mini-pig is also a high-quality local pig breed with high economic value in China because of its characteristics of high tolerance of rough feed, disease resistance, and good adaptability (17). Therefore, Bama mini-pigs play a key role in poverty alleviation through industrial development at their place of origin (18). However, the small nesting range and extensive management methods have resulted in slow growth and low lean meat rate, which may greatly decrease the economic value of Bama mini-pigs and inhibit the development of practical production. Our previous studies have found that the betaine (3,500 mg kg^{-1}) supplementation to sows during pregnancy and lactation could enhance the reproduction performance, plasma reproductive hormones, and colostrum composition in Bama mini-pigs (19–21). Moreover, we have also found that betaine addition in sow and offspring diets could improve the average daily gain, feed to gain (F/G), and final weight of Bama mini-piglets during the 125 days trial (unpublished results). Therefore, based on the roles of betaine in pig nutrition, we hypothesized that the betaine addition in sow and offspring diets might affect the carcass traits and meat quality by regulating the nitrogen metabolism of pigs. Thus, the present study was conducted to investigate the effects of dietary betaine addition in sow and offspring diets during pregnancy and lactation on the carcass traits, meat quality, and nitrogen metabolism of Bama mini-pigs.

MATERIALS AND METHODS

Animals, Diets, and Feeding

Twenty-six healthy pregnant Bama mini-pigs with similar body conditions with three to seven parities were selected and randomly divided into two groups as follows: (a) control group, sows fed a basal diet ($n = 12$) and (b) betaine group, sows fed a basal diet with 3,500 mg kg^{-1} betaine hydrochloric ($n = 14$). Dietary betaine (purity; $\geq$ 95%) was purchased from Sunwin Biotech Shandong Co., Ltd (Shandong, China). The sows were housed individually in gestation crates (2.2 × 0.6 m) from mating to day 104 of gestation. On day 105 of gestation, the sows were transferred to individual farrowing crates (2.2 × 1.8 m) with a heated floor pad for offspring piglets with freely accessible *ad libitum* water. After weaning, at 35-d-old, a total of 104 piglets close to the average body weight of litter were selected from the two experimental groups (48 piglets from the control group and 56 piglets from the betaine group) and divided into three dietary treatments as follows: (a) control group, piglets from the control group fed a basal diet ($n = 48$); (b) sow-betaine group, piglets from the experimental group fed the basal diet ($n = 28$); (c) sow-offspring-betaine group, piglets from the betaine group fed a basal diet with 2,500 mg kg^{-1} betaine hydrochloride ($n = 28$). Dietary betaine was mixed with the basal diet, and the weaned piglets were fed by group.

During the trial period, the sows were fed pregnant sow diets from day 3 after mating to day 104 of pregnancy and fed lactating diets from day 105 of pregnancy to weaning. The piglets were fed with pre-nursery diets from 35- to 95-d-old and fed with late-nursery diets from 96- to 125-d-old. The basal diets' nutrient levels for sows and piglets met the Chinese local swine nutrient requirements (NY/T 65-2004), and the premixes met the National Research Council diet requirements (22, 23). The composition and nutrient levels of basal diets for sows are presented in **Supplementary Table 1**, and the composition and nutrient levels of basal diets for piglets are listed in **Supplementary Table 2**.

Sample Collection

The animals were weighed on days 65, 95, and 125 after fasted 12 h, respectively. A total of 26 pigs (including 12, 7, and 7 pigs from the control group, sow-betaine group, and sow-offspring-betaine group, respectively), were selected randomly at every stage for plasma sampling and then sacrificed under commercial conditions via electrical stunning (120 V, 200 Hz) and exsanguination (24). After slaughter, *Longissimus thoracis et lumborum* (LTL), *biceps femoris* (BF), and *psoas major* (PM) muscles from the right side of each carcass were sampled. One part of LTL muscle for meat quality measurement and one part of LTL, BF, and PM muscles was stored at −20°C for chemical composition analysis (including measurement of crude protein and dry matter). After that, the remaining muscle samples were stored in sealed plastic bags at −80°C prior to quantitative analysis.

Carcass Traits Analysis

After slaughter, the carcass was weighed after removing the head, feet, tail, and internal organs and calculated the carcass yield (dividing the carcass weight by live body weight). The backfat thickness (between sixth and seventh ribs on the left side of the carcass) were measured immediately using a caliper, and the width and thickness of cross-section of the LTL muscle at left thoracolumbar junction were measured by a caliper to calculate the loin-eye area, following the Chinese guidelines on performance measurement technology and regulations for pigs (GB8467-87, 1988).

Meat Quality Analysis

Meat quality was evaluated by determining marbling score, muscle color, pH, shear force, and drip loss of the LTL muscle. The marbling score was scored according to the NPPC colorimetric plate. Muscle color was measured at 24 h after slaughter by a colorimeter (Minolta CR-400; Konica Minolta, Tokyo, Japan) using the CIELAB trichromatic system as lightness, redness, and yellowness (A 90° standard observer angle, 8 mm diameter aperture, and D65 standard illuminant). The pH value was measured by direct insertion of an electrode (HI9125; Hanna Instruments, Padova, Italy) at 45 min and 24 h after slaughter stored at 4°C (1 cm deep into the LTL muscle), and the meter was previously calibrated with two buffer solutions of pH 4.0 and pH 7.0 at 25°C. The drip loss was measured 4 h after slaughter as previously described (25). Briefly, the LTL muscle samples were trimmed of adjacent fat and weighed, then hung in a plastic bag, sealed, and stored at 4°C for 24 h. Then the meat was weighed again to calculate drip loss, and defined as the loss in weight after 24 h. For the shear force analyses, the samples were cooked in a boiled water until the internal temperature reached 70°C, then the cooked samples were cooled at room temperature and cut into six stripes per sample (1 cm thick). Finally, the shear force was measured by using a texture analyzer (model HOUNSFILD-H5KS, UE).

Chemical Composition Analysis of Muscle

The muscle samples were cut into thin slices, dried in a vacuum freeze dryer at (10 ± 5) Pa and –(45 ± 5)°C for 48 h, and then ground into powder. The crude protein and dry matter contents were measured according to the GB/T 9695.15-2008 and GB5009.5-2010, respectively. Hydrolyzed amino acid contents in muscle samples were analyzed by an amino acid analyzer (L-8900; Hitachi, Japan) as previously described by Liu et al. (26).

Plasma Free Amino Acid Contents

The plasma samples were obtained by centrifuging at 6,500 × *g* for 10 min, and 600 μL of supernatant was taken into a new centrifuge tube. Then an equal volume of 8% sulfosalicylic acid solution was mixed, and the protein was fully precipitated at 4°C. After centrifuging at 6,500 × *g* for 10 min, the supernatant was filtered through a 0.22-μm membrane. Finally, using the L-8900 amino acid analyzer we measured the contents of free amino acids, including alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), sarcosine (Sar), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val).

Plasma Biochemical Parameters

The levels of plasma biochemical parameters, including alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), ammonia (AMM), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total protein (TP), and urea nitrogen (UN) were analyzed using commercially available biochemical kits (Leadman Biochemistry Technology Company, Beijing, China) according to the manufacturers' instructions.

Real-Time PCR Analysis

Total RNA was isolated from the LTL, BF, and PM muscles using TRIzol (Invitrogen, Shanghai, China). Beta-actin and the target genes are based on the GenBank database for pigs in NCBI, and the gene-specific primers were synthesized by Invitrogen Biotech Co., Ltd. (Shanghai, China; **Supplementary Table 3**). The reverse transcription-polymerase chain reaction (RT-PCR) assays were conducted using the SYBR Premix Ex Taq™ Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China). The quality detection of RNA, the RT-PCR, and the synthesis of cDNA were used the methods described in the previous study (27). The RT-PCR conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s, finally extension at 72°C for 30 s. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (28).

Statistical Analysis

The data were analyzed by using the general linear model in SPSS 22.0 software (IBM Corporation, 2014) to perform the one-way analysis of variance (ANOVA). The means of the different treatment groups were compared by Duncan tests. The individual pigs were considered the experimental unit. All data are presented as means ± SE unless otherwise indicated. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Carcass Traits

To evaluate the effects of dietary betaine on carcass traits, we assessed the carcass weight, carcass yield, fat yield, lean meat rate, backfat thickness, and loin-eye area of pigs on 65-, 95-, and 125-d-old, respectively (**Table 1**). In the 65-d-old pigs, the lean meat rate was increased ($P < 0.05$) in the sow-offspring-betaine group compared with the control group. However, there were no significant differences observed in carcass weight, carcass yield, backfat thickness, and loin-eye area of the 65-d-old pigs among the three different groups. In the 95-d-old pigs, the carcass weight, carcass yield, and loin-eye area of pigs in the sow-offspring-betaine group were increased ($P < 0.05$) compared with the pigs in the control and sow-betaine groups. In the 125-d-old pigs, dietary betaine addition to the sow and sow-offspring diets increased ($P < 0.05$) the backfat thickness and loin-eye area of pigs compared with the pigs in the control group.

TABLE 1 | Effect of dietary betaine on carcass traits of Bama mini-pigs.

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
Carcass weight (kg)	65	4.93 ± 0.16	5.35 ± 0.18	5.30 ± 0.36
	95	8.05 ± 0.63^{b}	6.89 ± 0.65^{b}	10.37 ± 0.68^{a}
	125	13.56 ± 1.48^{b}	16.45 ± 1.32^{ab}	19.87 ± 1.57^{a}
Carcass yield (%)	65	54.05 ± 0.77	54.89 ± 1.77	54.49 ± 3.62
	95	54.86 ± 1.03^{b}	55.66 ± 4.90^{b}	60.78 ± 1.24^{a}
	125	56.12 ± 1.83	60.70 ± 1.58	61.21 ± 0.82
Fat yield (%)	65	5.82 ± 0.95	6.11 ± 2.57	5.68 ± 2.08
	95	7.65 ± 1.79	7.22 ± 1.84	7.60 ± 1.21
	125	17.90 ± 3.05	20.49 ± 2.54	20.64 ± 1.97
Lean meat rate (%)	65	10.23 ± 1.18^{b}	11.10 ± 0.91^{ab}	12.19 ± 1.74^{a}
	95	10.76 ± 1.10	11.31 ± 1.53	11.95 ± 1.12
	125	22.54 ± 2.62	24.39 ± 1.83	24.53 ± 0.25
Back fat thickness (mm)	65	13.27 ± 0.59	13.49 ± 1.20	12.18 ± 1.05
	95	18.01 ± 1.42	15.89 ± 1.00	19.11 ± 1.16
	125	25.00 ± 1.41^{b}	27.12 ± 1.22^{ab}	30.42 ± 1.09^{a}
Loin-eye area (cm^2)	65	4.12 ± 0.14	4.20 ± 0.11	4.98 ± 0.55
	95	4.43 ± 0.34^{b}	4.98 ± 0.62^{b}	7.89 ± 0.92^{a}
	125	6.69 ± 0.56	7.72 ± 0.56	8.95 ± 1.17

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of the control group were 12, 11, and 8; the sow-betaine group were 6, 7, and 6; and the sow-offspring-betaine group were 7, 7, and 6, respectively.

Meat Quality and Chemical Composition

The effects of dietary betaine addition in the sow and sow-offspring diets on meat quality and meat chemical composition are presented in **Tables 2**, **3**. Compared with the control group, betaine addition to sow diets reduced ($P < 0.05$) the marbling score of LTL muscle of 65- and 95-d-old pigs, whereas betaine addition in the sow-offspring diets increased ($P < 0.05$) the shear force of LTL muscle of 65-d-old pigs and the meat brightness of 95-d-old pigs (**Table 2**). There were no significant differences in marbling score, meat color, drip loss, and shear force of LTL muscle of 125-d-old pigs among the three dietary groups ($P > 0.05$). Furthermore, the crude protein content of LTL muscle of 65-d-old pigs was increased in the sow-offspring-betaine group compared with the pigs in the sow-betaine group, whereas dry matter content of PM muscle was decreased in the sow-offspring-betaine group compared with the control group ($P < 0.05$). However, dietary betaine addition in the sow and sow-offspring diets did not affect ($P > 0.05$) the crude protein and dry matter contents of LTL, BF, and PM muscles of 95- and 125-d-old pigs (**Table 3**).

Plasma Free Amino Acid Contents

Plasma free amino acid contents of pigs at three different stages are presented in **Table 4**. Dietary betaine addition in sow-offspring diets increased ($P < 0.05$) the plasma Asp content of 65-d-old pigs and the plasma Met content of 95- and 125-d-old pigs compared with the pigs in the control and sow-betaine groups. In addition, the plasma Glu content of 65-d-old pigs was increased ($P < 0.05$) in the sow-offspring-betaine group compared with the sow-betaine group. Moreover, the plasma Sar content of 125-d-old pigs was increased ($P < 0.05$) in the sow-offspring-betaine group compared with the other two groups.

Plasma Biochemical Parameters

The effects of dietary betaine addition in the sow and sow-offspring diets on plasma biochemical parameters at different stages are presented in **Table 5**. In the 65-d-old pigs, the sow-betaine group had higher TP activity than the pigs in the control group, whereas the sow-offspring-betaine group had higher AST activity than the pigs in the other two groups ($P < 0.05$). In the 95-d-old pigs, the sow-offspring-betaine group showed a lower ($P < 0.05$) AST activity than the pigs in the sow-betaine group. In addition, both sow-betaine and sow-offspring-betaine groups showed lower ($P < 0.05$) LDH activity compared with the control group. However, dietary betaine had no effects ($P > 0.05$) on the plasma biochemical parameters of 125-d-old pigs.

Hydrolyzed Amino Acid Contents in the Muscle

The changes of muscle amino acid contents in the LTL, BF, and PM muscles are presented in **Table 6**. In the LTL muscle, compared with the control group, betaine addition in the sow and sow-offspring diets decreased ($P < 0.05$) the Asp, Ile, and Lys contents, whereas betaine addition in the sow diets decreased ($P < 0.05$) the Phe, Val, and essential amino acid (EAA) contents of 65-d-old pigs. In the 95-d-old pigs, the contents of Asp and Ile in the sow-betaine and sow-offspring-betaine groups were increased, whereas the content of Pro was decreased compared with the control group ($P < 0.05$). Moreover, the contents of Glu, Gly, and Lys in the sow-betaine group and the content

TABLE 2 | Effect of dietary betaine on meat quality of Bama mini-pigs.

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
Marbling score	65	1.67 ± 0.14[a]	1.00 ± 0.00[b]	1.43 ± 0.20[ab]
	95	1.73 ± 0.19[a]	1.00 ± 0.00[b]	1.29 ± 0.18[ab]
	125	1.50 ± 0.19	1.67 ± 0.21	1.67 ± 0.21
Meat color				
	65	49.22 ± 1.00	50.60 ± 1.91	47.19 ± 0.50
L*	95	50.25 ± 0.75[b]	49.39 ± 1.75[b]	54.33 ± 1.33[a]
	125	51.22 ± 1.98	51.15 ± 2.36	47.86 ± 1.12
	65	19.47 ± 0.87	21.60 ± 1.63	19.43 ± 1.40
a*	95	18.69 ± 0.70	18.73 ± 0.39	18.85 ± 0.83
	125	18.03 ± 0.77	15.71 ± 0.70	16.42 ± 0.73
	65	6.99 ± 0.13	6.60 ± 0.14	6.34 ± 0.49
b*	95	6.57 ± 0.37	6.04 ± 0.19	6.22 ± 0.24
	125	6.99 ± 0.38	7.13 ± 0.71	6.19 ± 0.66
	65	6.54 ± 0.05	5.66 ± 0.68	6.18 ± 0.05
pH_{45min}	95	6.45 ± 0.08	6.56 ± 0.04	6.67 ± 0.10
	125	6.43 ± 0.08	6.51 ± 0.11	6.51 ± 0.04
	65	5.53 ± 0.05	5.60 ± 0.23	5.70 ± 0.10
pH_{24h}	95	5.47 ± 0.02	5.44 ± 0.01	5.54 ± 0.04
	125	5.46 ± 0.02	5.50 ± 0.02	5.51 ± 0.01
	65	5.85 ± 0.71	5.75 ± 1.13	5.33 ± 0.59
Drip loss (%)	95	3.48 ± 0.54	2.87 ± 0.60	3.12 ± 0.38
	125	4.44 ± 0.68	2.78 ± 0.51	3.11 ± 0.54
	65	62.37 ± 3.95[b]	77.83 ± 2.62[ab]	84.99 ± 9.77[a]
Shear force (*N*)	95	70.68 ± 5.32	64.81 ± 4.56	74.41 ± 6.12
	125	93.51 ± 8.42	90.11 ± 10.09	67.08 ± 5.04

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of the control group were 12, 11, and 8; the sow-betaine group were 6, 7, and 6; and the sow-offspring-betaine group were 7, 7, and 6, respectively.
L, lightness; a*, redness; b*, yellowness.*

of Leu in the sow-offspring-betaine group were increased ($P < 0.05$) compared with the control group. In the 125-d-old pigs, the contents of His, Met, Phe, Thr, and Tyr were increased in the sow-betaine and sow-offspring-betaine groups, whereas the content of EAA was increased in the sow-offspring-betaine group compared with the control group ($P < 0.05$).

The BF muscle analysis showed that betaine addition in diets can significantly affect the hydrolyzed amino acid contents. Compared with the control group, betaine addition in the sow and sow-offspring diets increased the contents of Ala, Asp, Glu, Gly, Ile, Lys, Ser, Val, non-essential amino acid (NEAA), and flavor amino acid (FAA), while decreased the content of Pro of 65-d-old pigs ($P < 0.05$). Moreover, betaine addition in the sow-offspring diets increased ($P < 0.05$) the content of total amino acids (TAA) of 65-d-old pigs compared with the pigs in the control group. In the 95-d-old pigs, the contents of Arg, Asp, Gly, Ile, Leu, Lys, Thr, TAA, and EAA were decreased in the sow-offspring-betaine group compared with the other two groups, whereas the content of Glu was increased in the sow-betaine group compared with the other two groups ($P < 0.05$). Moreover, the content of FAA was decreased ($P < 0.05$) in the sow-offspring-betaine group compared with the other two groups. In the 125-d-old pigs, the contents of Ala, Asp, Glu, Ile, Leu, Lys, Ser, TAA, EAA, and FAA were increased in the sow-offspring-betaine group compared with the other two groups, whereas the contents of Gly, Thr, Val, and NEAA were increased in the sow-offspring-betaine group compared with the control group ($P < 0.05$).

In the PM muscle, compared with the control group, betaine addition in the sow-offspring diets decreased ($P < 0.05$) the content of Asp and increased the content of Phe of 65-d-old pigs ($P < 0.05$). In addition, betaine addition in the sow and sow-offspring diets increased the content of Pro compared with the control group, whereas betaine addition in the sow-offspring diets decreased the content of Tyr compared with the other two groups in the 65-d-old pigs ($P < 0.05$). In the 95-d-old pigs, the contents of Ala, Asp, Glu, Gly, His, Leu, Met, Ser, Val, TAA, EAA, NEAA, and FAA were decreased in the sow-offspring-betaine group compared with the other two groups, whereas the contents of Arg, Ile, and Thr were decreased compared with the control group ($P < 0.05$). In the 125-d-old pigs, the contents of Met and Phe were increased in the sow-betaine group, whereas the content of Tyr was decreased compared with the other two groups ($P < 0.05$). Moreover, the contents of His and

TABLE 3 | Effect of dietary betaine on routine nutrient contents of Bama mini-pigs (fresh weight basis; %).

d-old	Nutrient ingredients	Control group	Sow-betaine group	Sow-offspring-betaine group
***Longissimus thoracis et lumborum* muscle**				
65	Dry matter	29.12 ± 2.30	27.33 ± 0.84	28.04 ± 0.63
	Crude protein	23.25 ± 2.06^{ab}	22.27 ± 0.99^{b}	24.57 ± 0.78^{a}
95	Dry matter	28.33 ± 1.94	28.21 ± 1.11	28.70 ± 2.28
	Crude protein	22.03 ± 1.69	22.80 ± 0.90	22.92 ± 1.30
125	Dry matter	27.82 ± 2.04	28.54 ± 3.92	26.00 ± 0.51
	Crude protein	22.20 ± 0.70	22.51 ± 0.66	22.57 ± 0.61
***Biceps femoris* muscle**				
65	Dry matter	26.89 ± 0.85	25.94 ± 1.11	26.53 ± 1.01
	Crude protein	22.23 ± 1.55	21.97 ± 0.85	22.78 ± 0.98
95	Dry matter	26.74 ± 1.43	25.85 ± 0.81	26.19 ± 0.79
	Crude protein	23.12 ± 1.63	22.27 ± 0.70	22.14 ± 0.77
125	Dry matter	27.44 ± 2.90	26.94 ± 1.54	26.10 ± 1.00
	Crude protein	22.07 ± 1.14	21.26 ± 1.01	20.65 ± 2.40
***Psoas major* muscle**				
65	Dry matter	28.22 ± 1.33^{a}	26.84 ± 1.77^{ab}	26.07 ± 1.25^{b}
	Crude protein	22.28 ± 3.29	21.62 ± 1.39	22.60 ± 0.72
95	Dry matter	25.92 ± 2.05	25.96 ± 0.87	24.95 ± 1.30
	Crude protein	22.92 ± 2.25	22.47 ± 0.66	21.58 ± 1.32
125	Dry matter	24.80 ± 1.17	24.58 ± 0.87	23.97 ± 0.74
	Crude protein	21.18 ± 0.61	21.51 ± 0.47	20.98 ± 0.56

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of control group were 12, 11, and 8; sow-betaine group was 6, 7, and 6; and sow-offspring-betaine group was 7, 7, and 6, respectively.

Pro in the sow-betaine and sow-offspring-betaine groups were increased ($P < 0.05$) compared with the control group in the 125-d-old pigs.

The mRNA Expression of Genes Related to MyHC Isoform and MRFs in the Muscle

Table 7 presents the level of mRNA gene expressions related to myosin heavy chain (MyHC) isoform and myogenic regulatory factors (MRFs) in LTL, BF, and PM muscles. In the LTL muscle, compared with the control group, the mRNA expression level of *MyHC-IIb* was up-regulated ($P < 0.05$) in the sow-betaine group and the mRNA expression level of myogenic degradation factor 5 (*Myf5*) was up-regulated in the 65-d-old pigs. In the 95-d-old pigs, the mRNA expression levels of *MyHC-I* and Myogenin (*MyoG*) were up-regulated in the sow-offspring-betaine group compared with the control group, whereas the mRNA expression levels of *MyHC-IIx* and *Myf5* up-regulated in the sow-offspring-betaine group compared with the other two groups ($P < 0.05$). Moreover, the mRNA expression level of *MyHC-IIb* in the sow-betaine and sow-offspring-betaine groups was up-regulated ($P < 0.05$) compared with the control group in the 95-d-old pigs. In the 125-d-old pigs, the mRNA expression level of *MyHC-IIx* was up-regulated in the sow-offspring-betaine group compared with the control group.

In the BF muscle, in the 95-d-old pigs, the mRNA expression level of *MyHC-IIb* was up-regulated ($P < 0.05$) in the sow-offspring-betaine group compared with the sow-betaine group. Moreover, in the 125-d-old pigs, the mRNA expression level of *MyHC-IIb* was up-regulated ($P < 0.05$) in the sow-betaine group compared with the other two groups.

In the PM muscle, in the 65-d-old pigs, the mRNA expression levels of *MyHC-I* and *MyHC-IIb* were up-regulated ($P < 0.05$) in the sow-betaine and sow-offspring-betaine groups compared with the control group, and the level of *MyHC-IIa* was up-regulated in the sow-betaine group. The mRNA level of *Myf5* of 65-d-old pigs in the sow-betaine group was up-regulated ($P < 0.05$) compared with the 65-d-old pigs in the other two groups. Moreover, in the 125-d-old pigs, the mRNA expression level of *MyoG* was down-regulated ($P < 0.05$) in the sow-offspring-betaine group compared with the other two groups. However, betaine supplementation had no impacts ($P > 0.05$) on the mRNA expression levels of PM muscle in the 95-d-old pigs.

DISCUSSION

As an antibiotic substitute in livestock production, dietary betaine has gained more attraction because of its growth-promoting effect. For example, betaine added to the diet can significantly increase the growth hormone (GH) level and total protein content in the blood to improve the utilization of the protein and the growth of finishing pigs (29). Betaine addition in the broiler chicken's diet could improve antioxidant defenses and decrease the breast muscles' lipid peroxidation (30). Furthermore, a recent study suggested that betaine (500, 1,000, and 2,000 mg kg^{-1}) can improve the nitrogen retention, growth performance, digestive function,

TABLE 4 | Effect of dietary betaine on plasma free amino acids of Bama mini-pigs (μmol L^{-1}).

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
Ala	65	418.20 ± 28.10	365.88 ± 4.03	408.44 ± 60.46
	95	340.23 ± 37.44	348.82 ± 68.59	251.02 ± 25.80
	125	284.27 ± 25.04	372.51 ± 30.33	302.42 ± 42.99
Arg	65	78.01 ± 6.84	77.68 ± 5.39	73.65 ± 13.23
	95	82.09 ± 9.84	94.02 ± 7.17	96.94 ± 6.38
	125	88.44 ± 6.68	98.39 ± 22.84	113.29 ± 11.8
Asp	65	7.63 ± 0.94^{b}	9.58 ± 0.66^{b}	14.84 ± 1.42^{a}
	95	12.95 ± 2.14	13.89 ± 2.27	9.44 ± 0.54
	125	11.44 ± 1.42	10.52 ± 0.48	10.01 ± 0.70
Cit	65	26.82 ± 1.17	29.23 ± 1.22	29.75 ± 4.17
	95	36.31 ± 2.46	37.50 ± 2.29	39.63 ± 1.95
	125	41.61 ± 3.78	32.39 ± 2.81	35.23 ± 2.71
Glu	65	215.96 ± 21.03^{ab}	178.54 ± 18.64^{b}	296.66 ± 35.11^{a}
	95	190.71 ± 17.09	222.11 ± 21.23	196.33 ± 19.22
	125	157.72 ± 13.03	183.79 ± 16.01	198.18 ± 14.02
Gly	65	551.72 ± 54.19	488.64 ± 88.64	364.64 ± 29.09
	95	483.85 ± 56.50	568.49 ± 60.88	625.81 ± 20.54
	125	546.26 ± 29.07	549.24 ± 72.80	690.19 ± 91.14
His	65	37.39 ± 1.80	40.06 ± 2.75	40.61 ± 3.12
	95	41.06 ± 2.37	36.42 ± 2.27	41.13 ± 2.95
	125	42.72 ± 2.42	43.69 ± 2.73	49.33 ± 4.02
Ile	65	100.93 ± 6.16	108.55 ± 9.33	110.45 ± 9.42
	95	89.86 ± 6.62	89.28 ± 2.39	100.88 ± 11.88
	125	86.29 ± 3.02	101.02 ± 8.80	133.51 ± 16.71
Leu	65	160.52 ± 9.45	167.17 ± 12.35	178.92 ± 14.11
	95	136.37 ± 9.49	137.69 ± 4.30	151.66 ± 18.55
	125	142.49 ± 4.53	144.58 ± 4.59	203.97 ± 24.05
Lys	65	141.13 ± 10.87	155.82 ± 17.66	134.42 ± 14.15
	95	120.29 ± 12.11	133.92 ± 11.65	126.11 ± 6.61
	125	129.19 ± 4.75	133.52 ± 5.85	174.85 ± 22.84
Met	65	12.75 ± 0.85	11.44 ± 0.46	10.14 ± 1.52
	95	6.03 ± 1.80^{b}	11.07 ± 0.38^{b}	12.56 ± 0.65^{a}
	125	14.01 ± 0.90^{b}	14.32 ± 1.56^{b}	20.41 ± 0.19^{a}
Orn	65	53.55 ± 4.68	55.48 ± 2.82	68.64 ± 5.32
	95	64.81 ± 8.56	71.09 ± 14.08	52.84 ± 3.38
	125	66.05 ± 6.37	50.73 ± 3.56	68.89 ± 16.00
Phe	65	78.14 ± 2.61	84.38 ± 9.68	84.83 ± 2.38
	95	80.88 ± 3.84	75.84 ± 5.65	83.18 ± 6.41
	125	89.31 ± 6.39	88.79 ± 6.85	91.25 ± 3.17
Pro	65	171.23 ± 9.61	178.72 ± 48.20	182.06 ± 27.55
	95	153.18 ± 13.21	160.59 ± 19.97	181.47 ± 14.77
	125	190.08 ± 14.21	208.01 ± 20.92	166.44 ± 20.78
Sar	65	8.45 ± 1.75	6.77 ± 0.87	13.09 ± 1.79
	95	4.92 ± 1.83	2.85 ± 0.55	2.52 ± 0.74
	125	2.26 ± 0.24^{b}	3.07 ± 0.37^{b}	5.37 ± 1.45^{a}
Ser	65	79.55 ± 5.83	83.43 ± 9.57	87.97 ± 7.28
	95	73.37 ± 6.20	85.57 ± 5.90	76.41 ± 5.50
	125	79.78 ± 1.91	88.48 ± 3.36	98.55 ± 8.40
Thr	65	121.52 ± 10.11	134.77 ± 22.12	113.67 ± 10.10
	95	110.77 ± 10.26	129.64 ± 16.82	112.06 ± 5.77
	125	122.96 ± 7.01	133.82 ± 14.29	126.65 ± 14.93

(Continued)

TABLE 4 | Continued

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
Tyr	65	41.76 ± 5.09	55.40 ± 4.31	46.91 ± 0.97
	95	50.56 ± 4.41	59.97 ± 5.33	65.60 ± 6.31
	125	62.65 ± 3.92	59.71 ± 4.97	68.97 ± 6.70
Val	65	243.90 ± 14.39	243.20 ± 17.59	276.04 ± 23.11
	95	229.92 ± 14.16	214.00 ± 4.55	247.18 ± 31.97
	125	237.37 ± 9.88	267.48 ± 17.08	345.17 ± 43.46

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of the control group were 12, 11, and 8; the sow-betaine group were 6, 7, and 6; and the sow-offspring-betaine group were 7, 7, and 6, respectively.
Ala, alanine; Arg, arginine; Asp, aspartic acid; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Sar, sarcosine; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

TABLE 5 | Effect of dietary betaine on plasma biochemical parameters of Bama mini-pigs.

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
ALB (g L^{-1})	65	40.63 ± 0.95	44.23 ± 1.40	42.33 ± 2.49
	95	41.89 ± 0.88	40.39 ± 1.35	40.26 ± 1.39
	125	45.05 ± 1.52	46.22 ± 1.30	46.10 ± 2.01
ALP (U L^{-1})	65	162.33 ± 9.59	165.17 ± 8.79	197.14 ± 20.76
	95	125.09 ± 13.56	118.43 ± 11.53	83.57 ± 17.32
	125	175.63 ± 18.27	138.33 ± 17.07	122.00 ± 11.67
ALT (U L^{-1})	65	61.33 ± 4.58	64.30 ± 3.25	62.71 ± 6.93
	95	55.88 ± 2.91	49.58 ± 4.83	50.51 ± 5.41
	125	51.77 ± 4.66	55.33 ± 7.00	65.90 ± 3.96
AMM (μmol L^{-})	65	213.48 ± 28.32	179.42 ± 26.33	182.50 ± 13.04
	95	465.63 ± 119.89	213.67 ± 9.99	217.04 ± 11.76
	125	301.85 ± 52.62	132.45 ± 33.80	214.86 ± 46.38
AST (U L^{-1})	65	61.08 ± 6.99^{b}	62.00 ± 4.52^{b}	93.71 ± 11.02^{a}
	95	73.30 ± 5.53^{ab}	91.43 ± 18.85^{a}	46.86 ± 2.55^{b}
	125	69.50 ± 12.02	56.67 ± 10.89	82.00 ± 24.09
LDH (U L^{-1})	65	410.92 ± 23.68	450.75 ± 25.03	521.57 ± 51.95
	95	474.55 ± 36.21^{a}	372.50 ± 32.82^{b}	355.43 ± 19.04^{b}
	125	508.88 ± 45.90	451.00 ± 28.76	411.80 ± 35.37
TP (g L^{-1})	65	65.61 ± 1.12^{b}	74.38 ± 2.71^{a}	70.17 ± 3.53^{ab}
	95	74.09 ± 1.60	72.59 ± 2.08	74.46 ± 2.12
	125	71.25 ± 2.08	68.98 ± 0.65	73.68 ± 2.61
UN (mmol L^{-1})	65	3.13 ± 0.22	4.13 ± 0.71	3.96 ± 0.37
	95	3.56 ± 0.28	2.66 ± 0.29	3.01 ± 0.47
	125	3.50 ± 0.47	3.37 ± 0.38	3.76 ± 0.35

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of the control group were 12, 11, and 8; the sow-betaine group were 6, 7, and 6; and the sow-offspring-betaine group were 7, 7, and 6, respectively.
ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMM, ammonia; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TP, total protein; UN, urea nitrogen.

carcass characteristics, and meat quality of broilers under heat stress (31). Similarly, we found that the betaine addition in the sow and offspring diets significantly changes the carcass traits, meat quality, and nitrogen metabolism of Bama-mini-pigs.

Carcass traits and meat quality can intuitively reflect the digestion, absorption, and deposition of the nutrients in the body. Dietary compositions can directly or indirectly affect the metabolism of nutrients in the body and thereby change the meat quality and carcass traits. For example, Cheng et al. (32) found that low amino acid content in diets can effectively improve the meat quality, such as muscle tender and fat content. In the present study, the betaine addition to the sow and offspring diets could effectively increase the carcass weight, carcass yield,

TABLE 6 | Effect of dietary betaine on hydrolyzed amino acid content in muscle of Bama mini-pigs (fresh weight basis; %).

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
***Longissimus thoracis et lumborum* muscle**	65	1.20 ± 0.09	1.14 ± 0.06	1.18 ± 0.10
Ala	95	1.09 ± 0.14	1.17 ± 0.10	1.23 ± 0.14
	125	1.17 ± 0.08	1.28 ± 0.11	1.22 ± 0.06
	65	1.35 ± 0.09	1.29 ± 0.05	1.28 ± 0.09
Arg	95	1.25 ± 0.15	1.38 ± 0.08	1.27 ± 0.10
	125	1.34 ± 0.09	1.45 ± 0.29	1.33 ± 0.13
	65	1.82 ± 0.14^{a}	1.62 ± 0.13^{b}	1.63 ± 0.13^{b}
Asp[1]	95	1.54 ± 0.16^{b}	1.80 ± 0.11^{a}	1.83 ± 0.15^{a}
	125	1.76 ± 0.11	1.81 ± 0.14	1.82 ± 0.07
	65	2.64 ± 0.20	2.49 ± 0.11	2.61 ± 0.17
Glu[2]	95	2.62 ± 0.28^{b}	2.92 ± 0.21^{a}	2.66 ± 0.24^{b}
	125	2.84 ± 0.23	3.03 ± 0.22	3.11 ± 0.20
	65	1.08 ± 0.10	0.93 ± 0.09	0.99 ± 0.22
Gly	95	0.94 ± 0.13^{b}	1.08 ± 0.05^{a}	0.93 ± 0.10^{b}
	125	0.98 ± 0.09	1.03 ± 0.11	0.94 ± 0.04
	65	0.80 ± 0.08	0.80 ± 0.14	0.88 ± 0.20
His	95	0.92 ± 0.18	0.79 ± 0.08	0.80 ± 0.05
	125	0.89 ± 0.04^{b}	1.07 ± 0.10^{a}	1.14 ± 0.08^{a}
	65	0.90 ± 0.07^{a}	0.83 ± 0.04^{b}	0.81 ± 0.03^{b}
Ile	95	0.83 ± 0.07^{b}	0.94 ± 0.08^{a}	0.93 ± 0.06^{a}
	125	0.99 ± 0.07	1.04 ± 0.07	1.08 ± 0.04
	65	1.75 ± 0.14^{a}	1.65 ± 0.07^{ab}	1.61 ± 0.08^{b}
Leu	95	1.48 ± 0.12^{b}	1.58 ± 0.10^{b}	1.75 ± 0.13^{a}
	125	1.67 ± 0.11	1.75 ± 0.13	1.77 ± 0.08
	65	1.78 ± 0.13^{a}	1.64 ± 0.09^{b}	1.64 ± 0.05^{b}
Lys	95	1.62 ± 0.14^{b}	1.83 ± 0.18^{a}	1.75 ± 0.15^{ab}
	125	1.89 ± 0.14	1.97 ± 0.14	1.97 ± 0.07
	65	0.53 ± 0.04	0.51 ± 0.03	0.53 ± 0.06
Met	95	0.54 ± 0.06	0.54 ± 0.04	0.54 ± 0.04
	125	0.62 ± 0.04^{b}	0.68 ± 0.04^{a}	0.71 ± 0.03^{a}
	65	0.87 ± 0.06^{a}	0.80 ± 0.05^{b}	0.89 ± 0.06^{a}
Phe	95	0.86 ± 0.08	0.86 ± 0.09	0.81 ± 0.06
	125	0.90 ± 0.07^{c}	1.00 ± 0.06^{b}	1.11 ± 0.09^{a}
	65	0.94 ± 0.09	1.01 ± 0.13	0.99 ± 0.13
Pro	95	1.18 ± 0.29^{a}	1.03 ± 0.14^{ab}	0.82 ± 0.12^{b}
	125	1.04 ± 0.15	0.98 ± 0.13	0.88 ± 0.08
	65	0.69 ± 0.05	0.65 ± 0.03	0.66 ± 0.05
Ser	95	0.72 ± 0.09	0.75 ± 0.06	0.68 ± 0.06
	125	0.75 ± 0.06	0.84 ± 0.08	0.79 ± 0.03
	65	0.94 ± 0.08	0.89 ± 0.07	0.88 ± 0.03
Thr	95	0.86 ± 0.10	0.92 ± 0.09	0.91 ± 0.07
	125	0.96 ± 0.08^{b}	1.08 ± 0.07^{a}	1.09 ± 0.05^{a}
	65	0.71 ± 0.05	0.67 ± 0.09	0.66 ± 0.07
Tyr	95	0.58 ± 0.24	0.68 ± 0.08	0.61 ± 0.08
	125	0.75 ± 0.10^{b}	0.91 ± 0.05^{a}	1.01 ± 0.13^{a}
	65	0.97 ± 0.07^{a}	0.90 ± 0.05^{b}	0.91 ± 0.05^{ab}
Val	95	0.95 ± 0.09	1.02 ± 0.07	0.98 ± 0.08
	125	1.08 ± 0.08	1.13 ± 0.09	1.15 ± 0.05
	65	18.98 ± 1.39	17.83 ± 0.78	18.16 ± 0.93
TAA	95	17.97 ± 2.00	19.30 ± 1.34	18.51 ± 1.38
	125	19.64 ± 1.37	21.04 ± 1.52	21.13 ± 0.63

(Continued)

TABLE 6 | Continued

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
	65	7.75 ± 0.59[a]	7.22 ± 0.34[b]	7.27 ± 0.27[ab]
EAA	95	7.15 ± 0.58	7.69 ± 0.61	7.68 ± 0.56
	125	8.12 ± 0.58[b]	8.64 ± 0.53[ab]	8.89 ± 0.27[a]
	65	11.23 ± 0.81	10.61 ± 0.46	10.90 ± 0.74
NEAA	95	10.83 ± 1.44	11.61 ± 0.74	10.83 ± 0.86
	125	11.52 ± 0.80	12.40 ± 1.04	12.24 ± 0.47
	65	9.31 ± 0.68	8.73 ± 0.35	8.94 ± 0.66
FAA	95	8.94 ± 1.06	9.67 ± 0.64	9.06 ± 0.75
	125	9.50 ± 0.69	10.05 ± 0.72	9.84 ± 0.40
***Biceps femoris* muscle**	65	1.05 ± 0.11[b]	1.15 ± 0.03[a]	1.16 ± 0.04[a]
Ala	95	1.27 ± 0.20	1.25 ± 0.13	1.09 ± 0.06
	125	1.12 ± 0.08[b]	1.19 ± 0.05[b]	1.33 ± 0.14[a]
	65	1.27 ± 0.08	1.31 ± 0.03	1.34 ± 0.05
Arg	95	1.27 ± 0.16[a]	1.29 ± 0.12[a]	1.09 ± 0.06[b]
	125	1.29 ± 0.13	1.33 ± 0.06	1.44 ± 0.13
	65	1.69 ± 0.13[b]	2.03 ± 0.08[a]	1.94 ± 0.24[a]
Asp	95	1.86 ± 0.23[a]	1.85 ± 0.17[a]	1.55 ± 0.07[b]
	125	1.70 ± 0.16[b]	1.82 ± 0.07[b]	2.06 ± 0.20[a]
	65	2.52 ± 0.24[b]	2.85 ± 0.10[a]	2.94 ± 0.15[a]
Glu	95	2.70 ± 0.32[b]	2.97 ± 0.18[a]	2.56 ± 0.15[b]
	125	2.71 ± 0.25[b]	2.95 ± 0.14[b]	3.26 ± 0.35[a]
	65	0.83 ± 0.06[b]	1.00 ± 0.04[a]	1.02 ± 0.09[a]
Gly	95	0.88 ± 0.10[a]	0.94 ± 0.05[a]	0.80 ± 0.04[b]
	125	0.85 ± 0.08[b]	0.91 ± 0.05[ab]	1.00 ± 0.11[a]
	65	0.79 ± 0.08	0.84 ± 0.04	0.87 ± 0.08
His	95	0.91 ± 0.20	0.90 ± 0.11	0.93 ± 0.03
	125	0.93 ± 0.15	0.95 ± 0.06	1.01 ± 0.08
	65	0.86 ± 0.06[b]	0.96 ± 0.04[a]	0.93 ± 0.05[a]
Ile	95	0.92 ± 0.10[a]	0.97 ± 0.08[a]	0.82 ± 0.04[b]
	125	0.95 ± 0.08[b]	0.99 ± 0.04[b]	1.10 ± 0.12[a]
	65	1.70 ± 0.13	1.76 ± 0.07	1.69 ± 0.14
Leu	95	1.78 ± 0.25[a]	1.76 ± 0.15[a]	1.52 ± 0.07[b]
	125	1.62 ± 0.12[b]	1.68 ± 0.06[b]	1.87 ± 0.18[a]
	65	1.71 ± 0.12[b]	2.01 ± 0.08[a]	1.93 ± 0.19[a]
Lys	95	1.77 ± 0.20[a]	1.75 ± 0.14[a]	1.51 ± 0.07[b]
	125	1.79 ± 0.18[b]	1.90 ± 0.09[b]	2.14 ± 0.28[a]
	65	0.51 ± 0.04	0.55 ± 0.03	0.55 ± 0.04
Met	95	0.55 ± 0.06	0.55 ± 0.03	0.51 ± 0.05
	125	0.58 ± 0.07	0.59 ± 0.03	0.68 ± 0.11
	65	0.84 ± 0.08	0.78 ± 0.02	0.82 ± 0.10
Phe	95	0.84 ± 0.11	0.86 ± 0.04	0.81 ± 0.08
	125	0.87 ± 0.10	0.90 ± 0.07	1.03 ± 0.21
	65	0.99 ± 0.08[a]	0.63 ± 0.02[c]	0.81 ± 0.26[b]
Pro	95	0.84 ± 0.22	0.90 ± 0.20	0.82 ± 0.10
	125	0.96 ± 0.23	0.96 ± 0.18	1.00 ± 0.17
	65	0.58 ± 0.08[c]	0.70 ± 0.03[b]	0.77 ± 0.04[a]
Ser	95	0.71 ± 0.09	0.78 ± 0.04	0.72 ± 0.05
	125	0.66 ± 0.07[b]	0.71 ± 0.04[b]	0.84 ± 0.11[a]
	65	0.99 ± 0.09	0.91 ± 0.03	0.93 ± 0.04

(Continued)

TABLE 6 | Continued

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
Thr	95	0.93 ± 0.12^{a}	0.97 ± 0.07^{a}	0.84 ± 0.05^{b}
	125	0.90 ± 0.09^{b}	0.93 ± 0.06^{ab}	1.03 ± 0.12^{a}
	65	0.70 ± 0.05	0.66 ± 0.02	0.67 ± 0.04
Tyr	95	0.61 ± 0.08	0.60 ± 0.17	0.49 ± 0.13
	125	0.72 ± 0.10	0.72 ± 0.09	0.75 ± 0.08
	65	0.92 ± 0.07^{b}	1.01 ± 0.04^{a}	1.00 ± 0.03^{a}
Val	95	0.99 ± 0.11	1.02 ± 0.06	0.92 ± 0.05
	125	1.03 ± 0.10^{b}	1.07 ± 0.05^{ab}	1.19 ± 0.14^{a}
	65	17.96 ± 1.44^{b}	19.14 ± 0.59^{ab}	19.35 ± 0.81^{a}
TAA	95	18.85 ± 2.28^{a}	19.36 ± 1.26^{a}	16.97 ± 0.84^{b}
	125	18.71 ± 1.69^{b}	19.59 ± 0.94^{b}	21.73 ± 2.49^{a}
	65	7.54 ± 0.58	7.97 ± 0.29	7.85 ± 0.43
EAA	95	7.79 ± 0.91^{a}	7.87 ± 0.51^{a}	6.91 ± 0.32^{b}
	125	7.74 ± 0.72^{b}	8.06 ± 0.37^{b}	9.04 ± 1.16^{a}
	65	10.42 ± 0.86^{b}	11.17 ± 0.31^{a}	11.50 ± 0.45^{a}
NEAA	95	11.05 ± 1.39	11.48 ± 0.79	10.06 ± 0.55
	125	10.97 ± 0.99^{b}	11.54 ± 0.57^{ab}	12.69 ± 1.33^{a}
	65	8.65 ± 0.75^{b}	9.28 ± 0.27^{a}	9.57 ± 0.37^{a}
FAA	95	9.19 ± 1.11^{ab}	9.66 ± 0.58^{a}	8.38 ± 0.44^{b}
	125	8.92 ± 0.76^{b}	9.47 ± 0.45^{b}	10.51 ± 1.19^{a}
***Psoas major* muscle**	65	1.19 ± 0.15	1.12 ± 0.06	1.11 ± 0.07
Ala	95	1.25 ± 0.15^{a}	1.16 ± 0.08^{a}	0.95 ± 0.06^{b}
	125	1.13 ± 0.08	1.11 ± 0.06	1.11 ± 0.08
	65	1.31 ± 0.17	1.32 ± 0.10	1.27 ± 0.11
Arg	95	1.33 ± 0.20^{a}	1.23 ± 0.10^{ab}	1.12 ± 0.04^{b}
	125	1.29 ± 0.07	1.26 ± 0.06	1.27 ± 0.07
	65	1.92 ± 0.31^{a}	1.76 ± 0.13^{ab}	1.61 ± 0.15^{b}
Asp	95	1.76 ± 0.23^{a}	1.67 ± 0.12^{a}	1.48 ± 0.07^{b}
	125	1.74 ± 0.13	1.63 ± 0.05	1.62 ± 0.10
	65	2.81 ± 0.44	2.87 ± 0.24	2.83 ± 0.30
Glu	95	2.95 ± 0.46^{a}	3.01 ± 0.26^{a}	2.43 ± 0.10^{b}
	125	2.95 ± 0.18	3.10 ± 0.10	2.90 ± 0.22
	65	0.96 ± 0.13	0.96 ± 0.12	0.88 ± 0.11
Gly	95	0.90 ± 0.15^{a}	0.89 ± 0.07^{a}	0.77 ± 0.06^{b}
	125	0.85 ± 0.05	0.82 ± 0.05	0.83 ± 0.05
	65	0.74 ± 0.10	0.73 ± 0.08	0.84 ± 0.19
His	95	0.86 ± 0.15^{a}	0.87 ± 0.16^{a}	0.65 ± 0.04^{b}
	125	0.74 ± 0.06^{c}	0.92 ± 0.06^{a}	0.83 ± 0.06^{b}
	65	0.93 ± 0.12	0.91 ± 0.08	0.86 ± 0.07
Ile	95	0.97 ± 0.15^{a}	0.91 ± 0.07^{ab}	0.83 ± 0.03^{b}
	125	0.94 ± 0.06	0.91 ± 0.04	0.91 ± 0.05
	65	1.71 ± 0.23	1.61 ± 0.10	1.53 ± 0.15
Leu	95	1.72 ± 0.20^{a}	1.66 ± 0.12^{a}	1.41 ± 0.06^{b}
	125	1.59 ± 0.12	1.56 ± 0.04	1.50 ± 0.07
	65	1.97 ± 0.27	1.78 ± 0.16	1.71 ± 0.15
Lys	95	1.77 ± 0.29	1.73 ± 0.14	1.58 ± 0.07
	125	1.81 ± 0.11	1.75 ± 0.05	1.75 ± 0.11
	65	0.56 ± 0.06	0.55 ± 0.05	0.57 ± 0.06

(Continued)

TABLE 6 | Continued

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
Met	95	0.59 ± 0.08[a]	0.62 ± 0.06[a]	0.52 ± 0.02[b]
	125	0.55 ± 0.03[b]	0.61 ± 0.01[a]	0.56 ± 0.03[b]
	65	0.81 ± 0.09[b]	0.84 ± 0.08[ab]	0.94 ± 0.12[a]
Phe	95	0.91 ± 0.13[b]	1.03 ± 0.10[a]	0.75 ± 0.05[c]
	125	0.85 ± 0.05[b]	1.01 ± 0.06[a]	0.90 ± 0.04[b]
	65	0.77 ± 0.14[b]	0.92 ± 0.16[a]	1.00 ± 0.12[a]
Pro	95	0.98 ± 0.26	0.94 ± 0.13	0.78 ± 0.14
	125	0.89 ± 0.07[b]	1.01 ± 0.14[a]	1.05 ± 0.08[a]
	65	0.77 ± 0.09	0.76 ± 0.07	0.75 ± 0.11
Ser	95	0.78 ± 0.12[a]	0.76 ± 0.07[a]	0.62 ± 0.03[b]
	125	0.74 ± 0.05	0.74 ± 0.05	0.74 ± 0.06
	65	0.94 ± 0.12	0.90 ± 0.07	0.89 ± 0.08
Thr	95	0.99 ± 0.16[a]	0.91 ± 0.07[ab]	0.79 ± 0.04[b]
	125	0.91 ± 0.06	0.92 ± 0.05	0.93 ± 0.04
	65	0.70 ± 0.09[a]	0.66 ± 0.08[a]	0.44 ± 0.31[b]
Tyr	95	0.66 ± 0.19	0.66 ± 0.05	0.66 ± 0.08
	125	0.70 ± 0.05[a]	0.32 ± 0.26[b]	0.76 ± 0.04[a]
	65	1.00 ± 0.11	0.99 ± 0.08	0.99 ± 0.11
Val	95	1.02 ± 0.16[a]	1.06 ± 0.08[a]	0.89 ± 0.04[b]
	125	1.00 ± 0.06	1.04 ± 0.03	0.99 ± 0.06
	65	19.06 ± 2.37	18.70 ± 1.52	18.24 ± 1.73
TAA	95	19.43 ± 2.78[a]	19.11 ± 1.53[a]	16.22 ± 0.61[b]
	125	18.68 ± 1.09	18.70 ± 0.74	18.66 ± 0.94
	65	7.90 ± 0.97	7.59 ± 0.63	7.49 ± 0.67
EAA	95	7.96 ± 1.11[a]	7.92 ± 0.63[a]	6.77 ± 0.26[b]
	125	7.66 ± 0.47	7.79 ± 0.14	7.55 ± 0.36
	65	11.16 ± 1.43	11.10 ± 0.93	10.74 ± 1.09
NEAA	95	11.47 ± 1.69[a]	11.19 ± 0.93[a]	9.45 ± 0.37[b]
	125	11.03 ± 0.62	10.91 ± 0.68	11.11 ± 0.58
	65	9.36 ± 1.22	9.29 ± 0.75	9.09 ± 0.86
FAA	95	9.60 ± 1.43[a]	9.34 ± 0.73[a]	7.82 ± 0.29[b]
	125	9.21 ± 0.55	9.33 ± 0.43	9.18 ± 0.54

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of the control group were 12, 11, and 8; the sow-betaine group were 6, 7, and 6; and the sow-offspring-betaine group were 7, 7, and 6, respectively.
[1]*includes aspartate plus asparagine;* [2]*includes glutamate plus glutamine. EAA includes Thr, Val, Met, Ile, Leu, Phe, Lys, His, Arg, and Pro; NEAA includes Asp, Ser, Glu, Gly, Ala, and Tyr.*

backfat thickness, and loin-eye area of 65-, 95-, and 125-d-old Bama mini-pigs. In light of the previous findings by Zhan et al. (33) in broiler chickens, dietary betaine could act as a methyl donor and increase the utilization of amino acids, thereby promote protein synthesis, increase the carcass lean meat rate, and improve carcass quality (34). Meat quality is generally measured by two parts, the sensory and nutrients indexes. The present study found that betaine addition to sows and offspring diets could significantly increase the shear force, brightness, and crude protein content of 125-d-old pigs, while betaine addition to sows could decrease the marbling score of LTL muscles of 65- and 95-d-old pigs, respectively. These findings are inconsistent with the results of Lawrence et al. (35). The possible explanation of the discrepancy may be caused by the protein deposition, which is faster than the lipid in the early life of pigs.

Protein is the most important and nutritious component of meat, and consumers are concerned most about it (36). The composition and content of amino acid shape the nutritional value and flavor of the meat. Therefore, we evaluated the amino acid content in the plasma and muscle to investigate how dietary betaine affects the metabolism of amino acid. The results showed that the betaine addition in the sow-offspring diets significantly improved the contents of various free amino acids in the plasma, such as Asp, Met, Ser, and Glu at different stages. Meanwhile, Glu plays an important role in intestinal, cardiovascular, and neurodevelopment, and Met and Ser are the important FAAs (37). Because of the methyl donor properties, dietary betaine

TABLE 7 | Effect of dietary betaine on the expression of genes related to MyHC isoform and MRFs in the muscle of Bama mini-pigs.

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
***Longissimus thoracis et lumborum* muscle**	65	1.00 ± 0.25	0.84 ± 0.20	1.25 ± 0.26
MyHC-I	95	1.00 ± 0.36^{b}	1.93 ± 0.26^{ab}	3.31 ± 1.05^{a}
	125	1.00 ± 0.19	0.74 ± 0.18	1.60 ± 0.36
	65	1.00 ± 0.20	0.75 ± 0.07	0.99 ± 0.12
MyHC-IIa	95	1.00 ± 0.10	1.01 ± 0.08	1.14 ± 0.25
	125	1.00 ± 0.28	0.82 ± 0.06	1.20 ± 0.31
	65	1.00 ± 0.06	1.59 ± 0.33	1.52 ± 0.23
MyHC-IIx	95	1.00 ± 0.15^{b}	1.25 ± 0.27^{b}	2.49 ± 0.15^{a}
	125	1.00 ± 0.22^{b}	2.21 ± 0.53^{ab}	3.81 ± 1.22^{a}
	65	1.00 ± 0.11^{b}	1.67 ± 0.20^{a}	1.51 ± 0.29^{ab}
MyHC-IIb	95	1.00 ± 0.10^{b}	2.03 ± 0.40^{a}	2.14 ± 0.26^{a}
	125	1.00 ± 0.17	1.26 ± 0.14	1.20 ± 0.28
	65	1.00 ± 0.09	1.00 ± 0.22	0.65 ± 0.13
MSTN	95	1.00 ± 0.15	0.89 ± 0.18	1.62 ± 0.45
	125	1.00 ± 0.15	1.06 ± 0.13	1.28 ± 0.19
	65	1.00 ± 0.17^{b}	0.81 ± 0.11^{b}	1.94 ± 0.49^{a}
Myf5	95	1.00 ± 0.08^{b}	1.15 ± 0.23^{b}	2.81 ± 0.67^{a}
	125	1.00 ± 0.21	0.60 ± 0.07	0.90 ± 0.26
	65	1.00 ± 0.13	1.12 ± 0.08	1.02 ± 0.14
MyoG	95	1.00 ± 0.21^{b}	1.64 ± 0.29^{ab}	2.27 ± 0.39^{a}
	125	1.00 ± 0.12	0.83 ± 0.09	0.66 ± 0.10
***Biceps femoris* muscle**	65	1.00 ± 0.50	1.64 ± 0.68	1.46 ± 1.15
MyHC-I	95	1.00 ± 0.72	0.93 ± 0.34	1.61 ± 0.90
	125	0.83 ± 0.17	1.36 ± 0.51	1.14 ± 0.37
	65	1.00 ± 1.91	0.71 ± 0.53	0.55 ± 0.49
MyHC-IIa	95	1.01 ± 0.46	0.70 ± 0.18	1.27 ± 0.50
	125	1.13 ± 0.48	0.93 ± 0.54	1.33 ± 0.51
	65	1.01 ± 0.41	2.04 ± 0.85	1.67 ± 1.61
MyHC-IIx	95	1.00 ± 0.78	0.61 ± 0.10	0.89 ± 0.27
	125	1.12 ± 0.63	0.92 ± 0.72	1.06 ± 0.35
	65	1.00 ± 0.43	1.32 ± 0.24	26.54 ± 58.19
MyHC-IIb	95	1.00 ± 0.45^{ab}	0.67 ± 0.31^{b}	2.00 ± 1.74^{a}
	125	0.64 ± 0.45^{b}	1.80 ± 1.06^{a}	0.55 ± 0.24^{b}
	65	1.00 ± 1.08	0.68 ± 0.44	2.23 ± 3.48
MSTN	95	1.00 ± 0.73	0.60 ± 0.30	1.03 ± 0.41
	125	1.00 ± 0.42	1.34 ± 0.82	1.05 ± 0.33
	65	1.00 ± 0.36	1.62 ± 0.92	4.92 ± 6.02
Myf5	95	1.00 ± 1.18	0.41 ± 0.23	0.85 ± 0.73
	125	1.01 ± 0.95	1.49 ± 1.81	0.51 ± 0.25
	65	1.00 ± 1.99	0.55 ± 0.48	0.88 ± 1.21
MyoG	95	1.00 ± 0.69	0.67 ± 0.21	0.90 ± 0.25
	125	0.95 ± 0.36	1.03 ± 0.31	0.81 ± 0.23
***Psoas major* muscle**	65	1.00 ± 0.43^{b}	3.64 ± 2.20^{a}	2.58 ± 1.43^{a}
MyHC-I	95	1.00 ± 0.44	1.04 ± 0.47	0.69 ± 0.22
	125	1.00 ± 1.12	0.93 ± 0.64	0.51 ± 0.25
	65	1.00 ± 0.59^{b}	4.97 ± 4.54^{a}	2.47 ± 2.19^{ab}

(Continued)

TABLE 7 | Continued

Items	d-old	Control group	Sow-betaine group	Sow-offspring-betaine group
MyHC-IIa	95	1.00 ± 0.37	0.89 ± 0.35	0.85 ± 0.41
	125	1.00 ± 0.23	1.80 ± 1.43	1.29 ± 1.60
	65	1.00 ± 0.65	0.67 ± 0.47	0.50 ± 0.42
MyHC-IIx	95	1.00 ± 0.80	1.52 ± 1.05	1.37 ± 0.52
	125	1.00 ± 0.72	2.00 ± 2.29	1.02 ± 0.88
	65	1.00 ± 0.86^{b}	3.62 ± 2.88^{a}	3.22 ± 1.94^{a}
MyHC-IIb	95	1.00 ± 0.97	0.99 ± 0.48	0.79 ± 0.30
	125	1.00 ± 0.61	0.87 ± 0.44	0.78 ± 0.24
	65	1.00 ± 0.51	7.21 ± 11.44	1.54 ± 0.79
MSTN	95	1.00 ± 0.38	2.19 ± 2.65	2.23 ± 1.31
	125	1.00 ± 0.48	0.98 ± 0.51	1.15 ± 0.51
	65	1.00 ± 0.39	1.67 ± 0.70	1.20 ± 0.86
Myf5	95	1.00 ± 0.66	1.21 ± 1.35	1.68 ± 1.02
	125	1.00 ± 0.88	1.05 ± 1.27	0.91 ± 0.58
	65	1.00 ± 0.54^{b}	6.25 ± 6.75^{a}	1.88 ± 0.64^{b}
MyoG	95	1.00 ± 0.42	0.90 ± 0.37	0.63 ± 0.35
	125	1.00 ± 0.20^{a}	0.95 ± 0.30^{a}	0.60 ± 0.20^{b}

Data are presented as means ± SE. Values in the same row without a common superscript letter are significantly different ($P < 0.05$).
At 65-, 95-, and 125-d-old, the replicates of the control group were 12, 11, and 8; the sow-betaine group were 6, 7, and 6; and the sow-offspring-betaine group were 7, 7, and 6, respectively.
MyHC-IIa, Myosin heavy chain-IIa; MyHC-IIb, Myosin heavy chain-IIb; Myf5, Myogenic degradation factor; MyHC-I, Myosin heavy chain-I; MyHC-IIx, Myosin heavy chain-IIx; MyoG, Myogenin; MSTN, Myostation.

addition can enhance the methionine, and produce a variety of secondary metabolites to promote the synthesis of other EAAs, such as glycine and serine, eventually improves muscle flavor and meat quality (38). In the present study, the amino acids contents in different muscles (LTL, BF, and PM) and different stages (65-, 95-, and 125-d-old) of pigs were varied, several EAAs like Met, His, Ile, Lys, and Phe were significantly increased in the LTL muscle, whereas those EAAs were decreased in the BF and PM muscles. The reduction of these EAAs in the PM muscle may be caused by the different enzyme activities in different tissues, and further studies are necessary to reveal the specific mechanism.

The plasma biochemical parameters are the important indicator for judging the body's health, which can reflect the nutritional metabolism and the functions of various tissues and organs. Total protein content can reflect protein synthesis and metabolism to a certain extent and positively correlated with muscle rate (39). The plasma AST is an important transaminase in the process of amino acid metabolism; its activity is closely related to the body's liver physiological functions and the intensity of amino acid metabolism (40). The present study showed that the betaine addition in sow diets increased the plasma TP content in the 65-d-old pigs, while betaine addition in sow and offspring diets increased the plasma AST content. These findings indicated that dietary betaine could promote protein synthesis, including the low-density lipoproteins in the liver.

Skeletal muscle is the main meat-producing tissue of pigs. The number and diameter of muscle fibers are important factors in muscle formation (41). Moreover, the growth and development of muscle fibers also play an important role in meat quality. The type of muscle fiber has been determined during the fetal period. The muscle fiber types of pigs during the fetal period are mainly *MyHC-I* and *MyHC-IIa*, and the muscle fiber types of newborn piglets are mainly *MyHC-I*, *MyHC-IIa*, and *MyHC-IIx*. While *MyHC-IIb* is less, and *MyHC-IIb* muscle fibers will continue increasing with muscles developing (42, 43). In the present study, betaine addition in sow-offspring diets up-regulated the mRNA expression levels of *MyHC-I, MyHC-IIx*, *MyHC-IIb,* and *MyoG* genes of the muscles at different stages. The results were consistent with the previous findings by Zhuo et al. (44) who studied the effects of methyl donor supplementation in the sow diet on piglets' muscle growth. In addition, the present study also found that betaine addition to sows and their offspring' diets significantly up-regulated the *Myf5* and *MyoG* gene expressions in the LTL muscle. The *Myf5* and *MyoG* belong to the MRF genes family. The expression of MRF has great significance to the growth, development, and differentiation of skeletal muscle (45). Therefore, the present study indicated that dietary betaine can regulate the expression level of muscle-derived regulatory factors and genes related to muscle fiber types to influence the growth and development of skeletal muscle.

CONCLUSIONS

In summary, betaine addition in the sow and piglets' diets could increase the carcass weight, carcass yield, lean meat rate, meat color, shear force, and crude protein content of muscles at different stages. Furthermore, betaine addition in sow-offspring diets upregulated the expression level of *MyHC-I, MyHC-IIx*, *MyHC-IIb,* and *MyoG*, while dietary betaine addition

in the sow and sow-offspring diets improved the contents of muscle amino acids. However, betaine addition in the sow-offspring diet had more distinct effects than the betaine addition in sow diet. Therefore, these findings provide the reference for research into dietary betaine addition in the sow and piglets diets to improve the carcass traits and meat quality of Bama mini-pigs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All procedures were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (ISA-2018-071).

AUTHOR CONTRIBUTIONS

YC, MS, QZ, and MA performed sampling and nutrient measurements, analyzed data, interpreted the results, and drafted the manuscript. YC, MS, and QG conducted animal feeding and sampling. XK contributed to experimental concepts and design, provided scientific direction, and together with MA finalized the manuscript. All authors have read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant No. 31772613), Special Funds for Construction of Innovative Provinces in Hunan Province (2019RS3022), and Production and Research Talent Support Project of the CAS Wang Kuancheng Initiative Talent Program.

ACKNOWLEDGMENTS

The authors thank the staff and postgraduate students from the Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process for collecting samples and technicians from the CAS Key Laboratory of Agro-ecological Processes in Subtropical Region for providing technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.728477/full#supplementary-material

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frontiers in Nutrition

SYSTEMATIC REVIEW
published: 29 September 2021
doi: 10.3389/fnut.2021.746765

Effects of Polyunsaturated Fatty Acids Supplementation on the Meat Quality of Pigs: A Meta-Analysis

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Polyunsaturated fatty acids (PUFAs) supplementation has been widely discussed as a strategy for improving meat quality in pig production, but the effects are inconsistent. This meta-analysis was performed to comprehensively evaluate its effects on the meat quality and growth performance of pigs. We searched the PubMed and the Web of Science databases (articles published from January 1, 2000 to October 16, 2020) and compared PUFAs-supplemented diets with control diets. We identified 1,670 studies, of which 14 (with data for 752 pigs) were included in our meta-analysis. The subgroup analysis was classified as PUFA source [conjugated linoleic acid (CLA) or linseed], concentration (high or low concentration), and initial stage (growing or finishing pigs). Our analysis found that PUFA supplementation increased the intramuscular fat (IMF) content (WMD = 0.467%, 95% CI: 0.312–0.621, $p < 0.001$), decreased the meat color L* (WMD = −0.636, 95% CI: −1.225 to −0.047, $p = 0.034$), and pH 24 h (WMD = −0.021, 95% CI: −0.032 to −0.009, $p < 0.001$) but had no influence on drip loss, meat color a* and b*, pH 45 min, and growth performance. CLA supplementation improved IMF content (WMD = 0.542%, 95% CI: 0.343–0.741, $p < 0.001$) and reduced meat color b* (WMD = −0.194, 95% CI: −0.344 to −0.044, $p = 0.011$). Linseed supplementation increased IMF content (WMD = 0.307%, 95% CI: 0.047–0.566, $p = 0.021$), decreased meat color L* (WMD = −1.740, 95% CI: −3.267 to −0.213, $p = 0.026$), and pH 24 h (WMD = 0.034, 95% CI: −0.049 to −0.018, $p < 0.001$). We discovered an increase on the IMF content in both high and low concentration PUFA supplementation (WMD = 0.461%, 95% CI: −0.344 to −0.044, $p < 0.001$; WMD = 0.456%, 95% CI: 0.276–0.635, $p < 0.001$). Furthermore, we also found the effects of PUFA supplementation on meat color L* and pH 24 h are concentration- and stage-dependent. PUFA supplementation can improve the meat quality of pigs, which mainly emerges in greatly increasing IMF content.

Keywords: polyunsaturated fatty acids, meat quality, pig, meta-analysis, conjugated linoleic acid, linseed

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *24 July 2021*
Accepted: *30 August 2021*
Published: *29 September 2021*

Citation:
Wang L, Huang Y, Wang Y and Shan T (2021) Effects of Polyunsaturated Fatty Acids Supplementation on the Meat Quality of Pigs: A Meta-Analysis. Front. Nutr. 8:746765. doi: 10.3389/fnut.2021.746765

INTRODUCTION

There has been an increased interest in recent years in ways to produce high-quality pork. This is because pork is one of the most consumed animal proteins in the world and is an important source of dietary protein and fatty acid, especially saturated fatty acids, which is closely related to human health (1). The main traits by which pork quality is evaluated include intramuscular fat

(IMF) content, drip loss, meat color, pH, juiciness, tenderness, flavor, and fatty acid composition. IMF is mainly distributed in the epimysium, perimysium, and endomysium of skeletal muscle and is positively correlated with meat quality including flavor, tenderness, and juiciness (2). Multiple factors can influence pork quality, such as nutrition, genetics, environment, management practices, and production systems (3); hence, it is of great significance to improve pork quality *via* seeking effective strategies.

Dietary intervention is one of the most common methods to improve the meat quality of pigs. Previous studies have found that dietary fatty acid composition plays an important role in regulating the nutritional quality of pork not only in lean pig breeds but also in Chinese indigenous breeds pigs (4, 5). It is a consumer-acceptable and effective strategy for producers to improve the meat quality of pigs through added fatty acid supplementation in diet. Polyunsaturated fatty acids (PUFAs) are one of the essential fatty acids, including n-3 PUFAs and n-6 PUFAs. PUFAs play an irreplaceable role in regulating fat deposition, muscle development, and glycolipid metabolism (6–8). In recent years, many studies have conducted feeding trials on pigs to explore the effects of PUFAs on meat quality with inconsistent results. The most commonly used PUFA supplementation is conjugated linoleic acid (CLA) and linseed. CLA is a secondary derivative of linoleic acid, and linseed is the ripe seed of flax. Several factors lending to these treatment effect inconsistencies include several factors, such as different PUFA source supplementation (CLA or linseed), added concentration (high concentration or low concentration), and initial growth stage of pigs (growing or finishing pigs), led to the inconsistent results by further comparison.

The aim of our study was to reveal the main effects or the effect orientation of PUFA supplementation on the meat quality of pigs by performing a meta-analysis. We also elucidated the potential influential factors based on the outcomes including IMF content, drip loss, meat color, pH 45 min, and pH 24 h. This is the first comprehensive and systematic meta-analysis focused on this topic and providing useful strategies for producing high-quality pork in the pig industry.

MATERIALS AND METHODS

We conducted and reported the meta-analysis strictly following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement (9).

Search Strategy

We collected studies from the last 20 years published between January 1, 2000 and October 16, 2020 in the PubMed (https://www.thncbi.nlm.nih.gov/pubmed; accessed October 16, 2020) and Web of Science (http://webofknowledge.com; accessed October 16, 2020) databases. We applied no language restrictions. The complete search principles were as follows: (1) the term "pigs" was searched in the PubMed database beforehand and shown to be "swine," "suidae," "warthogs," "wart hogs," "hog, wart," "hogs, wart," "wart hog," and "phacochoerus"; (2) similarly, the terms related to PUFAs were extended to include "fatty acids, unsaturated," "acids, unsaturated fatty," "unsaturated fatty acids," "acids, unsaturated fatty," and "fatty acids, polyunsaturated"; and (3) meat quality was equal to pork quality and meat characteristic. The detailed search strategy and findings are shown in **Table 1**. We considered all potentially eligible studies instead of the primary outcome or language. We also did a manual search to obtain more studies. The complete search method was shown in **Table 1**.

TABLE 1 | Search strategy.

Search	Query	Items found
PubMed		
#1	Search: (((((((((Swine) or Suidae) or Pigs) or Warthogs) or Wart Hogs) or Hog, Wart) or Hogs, Wart) or Wart Hog) or Phacochoerus)); Filters: Publication date from 2000/01/01 to 2020/10/16	157,481
#2	Search: ((((((Fatty Acids, Unsaturated) or Acids, Unsaturated Fatty) or Unsaturated Fatty Acids) or Polyunsaturated Fatty Acids) or Acids, Polyunsaturated Fatty) or Fatty Acids, Polyunsaturated))	122,686
#3	Search: (((meat quality) or pork quality) or meat characteristic))	18,919
#1 AND #2 AND #3		274
Web of science		
#1	TS = (Swine or Suidae or Pigs or Warthogs or Wart Hogs or Hog, Wart or Hogs, Wart or Wart Hog or Phacochoerus)	411,441
#2	TS = (Fatty Acids, Unsaturated or Acids, Unsaturated Fatty or Unsaturated Fatty Acids or Polyunsaturated Fatty Acids or Acids, Polyunsaturated Fatty or Fatty Acids, Polyunsaturated)	102,584
#3	TS= (meat quality or pork quality or meat characteristic)	117,463
#1 AND #2 AND #3		1396

Selection Criteria and Procedure

We regarded studies as eligible for inclusion if they met the following criteria: (1) studies reported the effects of PUFAs on meat quality (IMF, drip loss, meat color, pH 45 min, and pH 24 h); (2) PUFAs, PUFA-rich compounds, PUFA supplements, or PUFA extracts were added to the feed throughout the experimental period; (3) the growth stage of pigs was growing or finishing; and (4) the concentration of PUFA supplements was reported. The exclusion criteria were as follows: (1) studies lacked a control group; (2) studies are proceedings papers; (3) studies lacked full-text online resources; (4) studies used mixed additives; and (5) studies investigated piglets. Based on these criteria, we screened eligible studies for subsequent meta-analysis (**Figure 1A**).

The following information was extracted from each selected study: author information (first author, year, and country), genetic background, PUFA source, experimental duration, added concentration, basal diet, energy difference, sum number of pigs included in the control and treatment groups, sex, growth stage

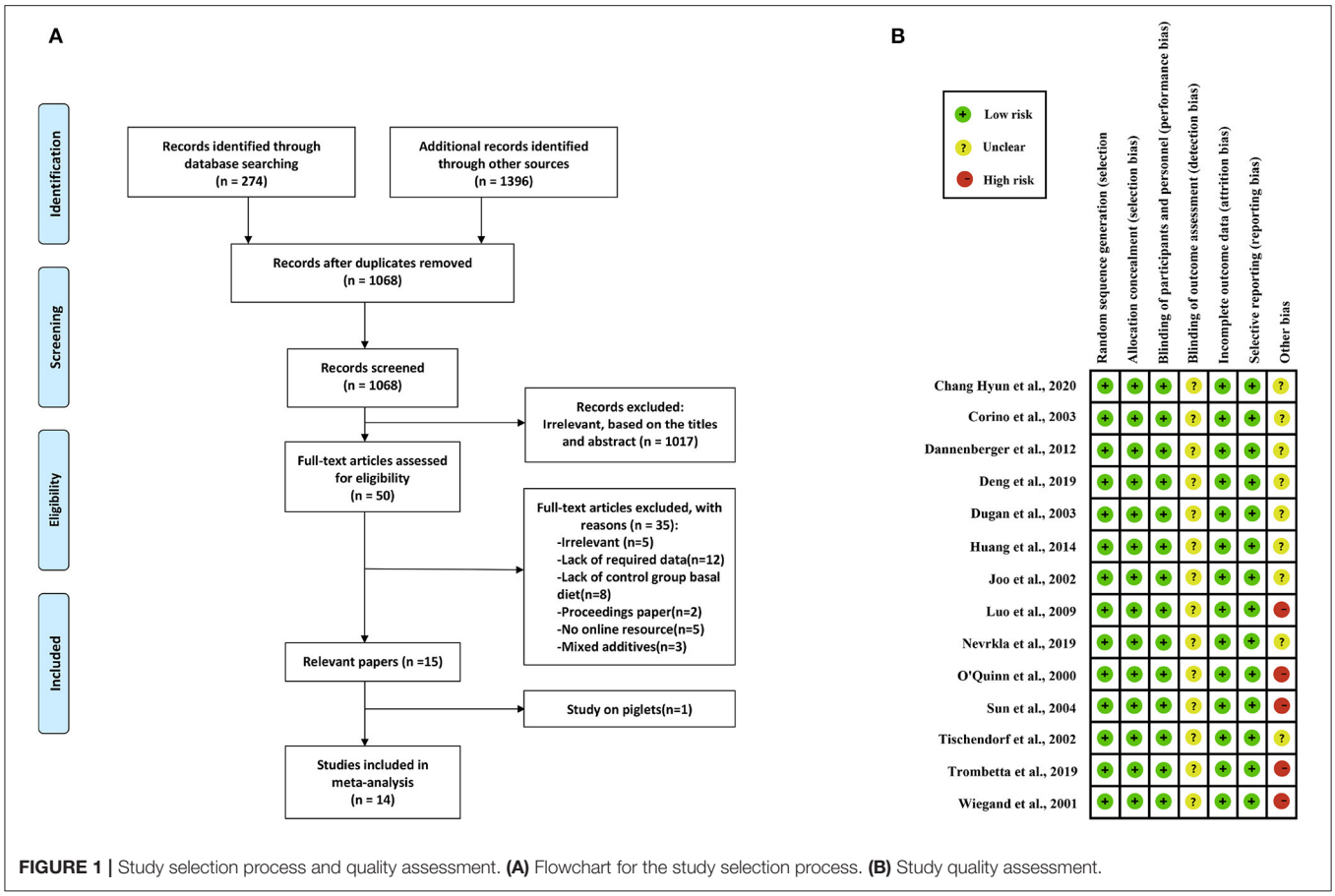

FIGURE 1 | Study selection process and quality assessment. **(A)** Flowchart for the study selection process. **(B)** Study quality assessment.

(growing, finishing, or growing-finishing), growth performance parameters [average daily gain (ADG), average daily feed intake (ADFI), and gain:feed (G:F) ratio], and outcomes of meat quality (IMF, drip loss, meat color, pH 45 min, and pH 24 h). One study might have more than one record due to the duration of the pigs and concentration of the supplemental substance.

The study selection procedure was as follow: (1) two investigators (L. Wang and Y. Huang) independently screened the titles and abstracts of the articles according to the inclusion criteria; (2) disagreements during independent selection were solved through consultation with a third author (T. Shan); and (3) after the included studies were verified and confirmed, one investigator (L. Wang) extracted the data and information from each study and the other investigator (Y. Huang) checked. The summarized information of included studies was shown in **Table 2**.

Study Quality Assessment

Two investigators (L. Wang and Y. Huang) independently assessed the quality assessment of included studies by using two methods: the Cochrane Handbook for Systematic Review of Interventions (24) and the Study Quality Assessment on Nonruminants (SQANR) (25), which is a new assessment method for feeding trials and included five detailed part: within-group differences, multiple reports, sample size, rationality of experimental design, and completeness of experimental information. Articles were judged as high risk, low risk, or unclear in the following aspects: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias, in which the assessment of other bias according to the final score of SQANR (high and moderate qualities were defined as unclear risk, and low quality was defined as high risk) (**Figure 1B** and **Supplementary Table 2**).

Within-Group Standard Deviation Estimate

We obtained the within-group SD by the following three approaches: (1) used the within-group SE to calculate; (2) contacted the authors *via* emails if the study has neither the within-group SD nor SE; and (3) used pooled SD as within-group SD, which was calculated from SEM, and pooled SD is equal to SEM multiplied by the square root of the number of groups (26).

Statistical Analysis

The statistical analysis was performed with Stata 15.1 (Stata Corp., College Station, TX, USA).

Meta-Analysis

For continuous outcomes, because the units of measure data are the same and the mean varies little, we used a random-effects

TABLE 2 | Characteristics of included studies[a].

Refernces	Country	Genetic background	PUFA source	Duration	Concentration	Basal diet	Energy difference[b]	N[c]	Sex	Growth stage	Growth performance parameters[d]	Outcomes[e]
O'Quinn et al. (10)	American	PIC L326 or 327 boars × C22 sows	CLA 60	37.6–106.4 kg	50%	Corn-soybean	NA	24	Barrows	Growing-finishing pigs	ADG, ADFI, G:F ratio	Drip loss, meat color
Wiegand et al. (11)	American	NA	Conjugated linoleic acid	40–106 kg	0.75%	Corn-soybean	→	20	Barrows	Growing-finishing pigs	ADG, ADFI, G:F ratio	IMF, meat color
Joo et al. (12)	Korea	Landrace × Large White × Duroc	Conjugated linoleic acid	4 weeks	1%, 2.5%, 5%	NA	NA	20	Gilts	Finishing pigs	NA	IMF, drip loss, meat color, pH 24 h
Tischendorf et al. (13)	Germany	Pietrain × (Landrace × Large White)	Conjugated linoleic acid	8 weeks	2%	Barley-soybean	→	40	20 female and 20 male-castrated	Growing-finishing pigs	ADG, ADFI, G:F ratio	IMF, drip loss, meat color, pH 45 min, pH 24 h
Corino et al. (14)	Italy	Large White	Conjugated linoleic acid	97–172 kg	0.25%, 0.5%	Corn-soybean	→	36	18 barrows and 18 gilts	Finishing pigs	NA	meat color, pH 45 min, pH 24 h
Dugan et al. (15)	Canada	NA	Conjugated linoleic acid	35–115 kg	0.25%, 0.5%	Barley-soybean	→	108	NA	Growing-finishing pigs	NA	IMF, drip loss, meat color, pH 24 h
Sun et al. (16)	China	Duroc × Landrace × Large White	Conjugated linoleic acid	3, 6 weeks	2%, 4%	Corn-soybean	→	54	Crossbred barrows	Finishing pigs	ADG, ADFI, G:F ratio	IMF, drip loss
Luo et al. (17)	China	Landrace × NewDamLine	Linseed	30, 60, 90 days	10%	Corn-soybean	→	24	Barrows	Growing-finishing pigs	NA	IMF, drip loss, pH 45 min
Dannenberger et al. (18)	Germany	Landrace	High, reduced protein diet with linseed oil	60~100 kg to 120 kg	4.5%	Barley-soybean	↑	24	Male-castrated	Finishing pigs	ADG, ADFI, G:F ratio	IMF, drip loss, meat color, pH 45 min, pH 24 h
Huang et al. (19)	China	Rongchang pigs	Conjugated linoleic acid	30–60 kg, 60–90 kg	0.5%, 1%, 1.5%, 2%	Corn-soybean	→	160	NA	Growing-finishing pigs	NA	IMF, meat color, pH 45 min, pH 24 h
Deng et al. (20)	China	NA	Flaxseed	72 days	5%, 10%	Corn-soybean	→	72	NA	Growing-finishing pigs	ADG, ADFI, G:F ratio	IMF, drip loss, meat color, pH 45 min, pH 24 h
Nevrkla and Vaclavkova (21)	Czech Republic	(Large White × Landrace) × (Duroc × Pietrain)	Linseed	57 days	7%	Barley-soybean	↑	40	Gilts	Finishing pigs	NA	IMF, drip loss, pH 45 min, pH 24 h
Trombetta et al. (22)	Brazil	50% Large White × 50% Landrace	Linseed oil	90 days	3%	Corn-soybean	NA	22	10 castrated males and 12 females	Finishing pigs	ADG	IMF, drip loss, meat color, pH 24 h
Chang Hyun et al. (23)	Korea	Landrace × Yorkshire × Duroc	Linseed (n-6: n-3 PUFA ratio)	NA	1.5% (4:1), 3% (2:1)	Corn-soybean	→	108	NA	Finishing pigs	ADG, ADFI, G:F ratio	Drip loss, meat color, pH 45 min, pH 24 h

[a] *NA, not available.*

[b] *↑, higher energy density in treatment group; → , similar energy density in treatment and control groups; ↓, lower energy density in treatment group.*

[c] *Number of pigs included in studies.*

[d] *ADG, average daily gain; ADFI, average daily feed intake; G: F ratio, gain: feed ratio.*

[e] *IMF, intramuscular fat; pH 45 min, pH value measured at 45 min postmortem; pH 24 h, pH value measured at 24-h postmortem.*

TABLE 3 | The summary of meta-analysis and publication bias analysis of the included studies.

Outcome[a]	N[b]	WMD (95% CI)[c]	*P*	I^2	$P_{heterogeneity}$	Begg's Test	Egger's test
IMF (%)	26	0.467 (0.312–0.621)	<0.001	87.0%	<0.001	0.005	0.085
Drip loss (%)	24	−0.191 (−0.458 to 0.075)	0.159	63.4%	<0.001	0.861	0.439
L*	25	−0.636 (−1.225 to −0.047)	0.034	65.5%	<0.001	0.110	0.509
a*	25	0.081 (−0.244 to 0.406)	0.625	67.5%	<0.001	1.000	0.614
b*	25	−0.123 (−0.268 to 0.022)	0.095	53.4%	0.001	0.158	0.136
pH 45 min	24	0.038 (−0.042 to 0.117)	0.351	71.9%	<0.001	0.053	0.114
pH 24 h	24	−0.021 (−0.032 to −0.009)	<0.001	11.6%	0.300	0.516	0.229

[a] *L*, lightness, a*, redness, b*, yellowness.*
[b] *N, number of comparisons.*
[C] *WMD, weighted mean difference; CI, confidence interval.*

model to calculate the overall effect as weighted mean difference (WMD) and 95% CI between the treatment and control groups. If the 95% CI contained a zero value, there was no difference. We also used Cochran's *Q*-test (significance level of $p \leq 0.1$) and the I^2 statistic to assess the degree of statistical heterogeneity among studies, with a value of <25, 25–50%, 51–75, and >75% considered as no, low, moderate, and high level of heterogeneity, respectively (27). Particularly, based on Cochrane Handbook Chapter 10, we changed into fixed-effects model to meta-analysis if we found the statistics of study have homogeneity which meant I^2 statistic <50%.

Regression Analysis

We performed a meta-regression analysis to explore the potential sources of heterogeneity and define the effects of covariates on outcomes (IMF, drip loss, meat color, pH 45 min, and pH 24 h) (28). The covariates were as follows: PUFA source (CLA or linseed), added concentration [high concentration (>2%) or low concentration (≤2%)], and initial growth stage (growing pigs or finishing pigs). The regression analysis was applied only to groups with 10 or more records to avoid a false positive result.

Subgroup Analysis and Sensitivity Analysis

To explore the sources of heterogeneity, we conducted a subgroup analysis if the study was regarded as having moderate or high heterogeneity ($I^2 > 50\%$). We classified the subgroups into three groups: CLA group or linseed group, high concentration group or low concentration group, and growing pigs group or finishing pigs group and foreign pigs or Chinese local pigs. If the heterogeneity was significant ($p < 0.05$), we also performed a sensitivity analysis to identify which study (or studies) contributing to the heterogeneity using the leave-one-out method. Heterogeneity and pooled analyses were recalculated after a single study was removed from the outcome at a time. We included data which the source of heterogeneity was identified and exclude these data did not influence the pooled estimates.

Publication Bias

The potential publication bias was investigated by funnel plot asymmetry (**Supplementary Figure 1**), Begg's and Egger's weighted regression test, for which the significance level was defined at $p < 0.05$ (29). We used Egger's test as a reference if funnel plot asymmetry, Begg's and Egger's tests disagreed. In addition, the trim-and-fill test was used to estimate the effect of publication bias on the interpretation of the results (30).

RESULTS

We identified 1,670 studies, of which 14 (with data for 752 pigs) were included in our meta-analysis (**Figure 1A**) (10–23). The 14 studies were all published between 2000 and 2020, and there was no repetition between studies (**Table 1**). These studies investigated the effects of PUFA supplementation on meat quality (IMF, drip loss, meat color, pH 45 min, and pH 24 h) and growth performance (ADG, ADFI, and G:F ratio). Among the selected studies, there are eight added CLA and six added linseed or linseed oil, seven studies (14 records) began at the grower phase, and seven studies (17 records) began at the finisher phase. The study quality assessment was shown in **Figure 1B**. We defined the risk of detection bias as unclear because the blinding of outcome assessment was not reported in the included studies. Other bias were assessed based on the final score of SQANR (**Supplementary Table 2**); there are nine studies: four have unclear risk and five have high risk. According to the funnel plot (**Supplementary Figure 1**), Begg's and Egger's tests, the publication bias was not significant ($p > 0.05$) in the current meta-analysis (**Table 3**), so the trim-and-fill test was not necessary to perform.

Effects of PUFA Supplementation on the Meat Quality and Growth Performance of Pigs

As shown in **Table 3**, we presented the effects of PUFA supplementation on the meat quality of pigs. PUFA supplementation increased the content of IMF by 0.467% (95% CI: 0.312–0.621, $p < 0.001$) with high heterogeneity ($I^2 = 87.0\%$, $p_{heterogeneity} < 0.001$), decreased the meat color L* by 0.636 (95% CI: −1.225 to −0.047, $p = 0.034$) with moderate heterogeneity ($I^2 = 65.5\%$, $p_{heterogeneity} < 0.001$) and decreased the pH 24 h by 0.021 (95% CI: −0.032 to −0.009, $p < 0.001$) with no heterogeneity ($I^2 = 11.6\%$, $p_{heterogeneity} = 0.300$). However, PUFA supplementation had no effect on the drip loss (WMD = −0.191, 95% CI: −0.458 to 0.075, $p = 0.159$) with moderate

TABLE 4 | Regression and subgroup analysis of studies included in the meta-analysis.

Outcome	Subgroup		N[a]	$P^{b}_{regression}$	WMD (95% CI)	P	I^2	$P_{heterogeneity}$
IMF (%)	PUFA source	CLA	193	0.020	0.542 (0.343–0.741)	<0.001	86.4%	<0.001
		Linseed	81		0.307 (0.047–0.566)	0.021	86.6%	<0.001
	Concentration	High concentration	97	0.021	0.461 (0.208–0.715)	<0.001	92.2%	<0.001
		Low concentration	177		0.456 (0.276–0.635)	<0.001	72.0%	<0.001
	Initial growth stage	Growing pigs	200	0.006	0.563 (0.395–0.731)	<0.001	75.7%	<0.001
		Finishing pigs	74		0.254 (−0.061 to 0.569)	0.114	93.6%	<0.001
Drip loss (%)	PUFA source	CLA	163	0.828	−0.147 (−0.314 to 0.021)	0.086	0.0%	0.857
		Linseed	105		−0.299 (−0.959 to 0.361)	0.374	81.9%	<0.001
	Concentration	High concentration	147	0.891	−0.268 (−0.775 to 0.240)	0.301	74.9%	<0.001
		Low concentration	121		−0.128 (−0.307 to 0.050)	0.159	0.0%	0.878
	Initial growth stage	Growing pigs	170	0.868	−0.135 (−0.301 to 0.031)	0.111	0.0%	0.777
		Finishing pigs	98		−0.258 (-0.955 to 0.440)	0.469	81.6%	<0.001
L*	PUFA source	CLA	205	0.226	−0.155 (−0.590 to 0.280)	0.485	14.0%	0.287
		Linseed	67		−1.740 (−3.267 to −0.213)	0.026	81.9%	<0.001
	Concentration	High concentration	65	0.932	−1.366 (−2.717 to −0.015)	0.047	78.9%	<0.001
		Low concentration	207		−0.172 (−0.627 to 0.283)	0.459	17.4%	0.254
	Initial growth stage	Growing pigs	182	0.266	−0.091 (−0.652 to 0.470)	0.750	34.5%	0.092
		Finishing pigs	90		−1.331 (−2.354 to −0.308)	0.011	72.5%	<0.001
a*	PUFA source	CLA	205	0.500	0.137 (−0.265 to 0.538)	0.504	73.3%	<0.001
		Linseed	67		−0.066 (−0.578 to 0.446)	0.800	36.8%	0.148
	Concentration	High concentration	65	0.269	0.146 (−0.364 to 0.656)	0.574	54.7%	0.024
		Low concentration	207		0.053 (−0.370 to 0.476)	0.806	73.0%	<0.001
	Initial growth stage	Growing pigs	182	0.179	0.227 (−0.141 to 0.595)	0.226	61.7%	0.001
		Finishing pigs	90		−0.255 (−0.900 to 0.391)	0.439	75.8%	<0.001
b*	PUFA source	CLA	205	0.187	−0.194 (−0.344 to −0.044)	0.011	52.7%	0.005
		Linseed	67		0.184 (−0.140 to 0.508)	0.265	26.7%	0.225
	Concentration	High concentration	65	0.890	0.032 (−0.209 to 0.273)	0.794	17.1%	0.290
		Low concentration	207		−0.180 (−0.351 to −0.009)	0.039	60.3%	0.001
	Initial growth stage	Growing pigs	182	0.686	−0.163 (−0.295 to −0.030)	0.016	32.0%	0.112
		Finishing pigs	90		−0.104 (−0.475 to 0.267)	0.583	70.0%	<0.001
pH 45 min	PUFA source	CLA	108	0.772	0.019 (−0.095 to 0.132)	0.749	64.1%	0.003
		Linseed	105		0.056 (−0.059 to 0.172)	0.337	77.7%	0.002
	Concentration	High concentration	93	0.774	0.058 (−0.064 to 0.179)	0.352	79.9%	<0.001
		Low concentration	120		0.021 (−0.086 to 0.128)	0.704	60.6%	0.003
	Initial growth stage	Growing pigs	118	0.185	−0.003 (−0.078 to 0.072)	0.940	31.6%	0.123
		Finishing pigs	95		0.106 (−0.054 to 0.266)	0.195	87.5%	<0.001
pH 24 h	PUFA source	CLA	195	0.742	−0.006 (−0.023 to 0.11)	0.486	0.0%	0.568
		Linseed	87		−0.034 (−0.049 to −0.018)	<0.001	1.2%	0.420
	Concentration	High concentration	85	0.673	−0.033 (−0.049 to −0.018)	<0.001	0.0%	0.526
		Low concentration	197		−0.006 (−0.023 to 0.011)	0.479	0.0%	0.486
	Initial growth stage	Growing pigs	172	0.108	−0.002 (−0.019 to 0.015)	0.813	0.0%	0.783
		Finishing pigs	110		−0.035 (−0.051 to −0.020)	<0.001	0.7%	0.434

[a] *N, total number of pigs.*

[b] $P_{regression}$, *P value of regression, significance level* $P_{regression} < 0.05$. *L*, lightness; a*, redness; b*, yellowness.*

heterogeneity (I^2 = 63.4%, $p_{heterogeneity}$ <0.001), meat color a* (WMD = 0.081, 95% CI: −0.244 to 0.406, p = 0.625) with moderate heterogeneity (I^2 = 67.5%, $p_{heterogeneity}$ < 0.001), meat color b* (WMD = −0.123, 95% CI: −0.268 to 0.022, p = 0.095) with moderate heterogeneity (I^2 = 53.4%, $p_{heterogeneity}$ = 0.001), and pH 45 min (WMD = 0.038, 95% CI: −0.042 to 0.117, p = 0.351) with moderate heterogeneity (I^2 = 71.9%, $p_{heterogeneity}$ < 0.001). Furthermore, we also presented the effects of PUFA supplementation on the growth performance of pigs in **Supplementary Table 3**. We found there is no significant difference in ADG, ADFI, and G:F ratio between the control and the PUFA supplementation group ($p > 0.05$).

Regression Analysis and Sources of Heterogeneity

To explore the potential sources of heterogeneity and define the effects of covariates on meat quality and growth performance, we performed a meta-regression analysis (**Table 4** and **Supplementary Table 4**). We found PUFA source, added concentration, and initial growth stage might play an important role in affecting the meat quality and growth performance, especially IMF content, because $p_{regression}$ was 0.020, 0.021, and 0.006, respectively. Therefore, we performed subgroup analysis of PUFA source, added concentration, initial growth stage, and breeds in the subsequent research to explore detailed sources of heterogeneity (**Table 4**, **Supplementary Tables 4, 5**). We figured out the significant heterogeneity of drip loss and L* came from linseed, high concentration, finishing pigs, and foreign pigs subgroup. CLA, low concentration, finishing pigs, and foreign pigs subgroup are sources of b* heterogeneity. The PUFA source and breeds are the source of a*, an initial growth stage is the source of pH 45 min, and the concentration is the source of ADFI. We used sensitivity analysis through the leave-one-out method to explore the heterogeneity in IMF as we did not find the source of IMF heterogeneity according to the subgroup

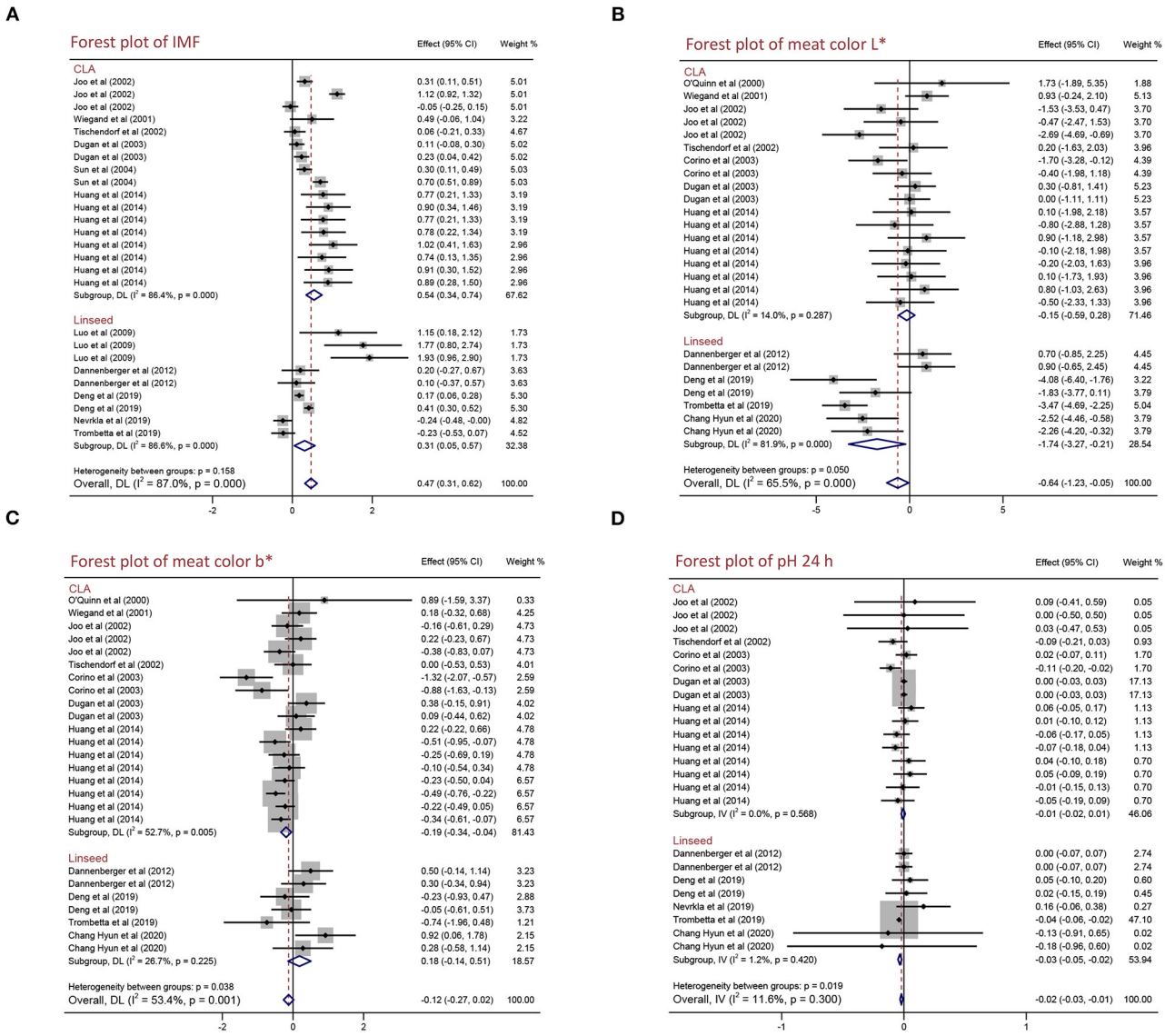

FIGURE 2 | Forest plot of the effects of CLA or linseed on the meat quality of pigs. **(A)** IMF, **(B)** meat color L*, **(C)** meat color b*, and **(D)** pH 24 h. WMD, weighted mean difference; CLA, conjugated linoleic acid. The small solid diamond represents the point estimate for each individual trial, and the horizontal line extending from each solid diamond represents the upper and lower limits of the 95% CI. The size of the shaded square indicates the relative weight of the trial in the meta-analysis. The hollow diamond represents the WMD and 95% CI of the trials, no intersection of the diamond and the solid black line in the middle indicates a significant difference ($p < 0.05$), vice versa.

analysis. The significant heterogeneity had no alteration after each included study was removed, so we assume that the meta-analysis results are robust and the heterogeneity did not interfere with the direction and significance of the final results.

Effects of CLA and Linseed Supplementation on the Meat Quality and Growth Performance of Pigs

To explain the effects of CLA and linseed supplementation on the meat quality and growth performance of pigs, we performed a subgroup analysis of different PUFA source [CLA and linseed (linseed or linseed oil)]. As shown in **Figure 2** and **Table 4**, both CLA and linseed supplementation increased IMF content by 0.542% (95% CI: 0.343–0.741, $p < 0.001$) with high heterogeneity ($I^2 = 86.4\%$, $p_{\text{heterogeneity}} < 0.001$) and 0.307% (95% CI: 0.047–0.566, $p = 0.021$) with high heterogeneity ($I^2 = 86.6\%$, $p_{\text{heterogeneity}} < 0.001$). CLA supplementation can decrease meat color b* by 0.194 (95% CI: −0.344 to −0.044, $p = 0.011$) with moderate heterogeneity ($I^2 = 52.7\%$, $p_{\text{heterogeneity}} = 0.005$). However, there are no effects on drip loss, meat color L*, meat color a*, pH 45 min, and pH 24 h ($p > 0.05$) (**Table 4**). Linseed and linseed oil decreased meat color L* (WMD = −1.740, 95% CI: −3.267 to −0.213, $p = 0.026$) with high heterogeneity ($I^2 = 81.9\%$, $p_{\text{heterogeneity}} < 0.001$) and pH 24 h (WMD = −0.034, 95% CI: −0.049 to −0.018, $p < 0.001$) with no heterogeneity ($I^2 = 1.2\%$, $p_{\text{heterogeneity}} = 0.420$). To sum up, CLA supplementation increased IMF content and decreased meat color b*, whereas linseed supplementation reduced meat color L* and pH 24 h. Furthermore, we found no significant differences in other meat quality and growth performance indexes ($p > 0.05$) (**Table 4** and **Supplementary Table 4**).

Effects of Different PUFA Supplementation Concentration on the Meat Quality and Growth Performance of Pigs

To explore the effects of different PUFA supplementation concentration on the meat quality and growth performance of pigs, we performed a subgroup analysis of different concentration [high concentration (>2%) and low concentration (≤2%)]. As presented in **Figure 3** and **Table 4**, not only high concentration but also low concentration improved IMF content by 0.461% (95% CI: 0.208–0.715, $p < 0.001$) and 0.456% (95% CI: 0.276–0.635, $p < 0.001$) with high heterogeneity ($I^2 = 92.2\%$, $p_{\text{heterogeneity}} < 0.001$) and moderate heterogeneity ($I^2 = 72.0\%$, $p_{\text{heterogeneity}} < 0.001$). High PUFA supplementation concentration decreased meat color L* (WMD = −1.366, 95% CI: −2.717 to −0.015, $p = 0.047$) with high heterogeneity ($I^2 = 78.9\%$, $p_{\text{heterogeneity}} < 0.001$) and pH 24 h (WMD = −0.033, 95% CI: −0.049 to −0.018, $p < 0.001$) with no heterogeneity ($I^2 = 0.0\%$, $p_{\text{heterogeneity}} = 0.526$). Additionally, we found low concentration reduced meat color b* by 0.180 (95% CI: −0.351 to −0.009, $p = 0.039$) with moderate heterogeneity ($I^2 = 60.3\%$, $p_{\text{heterogeneity}} = 0.001$) but increased ADFI by 50.000 g/day (95% CI: 49.957–50.043, $p < 0.001$) with no heterogeneity ($I^2 = 0.0\%$, $p_{\text{heterogeneity}} = 0.768$). In conclusion, high PUFA supplementation concentration improved IMF content and reduced meat color L* and pH 24 h, whereas low concentration decreased meat color b* and increased ADFI. There are no significant differences on other indexes (drip loss, meat color a*, pH 45 min, ADG, and G:F ratio; $p > 0.05$) (**Table 4** and **Supplementary Table 4**).

Effects of PUFA Supplementation on the Meat Quality and Growth Performance of Growing and Finishing Pigs

As shown in **Figure 4** and **Table 4**, for growing pigs, PUFA supplementation increased IMF content by 0.563% (95% CI: 0.395–0.731, $p < 0.001$) with high heterogeneity ($I^2 = 75.7\%$, $p_{\text{heterogeneity}} < 0.001$), whereas decreased meat color b* by 0. 163 (95% CI: −0.295 to −0.030, $p = 0.016$) with low heterogeneity ($I^2 = 32.0\%$, $p_{\text{heterogeneity}} = 0.112$). Moreover, PUFA supplementation reduced meat color L* by 1.331 (95% CI: −2.354 to −0.308, $p = 0.011$) with moderate heterogeneity ($I^2 = 72.5\%$, $p_{\text{heterogeneity}} < 0.001$), pH 24 h by 0.035 (95% CI: −0.051 to −0.020, $p < 0.001$) with no heterogeneity ($I^2 = 0.7\%$, $p_{\text{heterogeneity}} = 0.434$), and improved ADFI by 103.847 g/day (95% CI: −36.922 to 170.772, $p < 0.002$) with high heterogeneity ($I^2 = 100.0\%$, $p_{\text{heterogeneity}} < 0.001$) in finishing pigs. In a word, PUFA supplementation increased IMF content and decreased meat color b* in growing pigs, whereas reduced meat color L*, pH 24 h but improved ADFI in finishing pigs. Additionally, we found PUFA supplementation had no influence on drip loss, meat color a*, pH 45 min, ADG, and G:F ratio in both growing pigs and finishing pigs ($p > 0.05$) (**Table 4** and **Supplementary Table 4**).

DISCUSSION

Pork is one of the most popular meats among people because of its rich nutrition, good flavor, and good economic benefits, and pork has been the first meat for many years. However, in pig production, meat quality has been declining in recent years due to the blind pursuit of production efficiency and increase of backfat thickness. The evaluation index of pork quality includes meat color, tenderness, pH, flavor, IMF, drip loss, and so on (31, 32). Pork quality is affected by many factors, such as genetics (breed), nutrition sex, and environment (feeding management conditions, pre-slaughter conditions, etc.). Nutritional intervention is one of the most common methods to improve pork quality. Recent studies have found that PUFA supplementation in diet had an effect on the meat quality of pigs, but the results are inconsistent. Our meta-analysis demonstrated that PUFA supplementation can significantly increase IMF content but decrease meat color L* and pH 24 h in pigs (**Table 3**). Furthermore, we also found there is a little energy level difference between control and treatment groups in dietary diet. Hence, the effects of different diets on meat quality mainly are due to the PUFA supplementation rather than energy level. Overall, these data support that PUFA supplementation is a benefit for improving meat quality in pigs.

It has been reported that the content of IMF is positively related to pork quality including tenderness, flavor, and juiciness (33). How to improve IMF content is one of the most urgent

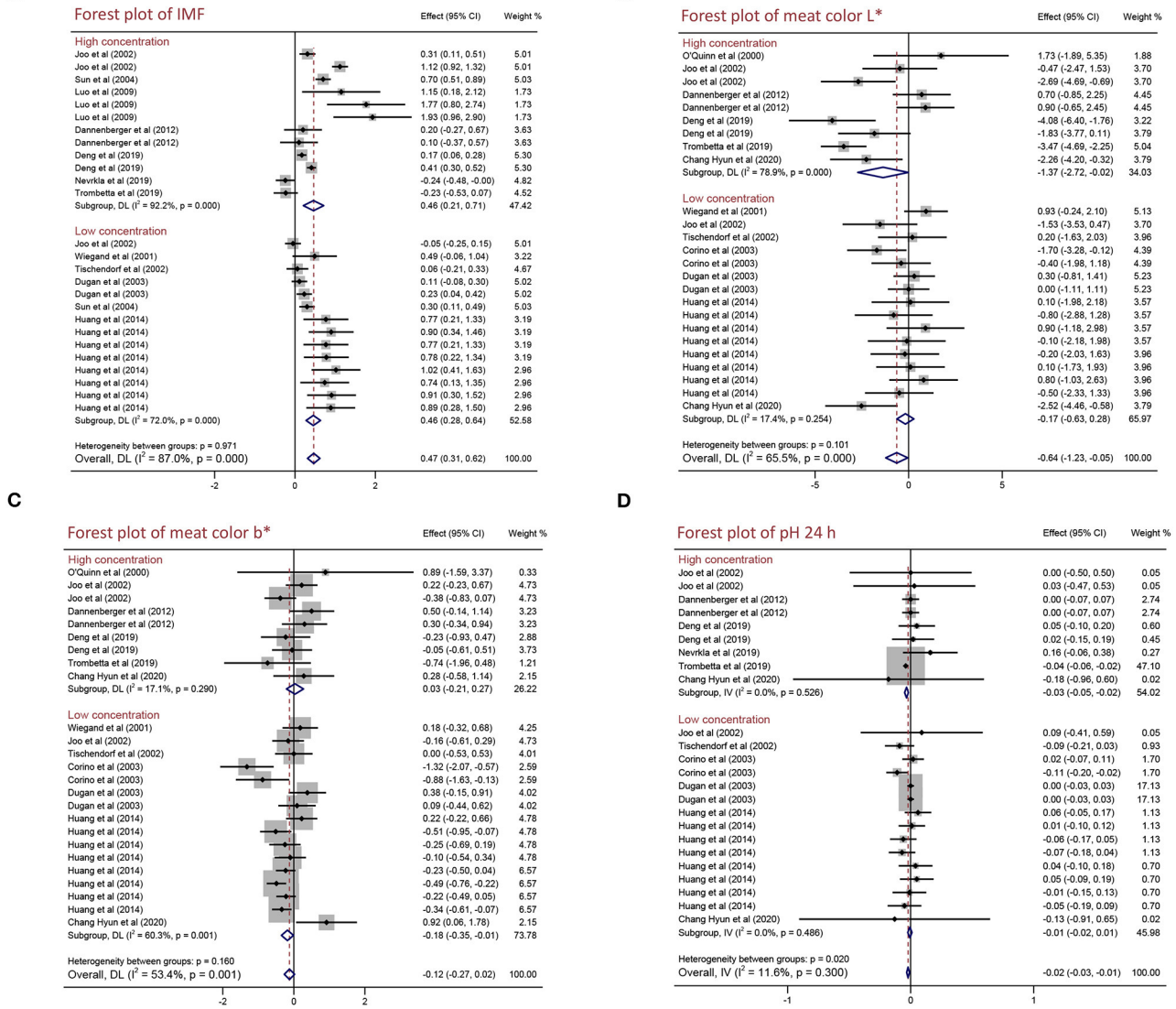

FIGURE 3 | Forest plot of the differences in the meat quality of pigs fed high/low PUFA supplementation concentration. **(A)** IMF, **(B)** meat color L*, **(C)** meat color b*, and **(D)** pH 24 h. WMD, weighted mean difference; CLA, conjugated linoleic acid. The small solid diamond represents the point estimate for each individual trial, and the horizontal line extending from each solid diamond represents the upper and lower limits of the 95% CI. The size of the shaded square indicates the relative weight of the trial in the meta-analysis. The hollow diamond represents the WMD and 95% CI of the trials, no intersection of the diamond and the solid black line in the middle indicates a significant difference ($p < 0.05$), vice versa.

problems in the pig industry. IMF is mainly distributed in the epimysium, perimysium, and endomysium of skeletal muscle in which the main components are phosphoric acid and triglyceride. Previous studies found that IMF content is related to breed, sex, diet, and weight at slaughter in pigs (34–36). PUFAs are one of the essential fatty acids and play an important role in regulating fat deposition, muscle development, and glycolipid metabolism (7, 8). CLA is a group of positional and geometric isomers of linoleic acid with a conjugated double bond, which is generally found in ruminant animals and dairy products and has many physiological functions including anti-obesity, anti-diabetic, anti-cancer, and anti-hypertension (37). Linseed is the ripe seed of flax, which is rich in n-3 PUFAs and has anti-obesity, anti-inflammatory, anti-cancer, and regulating glucose and lipid metabolism effects (38). In our meta-analysis, we observed that the dietary PUFA supplementation can increase IMF content, not only CLA but also linseed supplementation significantly improved IMF content (**Figure 2A**). The concentration of PUFA supplementation in diet might influence the effects on pork quality. However, we found the benefit of PUFA supplementation on IMF content is not dependent on concentration and PUFA supplementation has positive effects in different breeds (foreign and Chinese local

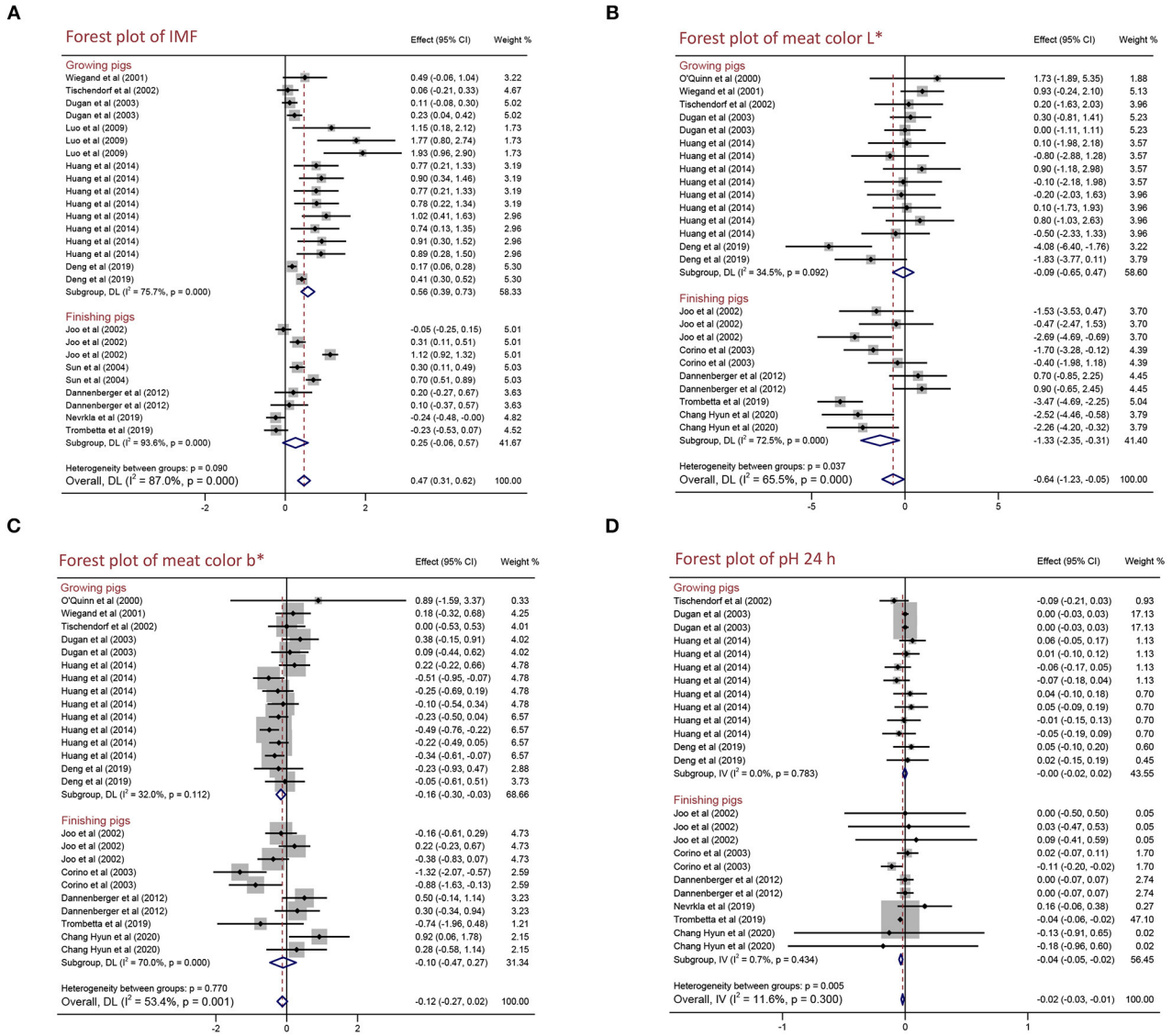

FIGURE 4 | Forest plot of the effects of polyunsaturated fatty acid (PUFA) supplementation on the meat quality of growing and finishing pigs. **(A)** IMF, **(B)** meat color L*, **(C)** meat color b*, and **(D)** pH 24 h. WMD, weighted mean difference; CLA, conjugated linoleic acid. The small solid diamond represents the point estimate for each individual trial, and the horizontal line extending from each solid diamond represents the upper and lower limits of the 95% CI. The size of the shaded square indicates the relative weight of the trial in the meta-analysis. The hollow diamond represents the WMD and 95% CI of the trials, no intersection of the diamond and the solid black line in the middle indicates a significant difference ($p < 0.05$), vice versa.

pigs) (**Figure 3A** and **Supplementary Table 5**). Hence, PUFA supplementation can be a nutritional measure to regulate IMF content. However, only growing pigs had an increased IMF content after being fed PUFA supplementation, and finishing pigs had an insignificant effect (**Figure 4A**). It might because there are nutritional requirements (energy, amino acids, minerals, etc.) that differ in pigs at different growth stages.

Meat color and pH are some of the most important factors that affect the sensory quality of pork (39, 40). However, current studies on the effects of PUFA supplementation on meat color and pH are controversial. Meat color will turn bright red into dark red when pork is placed for a certain time. The difference in myoglobin content influences meat color, and the ratio of the three forms of myoglobin (deoxy myoglobin, oxygen myoglobin, and ferric myoglobin) determine meat color (41, 42). Currently, people use a flesh-color meter to determine the color of meat, including lightness (L*), redness (a*), and yellowness (b*). In our analysis, dietary PUFA supplementation significantly decreased L*, but a* and b* were not influenced (**Table 3**). We conjectured it might be because PUFA supplementation led to myoglobin oxidation and decreased the ratio of oxygen myoglobin to reduce lightness but had no influence on redness and yellowness. We also discovered that the effects of PUFA supplementation on L* and b* are dependent on concentration, growth stage,

and breeds (**Figures 3**, **4** and **Supplementary Table 5**). The pH value is an important index to reflect the muscle contraction and glycolysis rate of pigs after slaughter. After slaughter, pH decreased rapidly from 7.0–7.2 to 5.5–6.5, which was mainly due to muscle glycolysis and lactic acid production. Furthermore, the alteration of meat color and pH results from the different post-mortem processes are affected by environmental factors, such as nutrition, breeding conditions, transport conditions, stress, weather conditions, and the methods of slaughter (43). A previous study summarized those differences in pork quality including flavor, tenderness, odor, and acidity resulted from the environment of delivery, the feeding environment (temperature, humidity, breeding density, etc.), and the pre-slaughter environment (excessive stress and excessively hungry before slaughter) (39). Additionally, some genetic genes are also reported to affect meat color and pH value following influence on pork quality. The recessive homozygote of *halothane* gene causes stress in pigs and reduces pork quality, and the adverse allele of *rendement napole* gene can significantly increase muscle glycogen content, produce more lactic acid, and decrease pH value (44). Particularly, pigs are subjected to excessive stress and severe hypoxia before slaughter, and a large amount of lactic acid was produced by glycolysis in the body lead to low pH 45 min value (below 5.5), which is often associated with pale meat color, resulting in pale, soft and exudative (PSE) pork. In contrast, pigs are excessively hungry before slaughter and a large amount of glycogen in the body is used for energy, resulting in insufficient glycogen in the body after slaughter lead to high meat pH 24 h (above 6.4) often causes dark, firm, and dry (DFD) pork (45). Post-slaughter glycolysis produces lactic acid and reduces the pH of meat, and the speed and duration of the post-slaughter glycolysis determine the development of PSE, DFD, or normal meat. Our results showed PUFA supplementation significantly decreased pH 24 h but did not affect pH 45 min (**Table 3**). Due to the pH 45 min is an indicator of the speed of the glycolysis and pH 24 h is the consequence of the whole period glycolysis, we assume PUFAs might affect muscle contraction and production of lactic acid but not affect glycolysis rate after slaughter. Even though CLA and linseed are all PUFAs, they had different effects on L*, b*, and pH 24 h (**Figure 2**), and it might result from different fatty acids composition. Furthermore, we demonstrated that PUFA supplementation significantly decreased pH 24 h in foreign pigs but not significantly reduced in Chinese local pigs (**Supplementary Table 5**) because different breeds have different nutritional requirements. In addition, we found neither pH 24 h values above 6.4 nor pH 45 min below 5.5 in any studies, and there is no significant effect on growth performance parameters including ADG, ADFI, and G:F ratio in pigs. Hence, PUFA supplementation might provide a safe and useful strategy to improve pork quality.

As shown in **Table 4**, the significant heterogeneity in the drip loss and L*of pigs was primarily driven by the linseed, high concentration, and finishing pigs subgroup. Differently, the CLA, low concentration, and finishing pigs subgroup are sources of b* heterogeneity. CLA and finishing pigs subgroup are the source of a* and pH 45 min heterogeneity, respectively. Additionally, we found ADG and ADFI had high heterogeneity. Although we demonstrated that the significant heterogeneity in ADFI is driven by the high concentration group, we still thought the high heterogeneity was due to the number of included studies for growth performance analysis was too small. Because we did not find the source of IMF heterogeneity according to the subgroup analysis, we performed a sensitivity analysis by using the leave-one-out method on IMF. However, the significant heterogeneity had no change after each included study was removed; thus, we assume that the meta-analysis results are robust, and the heterogeneity did not influence the significance of pooled estimates. Furthermore, we used a fixed-effects model to analyze pH 24 h and G:F ratio due to the homogeneity ($I^2 < 50\%$).

A limitation of this meta-analysis is that the effects of PUFA supplementation duration and actual grams of PUFA intake on meat quality of pigs and whether PUFA supplementation could affect sex of pigs are unknown as a result of the incomplete data, and we assume that further studies should focus on these questions. Furthermore, as SD values are important for meta-analysis and they affect many estimates, including the weight of an individual study, the 95% CI, and heterogeneity, so the lack of within-group SD might influence the results of the meta-analysis. We used pooled SD as within-group SD, and it might be impacted by the number of groups and SEM. To verify our findings are reliable, we checked the consistency between 95% CI pooled estimate and the significance and tendency of included studies. Hence, our results are valid, and this method is appropriate for analyzing non-ruminant studies, which lack within-group SD. Furthermore, there is another method that can be used for estimating within-group SD, which is suitable for studies that reported the median, range, and size of a sample (46). In a word, different approaches can be adopted to estimate within-group SD and accordingly ensure the results of the meta-analysis are reliable and robust.

CONCLUSIONS

Our results indicate that PUFA supplementation increases IMF content, decreases meat color L* and pH 24 h but has no effect on other meat quality and growth performance indexes in pigs; this result is related to PUFA supplementation in the diet rather than the energy level in the basal diet. Our systemic analysis suggests that PUFA supplementation has beneficial influences on improving the meat quality of pigs, which mainly emerges in increasing IMF content in finishing pigs without considering breeds. Hence, we assume that using PUFA supplementation (both CLA and linseed have a function) in the diet is a safe and useful strategy to improve pork quality and without concentration- and breed-dependent to get the best results in increasing IMF content according to our meta-analysis. This may become an effective method for producing high-quality pork in the pig industry, but the optimal PUFA supplementation concentration needs to be further studied.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LW and YH participated in study quality assessment and study criteria selection. LW extracted data, conducted the statistical analysis, and wrote the final version of the manuscript. YH checked the data and assisted in the interpretation and revising of the article. YW and TS oversaw the development of the study and resolved conflicts in the meta-analysis. All authors have read and approved the final manuscript.

FUNDING

The project was partially supported by the Key Research and Development Program of Zhejiang Province (2021C02008), the Zaozhuang Talent Program Funding, the "Hundred Talents Program" funding from Zhejiang University to TS.

ACKNOWLEDGMENTS

We thank the members of the Shan laboratory for their comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.746765/full#supplementary-material

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frontiers in Nutrition

ORIGINAL RESEARCH
published: 23 November 2021
doi: 10.3389/fnut.2021.753643

Aronia Upregulates Myogenic Differentiation and Augments Muscle Mass and Function Through Muscle Metabolism

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OPEN ACCESS

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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: 05 August 2021
Accepted: 11 October 2021
Published: 23 November 2021

Citation:
Yun C-E, So H-K, Vuong TA, Na MW, Anh S, Lee H-K, Kim KH, Kang J-S, Bae G-U and Lee S-J (2021) Aronia Upregulates Myogenic Differentiation and Augments Muscle Mass and Function Through Muscle Metabolism. Front. Nutr. 8:753643. doi: 10.3389/fnut.2021.753643

Black chokeberry or aronia (the fruit of *Aronia melanocarpa*) has been reported to having pharmacological activities against metabolic syndrome, such as hypertension, obesity, diabetes, and pro-inflammatory conditions. However, the effects of aronia on myogenic differentiation and muscle homoeostasis are uncharacterized. In this study, we investigated the effects of aronia (black chokeberry) on myogenic differentiation and muscle metabolic functions in young mice. Aronia extract (AR) promotes myogenic differentiation and elevates the formation of multinucleated myotubes through Akt activation. AR protects dexamethasone (DEX)-induced myotube atrophy through inhibition of muscle-specific ubiquitin ligases mediated by Akt activation. The treatment with AR increases muscle mass and strength in mice without cardiac hypertrophy. AR treatment enhances both oxidative and glycolytic myofibers and muscle metabolism with elevated mitochondrial genes and glucose metabolism-related genes. Furthermore, AR-fed muscle fibers display increased levels of total OxPHOS and myoglobin proteins. Taken together, AR enhances myogenic differentiation and improves muscle mass and function, suggesting that AR has a promising potential as a nutraceutical remedy to intervene in muscle weakness and atrophy.

Keywords: ***aronia melanocarpa*, muscle atrophy, muscle differentiation, muscle mass and function, myofiber types**

INTRODUCTION

In aging, skeletal muscles undergo a progressive decline in mass, strength, and functionality related to a condition called aging-related sarcopenia (1). Sarcopenia represents a risk factor for frailty, the loss of independence, and physical disability in the elderly (2). Since skeletal muscle constitutes about 40% of body mass in the healthy young person and contributes to body metabolic health, declines in muscle mass, and functionality will have consequences in the incidence of secondary aging-related diseases, such as metabolic syndrome, cardiovascular diseases, or chronic inflammation (3, 4). These secondary diseases will further exacerbate muscle loss, contributing to the increase in morbidity and mortality (5). Thus, the prevention of sarcopenia through enhancing muscle mass and function appears to be critical for the extension of a healthy life

span in the elderly population. Multiple mechanisms, such as declines in mitochondrial function and muscle regenerative capacity or neuromuscular dysfunction, have been implicated in diverse muscle wasting conditions, such as sarcopenia (6–9). Thus, improving mitochondrial function, muscle regeneration, and motor neuron function is critical to prevent muscle wasting related to diverse conditions. Much attention has been paid to develop therapeutic tools to prevent muscle wasting and so far, the most effective intervention appears to be exercise (10). One of the major mechanisms of exercise is improving mitochondrial biogenesis and function through a transcription coactivator, called peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). The increase in mitochondrial function through exercise or mimetics can protect muscle atrophy and weakness caused by aging or other conditions, such as denervation (11). Improved muscle stem cell function has been also associated with the protective effect of exercise-mediated mechanisms (12).

Other pathways more directly linked to the control of muscle mass are IGF1/Akt/mTOR and myostatin-Smad2/3 pathways controlling protein synthesis and degradation, respectively (13, 14). In diverse condition-related sarcopenia, the imbalance in this protein metabolism is one of the major causative mechanisms (15, 16). The excessive protein degradation relative to protein synthesis causes muscle atrophy related to a variety of conditions, such as starvation, denervation, cancer cachexia, and aging (11, 14, 17, 18). Two major protein breakdown pathways, the proteasomal and the autophagic-lysosomal pathways, are activated during muscle atrophy and variably contribute to the loss of muscle mass (19). Two muscle-specific E3 ligases, muscle RING finger containing protein 1 (MuRF1) and muscle atrophy F-box protein (Atrogin-1), are associated with the muscle protein degradation pathway (20–22). These E3 ligases are regulated by transcription factors, such as Forkhead box O3 (FoxO3), which in turn is negatively regulated by Akt signaling (19, 22, 23). On the other hand, Akt/mammalian target of rapamycin (mTOR) signaling promotes protein synthesis leading to muscle hypertrophy (24). Akt can repress the activity of FoxO transcription factors by phosphorylation and consequently the expression of MuRF1 and Atrogin-1 (25). Thus, Akt/mTOR signaling attenuates protein degradation by blocking FoxO action and increases protein synthesis through S6K activation, leading to muscle hypertrophy.

Black chokeberry or aronia (the fruit of *Aronia melanocarpa, A. melanocarpa*) is a shrub of the *Rosaceae* family, which is a plant native to North America, and was transferred to Europe about a century ago (4, 26). Aronia is traditionally used by Potawatomi Native Americans to cure colds and is also used as a tonic and adaptogen activity (27). Aronia has various biological activities based on phenolic compounds, such as anthocyanins, flavonols, flavanols, proanthocyanidins, and phenolic acids (28, 29). Aronia and its products have a great health-promoting potential related to reducing risk factors of metabolic syndrome, such as dyslipidemia, hypertension, obesity, glucose metabolism disorders, pro-inflammatory conditions, and thrombosis (4, 30–34). In addition, it is shown to have effects to inhibit the development of various types of cancers, such as leukemia, breast and intestinal cancer, and cancer stem cells (33, 35). In this study, we investigated the effects of aronia extract (AR) on myoblast differentiation and myotube atrophy triggered by dexamethasone (DEX). In addition, the effect of AR on muscle function and metabolism in mice was determined.

MATERIALS AND METHODS

Reagents

Wild-type C57BL/6 male mice were purchased from (Orient-Bio, Seongnam, Korea). Fetal bovine serum (FBS), horse serum (HS), and Dulbecco modified Eagle's medium (DMEM) were purchased from Thermo Scientific (Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DEX, and all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies were purchased as follows: myosin heavy chain (MHC, Developmental Studies Hybridoma Bank (DSHB), Iowa, IA, USA) Myogenin, Myoglobin, total-OxPHOS (Abcam, Cambridge, MA, USA), total-Akt, phospho-Akt, phospho-mTOR, mTOR, phospho-S6K, S6K (Cell Signaling Technology, Beverly, MA, USA), MuRF1, Atrogin-1, HSP90 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-tubulin (Zymed, South San Francisco, CA, USA).

Aronia Material and Extraction Procedure

The fresh fruits of *A. melanocarpa* were collected at Danyang-gun, Chungcheongbuk-do, Korea, in August 2016 and were identified by one of the authors (K. H. Kim). A voucher specimen (SKKU AR-2016-08) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. Dried fruits (350 g) of *A. melanocarpa* were extracted with 80% MeOH for 3 days twice at room temperature. The extracts were then filtered, and the filtrate was concentrated under vacuum pressure, generating an AR (21.8 g). The AR was stored at −20°C until use.

Animal Studies

All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at Sungkyunkwan University School of Medicine (SUSM) and complied with the regulations of the institutional ethics committee. All mice were maintained at 23°C with a 12:12 light-dark cycle and free access to food and water. To examine the effect of AR, these mice were orally administered a daily dose of 3.3 mg/kg AR for 8 weeks (8-month-old mice). Control mice were administrated the same amount of vehicle (dimethylsulfoxide, DMSO) dissolved in saline. All animals were sacrificed after fasting for 6 h with *ad libitum* to water.

Cell Culture

C2C12 myoblasts were cultured as previously described (36). They were grown in Dulbecco's Modified Eagle Medium high glucose (DMEM; Thermo Scientific, Waltham, MA, USA) containing 15% FBS (growth medium, GM), 10 units/ml penicillin, and 10 μg/ml streptomycin (Welgene, Daegu, Korea) at 37°C, 5% CO_2. To induce differentiation of C2C12 myoblasts, cells at near confluence were changed growth medium into

DMEM containing 2% HS (differentiation medium, DM), and myotube formation was observed at 2 or 3 days after differentiation. For the DEX-induced atrophy study, C2C12 cells were induced to differentiate in DM for 3 days (D3), followed by the treatment with 100 μM DEX along with vehicle DMSO or AR for an additional 1 day (D4) (37).

Western Blotting and Immunostaining

Western blot analysis was performed as previously described (38). Briefly, cells were lysed in cell extraction buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100) containing a complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and incubation with primary and secondary antibodies.

Immunostaining for MHC expression was carried out as previously described (39). Briefly, the differentiated cultures were then immunostained for MHC antibodies and Alexa 568-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). Images were captured and processed with a Nikon ECLIPSE TE-2000U microscope and NIS-Elements F software (Nikon, Tokyo, Japan). To analyze the efficiency of myotube formation, the MHC-positive myotubes containing two to five, or six or more nuclei, were quantified at least three times and measured using ImageJ software.

Cryosections, Staining Analysis, and Fiber Size Measurement

Muscle tissue was embedded in Tissue-Tek OCT Compound (Sakura Finetek, Nagano, Japan), and 7 mm thick serial sections for staining were cut using a cryomicrotome. To analyze the nicotinamide adenine dinucleotide (NADH) dehydrogenase activity, we dried the sectioned tissues for 10 min at room temperature and incubated them in 0.9 mM NADH and 1.5 mM nitro blue tetrazolium (NBT; Sigma-Aldrich) in 3.5 mM phosphate buffer (pH 7.4) for 30 min. To analyze the succinate dehydrogenase (SDH) activity, we incubated the sections for 30 min in 50 mM sodium succinate and 0.3 mM nitro blue tetrazolium in 114 mM phosphate buffer containing K-EGTA (Sigma-Aldrich). To analyze the glycerol-3-phosphate dehydrogenase (GPDH) activity, we used the dried tissue samples and incubated them in 1.2 mM NBT, 2 mM phenazine methosulfate, and 1.86 mM glycerol phosphate in sodium phosphate buffer (pH 7.4) for 40 min.

Myh immunostaining of muscle tissue sections was performed in the sequence of fixation, permeation, and incubation with primary antibodies against MyhIIa and MyhIIb (DSHB) and laminin (Abcam). Images were captured with a Nikon ECLIPSE TE-2000U using NIS-Elements F software. Myofiber area was measured with ImageJ software. For muscle histology, the cryosections were stained with Mayer's hematoxylin and eosin (BBC Biomedical, McKinney, TX, USA). The images were captured using a Nikon ECLIPS TE-2000U.

TABLE 1 | The primers used in this study.

Primer		Sequence
MyhIIa	Forward	5'-GGCTTCAGGATTTGGTGGATAA-3'
	Reverse	5'-GGATCTTGCGGAACTTGGATAG-3'
MyhIIb	Forward	5'-GATTGACGTGGAGAGGTCTAAC-3'
	Reverse	5'-CCTGAGTTTCCTCGTACTTCTG-3'
MTCO	Forward	5'-CTACTATTCGGAGCCTGAGC-3'
	Reverse	5'-GCATGGGCAGTTACGATAAC-3'
SDHB	Forward	5'-ACCCCTTCTCTGTCTACCG-3'
	Reverse	5'-AATGCTCGCTTCTCCTTGTAG-3'
Myoglobin	Forward	5'-CACCATGGGGCTCAGTGATG-3'
	Reverse	5'-CTCAGCCCTGGAAGCCTAGC-3'
HK	Forward	5'-GCTGGAGGTTAAGAGAAGGATG-3'
	Reverse	5'-TGGAGTGGCACACACATAAG-3'
PK	Forward	5'-CATGCAGCACCTGATAGC-3'
	Reverse	5'-AGCTGCTGCTAAACACTTAT-3'
PFK	Forward	5'-ACCAGAGCACGTTTGTGTTAG-3'
	Reverse	5'-GGCGGACACTCAGGAATAAA-3'
MuRF1	Forward	5'-GAGAACCTGGAGAAGCAGCT-3'
	Reverse	5'-CCGCGGTTGGTCCAGTAG-3'
Atrogin-1	Forward	5'-CAACATTAACATGTGGGTGTAT-3'
	Reverse	5'-GTCACTCAGCCTCTGCATG-3'
18S rRNA	Forward	5'-AGGGGAGAGCGGGTAAGAGA-3'
	Reverse	5'-GGACAGGACTAGGCGGAACA-3'

RNA Isolation and Quantitative Real-Time (RT)-PCR

Total RNA extraction and quantitative RT-PCR analysis were performed as previously described (40). Tissues were homogenized by FastPrepR-24 (MP Biomedicals, Santa Ana, CA, USA) and extracted using the easy-spin Total RNA Extract kit (iNtRON, Seongnam, Korea). Gene expression fold change was normalized against the expression of 18S ribosomal RNA. The sequences of the primers used in this study are provided in **Table 1**.

Grip Strength Test

Grip strength was measured using a grip strength meter (Bioseb, Pinellar Park, FL, USA). The animal was allowed to grab the grid with a fore and back limb through a blind test. The limbs are pulled gently with consistent force until the forelimb was detached from the grid. The maximal strength was recorded when the grid was detached. Each animal was tested in three trials.

Statistical Analysis

Values are expressed as mean ± SD for *in vitro* systems or ± SEM for *in vivo* systems, as indicated in the figure legends. The statistical significance was calculated using either Student's *t*-test (unpaired, two-tailed) or ANOVA by *post-hoc* Tukey's tests for multiple comparisons. Differences were considered statistically significant at or under values of $P < 0.05$.

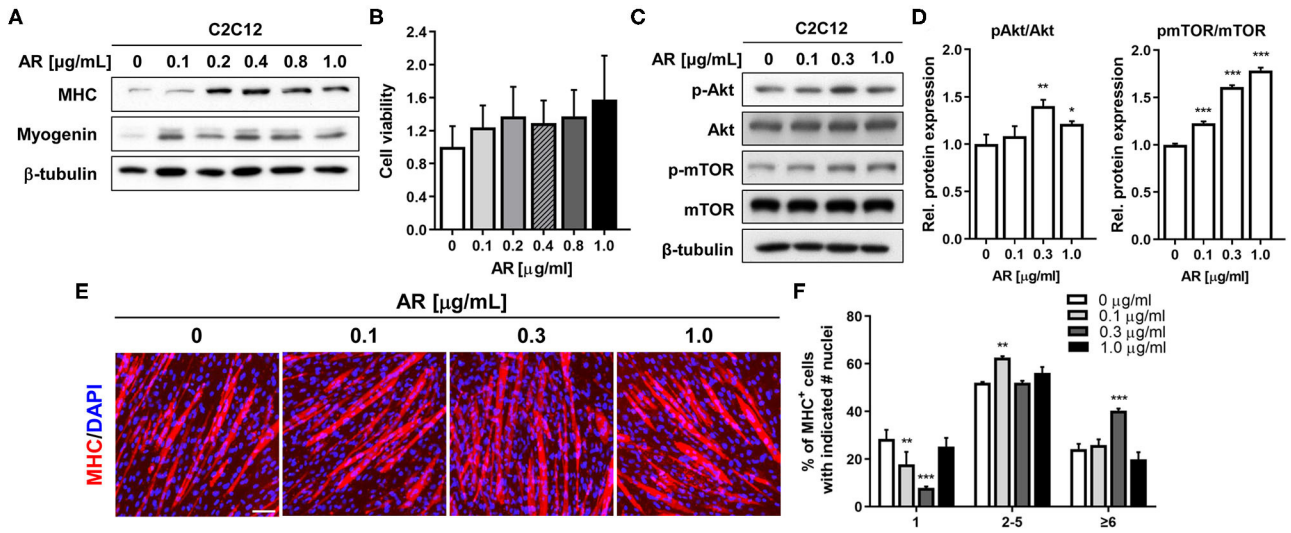

FIGURE 1 | AR promotes myoblasts differentiation in C2C12 cells without cytotoxicity. **(A)** C2C12 myoblasts were induced to differentiate for 3 days in the presence of DMSO or the indicated concentration of AR in DM. The lysates were subjected to immunoblotting analysis with muscle-specific proteins, MHC, and Myogenin, β-Tubulin was used as a loading control. The experiment was repeated three times with similar results. **(B)** Cell viability was determined with an MTT assay. **(C)** Immunoblot analysis of C2C12 cells for the expression of the phosphorylated form of Akt and total Akt and β-tubulin as a loading control treated with vehicle or AR for D3. **(D)** Quantification of blots from three experiments similarly performed as shown in panel C. The signal intensity of pAkt and pmTOR was quantified, and the values were normalized to total Akt and mTOR, respectively. **(E)** Immunostaining for MHC expression (red) and DAPI (blue) staining to visualize nuclei. C2C12 myoblasts were induced to differentiation for 3 days in the presence of DMSO or the indicated concentration of AR in DM. Scale bar: 50 μm. **(F)** The MHC-positive myocytes shown in panel E were quantified as a number of nuclei per myotube. The one-way ANOVA analysis with Tukey test. Data represent means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$. AR, Aronia extract; DMSO, DM, differentiation medium; MHC, myosin heavy chain; DAPI.

RESULTS

AR Enhances Myoblast Differentiation Through Akt Activation

To examine the effect of AR on myogenic differentiation, C2C12 myoblasts were induced to differentiate for 3 days (D3) in the presence of vehicle DMSO or AR with the indicated concentration ranging from 0.1 to 1 μg/ml in DM and subjected to the assessment of myoblast differentiation by immunoblotting analysis. The treatment with AR increased the expression levels of MHC and Myogenin in a dose-sensitive fashion and peaked at the concentration between 0.2 and 0.4 μg/ml (**Figure 1A**). This slight decrease at higher AR concentration was not due to any cytotoxicity as assessed by MTT assay (**Figure 1B**). To explore the molecular regulatory pathways of AR-mediated myogenic promotion, C2C12 myoblasts were treated with AR at the concentration of 0.1, 0.3, and 1.0 μg/ml for 48 h. Cell lysates were subjected to immunoblotting analysis for the active phosphorylated Akt (pAkt) to assess for the activation status of a promyogenic kinase Akt. The treatment of AR, especially 0.3 μg/ml concentration, increased the level of pAkt without altering the total Akt protein level (**Figures 1C,D**). In addition, AR treatment induced the phosphorylation of mTOR, a downstream target of Akt, in a dose-dependent manner (**Figures 1C,D**). These results indicate that AR promotes myogenic differentiation with enhanced Akt activation.

To confirm the promyogenic effects of AR, myotube formation was assessed by MHC immunostaining. As a result, AR treatment in C2C12 myoblasts elicited the formation of larger MHC-positive multinucleated myotubes, compared to control cells (**Figure 1E**). To quantify the myotube formation, MHC-positive myocytes were counted as mononucleate, myotubes containing two to five nuclei or containing six or more nuclei and plotted as a percentile (**Figure 1F**). The treatment with AR decreased the proportion of mononucleate myocytes, while it substantially elevated the proportion of larger myotubes containing six or more nuclei in a dose-sensitive manner. Our current data further support the positive effect of AR on myoblast differentiation at a morphological and at a biochemical level. AR can exert its promyogenic effect through Akt activation without overt cytotoxicity.

AR Protects DEX-Induced Myotube Atrophy Through Activation of Akt Signaling

The synthetic glucocorticoid DEX induces muscle-specific ubiquitin ligase expression contributing to muscle atrophic phenotypes with reduced myotube diameter (37). Akt activation is one of the key events to suppress the induction of muscle-specific ubiquitin ligase and muscle atrophy triggered by DEX (25). Since AR can activate Akt, the effects of AR at the concentration of 0.1 or 1.0 μg/ml on DEX-induced C2C12 myotube atrophy were examined. C2C12 cells were induced to differentiate in DM for 3 days (D3), followed by the treatment with DEX along with vehicle DMSO or AR for

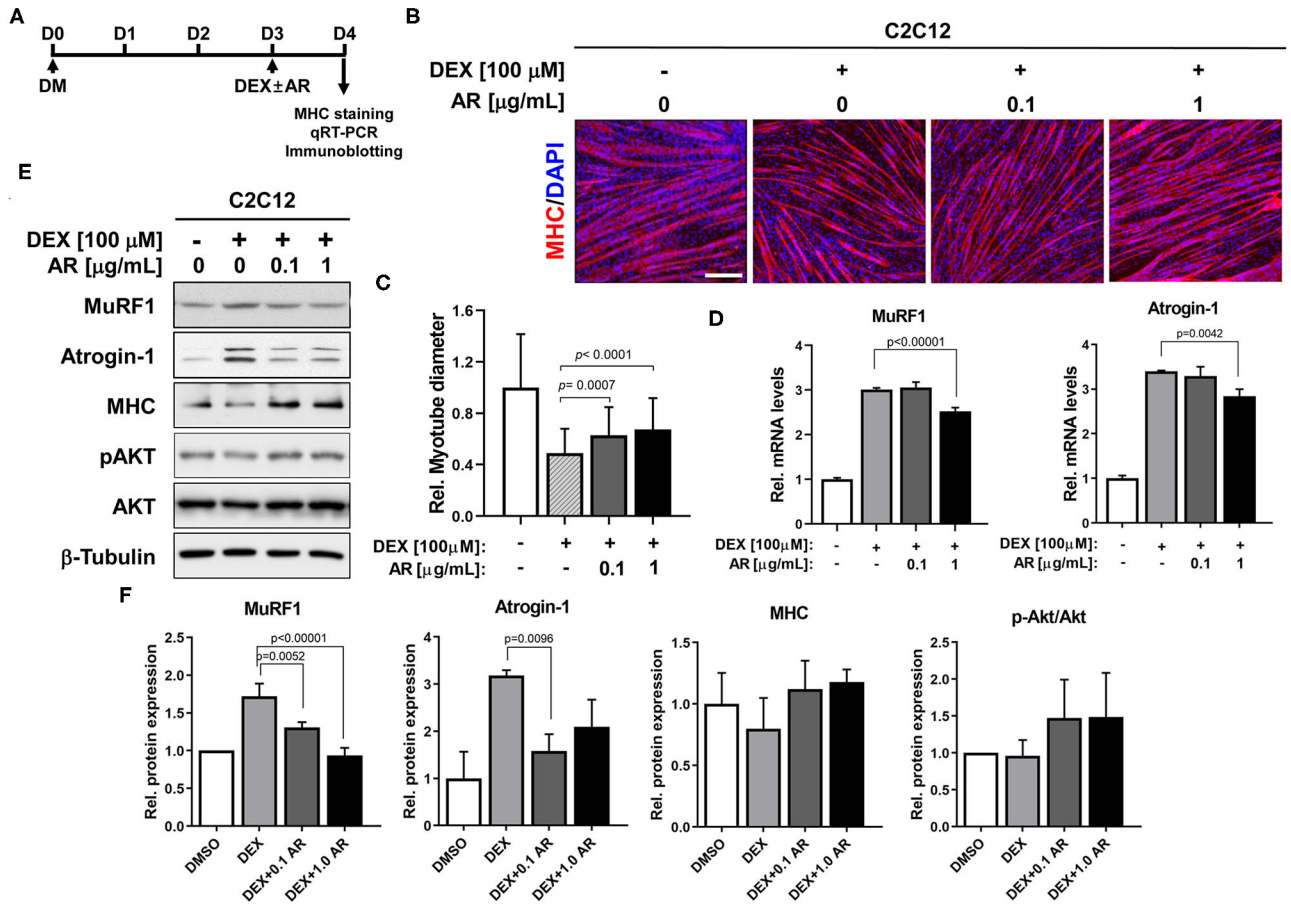

FIGURE 2 | AR prevents DEX-induced muscle atrophy. **(A)** The procedure diagram for atrophy study. **(B)** Immunostaining for MHC expression (red) and DAPI (blue) staining to visualize nuclei. C2C12 myoblasts were induced to differentiate for 3 days in DM and treated with vehicle DMSO or indicated concentration of AR with DEX. Scale bar, 60 μm. **(C)** The relative diameter for panel B was quantified to show effect of AR from DEX-induced muscle atrophy. $N = 123$ myotubes/each sample. **(D)** qRT-PCR analysis for expression of MuRF1 and Atrogin-1 in C2C12 cells of the vehicle or DEX-treated AR. **(E)** Immunoblot analysis of C2C12 cells for the expression of MHC, MuRF1, Atrogin-1, pAkt and Akt, and β-tubulin as a loading control treated with vehicle or DEX-treated AR for D3. **(F)** Quantification of blots from three experiments similarly performed as shown in panel E. The signal intensity of MuRF1, Atrogin-1, and MHC was quantified, and the values were normalized to β-tubulin. The signal intensity of pAkt was quantified, normalized to total Akt. The one-way ANOVA analysis with Tukey test. AR, Aronia extract; DEX, dexamethasone; MHC, myosin heavy chain; DAPI.

an additional 1 day (D4) (**Figure 2A**). Myotubes were then subjected to MHC immunostaining to access the thickness of myotubes. The treatment with DEX-elicited myotube atrophy, which was suppressed by AR treatment, was evident by the presence of larger multinucleated myotubes in AR-treated cultures (**Figure 2B**). The quantification of myotube diameter revealed that DEX treatment caused declines in myotube diameter, which was significantly recovered by AR treatment (**Figure 2C**). In addition, the qRT-PCR analysis showed that DEX treatment greatly elevated muscle-specific E3 ubiquitin ligases, MuRF1, and Atrogin-1 and AR treatment attenuated this induction (**Figure 2D**). To further define, the protein levels of muscle-specific proteins and ubiquitin ligases were determined by immunoblotting analysis. Consistent with qRT-PCR results, the treatment with AR in DEX-treated myotubes reduced the level of MuRF1 and Atrogin-1 proteins, compared to control (**Figures 2E,F**). The treatment of DEX elicited a reduction in MHC protein levels and the co-treatment of DEX with AR attenuated the decline in MHC proteins in myotubes. In addition, DEX-treated myotubes exhibited decreased levels of pAkt while AR treatment in DEX-treated myotubes abrogated this decrease. Taken together, these results suggest that AR protects DEX-induced myotube atrophy through inhibition of muscle-specific ubiquitin ligases mediated by Akt activation.

AR Enhances Muscle Mass and Functions in Young Mice

To assess the *in vivo* effects of AR on muscles, 8-month-old-mice were fed with control or AR for 8 weeks, followed by muscle functional analysis and biopsy. The hindlimb muscles of AR-ingested mice appeared darker than those of control-fed mice, which can be readily detected in gastrocnemius (GAS)

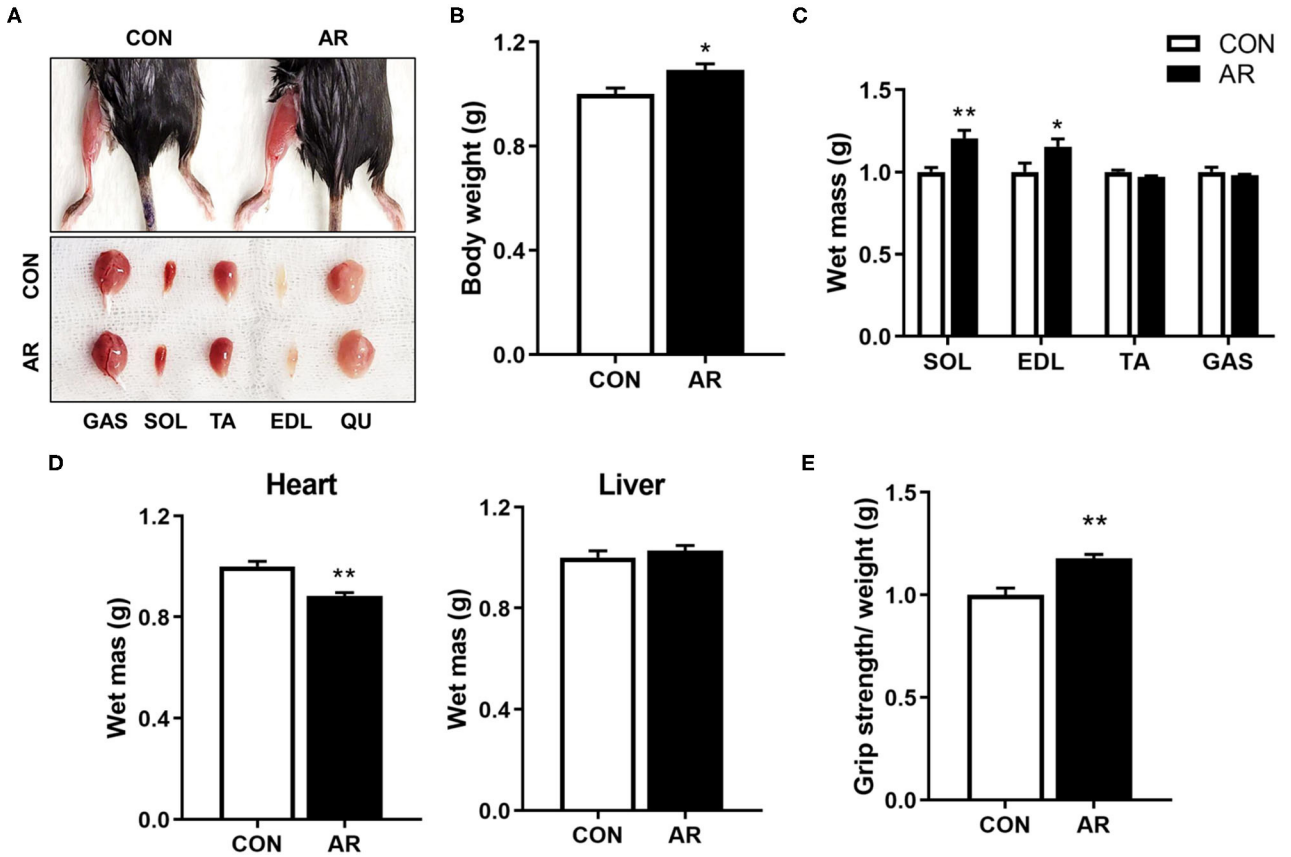

FIGURE 3 | AR enhances muscle mass and strength. **(A)** Photographs of isolated muscle types from hindlimbs from 8-month-old mice ingested with control or 3.3 mg/kg AR for 8 weeks. **(B)** Body weight of 8-month-old control-fed and AR-fed mice for 8 weeks. **(C, D)** Weights of **(C)** four muscle types and **(D)** heart and liver from control or 3.3 mg/kg AR-ingested mice for 8 weeks, $n = 3$. **(E)** Grip strength depicted as the force (gram) that animals in each group pulled, n = 5. An unpaired two-tailed student's *t*-test. The data represent the mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$. AR, Aronia extract.

muscles among five hindlimb muscle groups, such as soleus (SOL), tibialis anterior (TA), extensor digitorum longus (EDL), and quadriceps (QU) (**Figure 3A**). AR treatment mildly but significantly elevated body weights, compared with the vehicle-treated group (**Figure 3B**). Among four hindlimb muscles, the weights of SOL and EDL muscles were increased an approximately 20.0 and 15.3% in AR-fed mice, compared to vehicle-fed mice, respectively (**Figure 3C**). The heart mass was slightly reduced but the liver mass was not changed (**Figure 3D**). One week prior to harvesting muscles, muscle strength was examined by measuring the grip strength. Mice fed with AR displayed approximately 14.5% increase in grip strength relative to control mice (**Figure 3E**). These data collectively suggest that AR treatment improves muscle mass and function without overt hypertrophic effect on the cardiac muscle.

AR Increases Both Glycolytic and Oxidative Myofibers in Young Mice

To examine the detailed histology of muscles, control and AR-treated TA muscles were cryosectioned and subjected to hematoxylin and eosin staining, followed by the measurement of the cross-sectional area of myofibers (**Figures 4A,B**). AR-treated muscles had more lager myofibers, compared to the control-treatment muscles. To examine the effect of AR on myofiber types, muscle sections were analyzed by immunostaining with antibodies to MyhIIb and MyhIIa (**Figure 4C**). AR treatment elicited a shift toward larger MyhIIa- and MyhIIb-positive myofibers, compared to the vehicle treatment (**Figures 4D,E**). Consistently, qRT-PCR analysis of AR-treated muscles had elevated expression of MyhIIa and MyhIIb, compared to the control muscles (**Figure 4F**). These data suggest that AR treatment enhances MyhIIa and MyhIIb muscles.

AR Improves Both Muscle Oxidative and Glycolytic Metabolism in Young Mice

Myofibers have different metabolic characteristics. Myofiber type IIa has higher mitochondrial content, compared to glycolytic myofiber type IIb (41, 42). Since AR substantially elevated both MyhIIa and MyhIIb expressions, we next examined the muscle metabolism by measuring activities of SDH and nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) for muscle oxidative metabolism and GPDH for

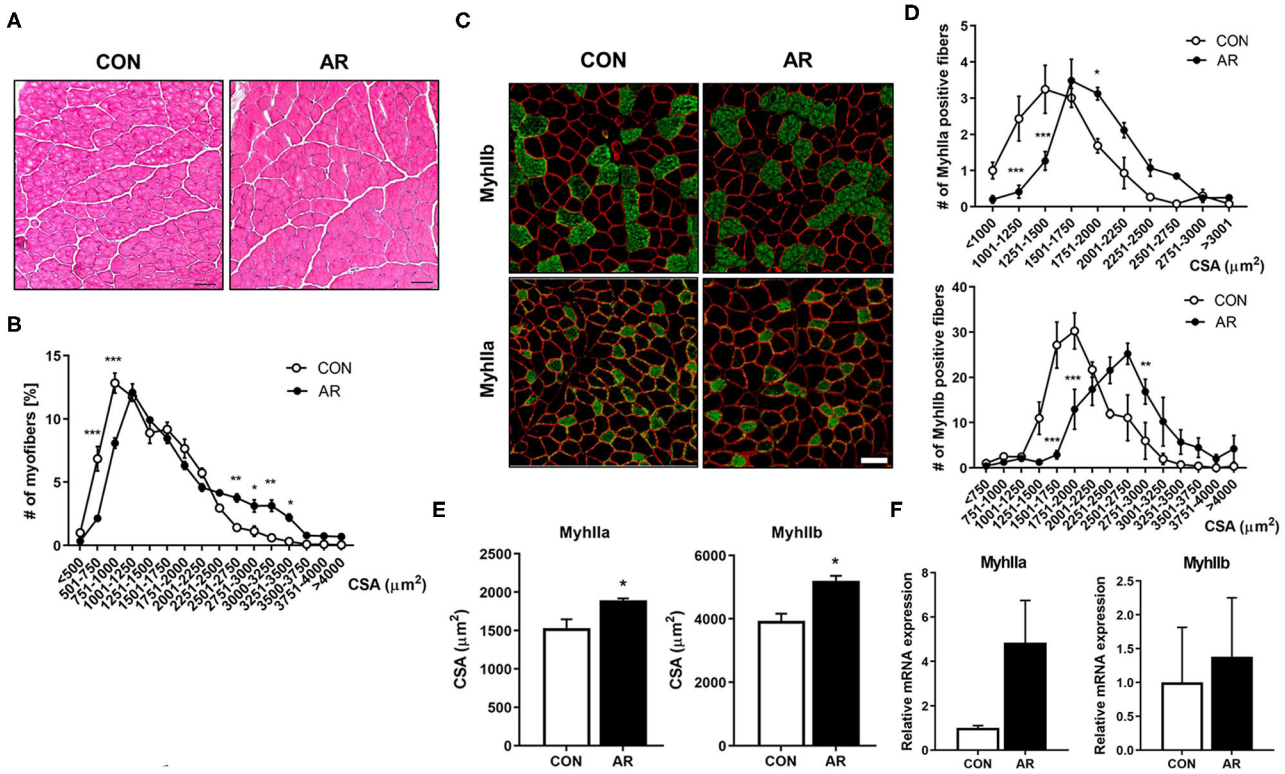

FIGURE 4 | AR induces the formation of larger MyhIIa- and MyhIIb-positive myofibers. **(A)** Histological staining of TA muscles of 8-month-old mice ingested with control or 3.3 mg/kg AR for 8 weeks. H&E staining shows the structural recovery. Scale bar, 50 μm. **(B)** Quantification of the cross-sectional area of total myofibers in TA. **(C)** Immunostaining of MyhIIb (green), MyhIIa (green), and laminin (red) in the TA muscles of control or 3.3 mg/kg AR-ingested mice for 8 weeks. Scale bar, 50 μm. **(D, E)** Quantification of MyhIIb- and MyhIIa-positive myofibers in panel **(C)** ($n = 3$). **(F)** qRT-PCR analysis for expression of MyhIIa and MyhIIb in the TA muscles of control or 3.3 mg/kg AR-ingested mice for 8 weeks ($n = 3$). An unpaired two-tailed student's *t*-test. The data represent the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. AR, Aronia extract; TA, tibialis anterior.

glycolytic metabolism. In agreement with the myofiber type staining, AR treatment elevated the proportion of myofibers with strong (dark) activities for SDH, NADH-TR, and GPDH, compared to the control muscles (**Figures 5A,B**), indicating that AR enhances both oxidative and glycolytic muscle metabolism. Consistently, AR-treated muscles expressed higher levels of mitochondrial genes (MTCO1, Sdhb), myoglobin, and glucose metabolism-related genes [hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK)], compared to the control muscles (**Figures 5C,D**). To further confirm, we examined the protein levels of total OXPHOS [ATP5A (CV), MTCO1 (CIII), SDHB (CII) and NDUF88 (CI)] proteins, myoglobin and S6K in Qu muscle tissues. In consistent with the RNA expression data, AR-treated muscles had elevated levels of total OXPHOS, myoglobin, and phosphorylated S6K proteins (**Figures 5E,F**). These data suggest that AR enhances the expression of muscle-specific genes and muscle metabolic genes, such as mitochondrial components.

DISCUSSION

Much attention has been paid to the discovery of effective pharmacological supplements to improve muscle mass and function in pathological conditions or the aging process. Recent advances in muscle biology led to new interests in pharmacological or nutraceutical treatment to prevent muscle atrophy and weakness. In this study, we investigate the effects of AR on myogenic differentiation and muscle function in mice. Our results demonstrate that AR elicits myoblast differentiation and the formation of multinucleated myotubes through Akt activation without overt cytotoxicity. The treatment of AR in DEX-induced atrophic myotubes restores myotube formation through suppression of muscle-specific ubiquitin ligases, likely mediated by Akt activation. In addition, AR treatment improves muscle mass and function through elevating the expression of muscle-specific genes and muscle metabolic genes without overt hypertrophic effect on the cardiac muscle.

Myoblast differentiation is initiated by the proliferation of myoblast and the subsequent cell cycle arrest, followed by a differentiation program, such as the expression of muscle-specific genes and the formation of multinucleated myofibers by myoblast fusion (43). Among diverse signaling pathways implicated in myoblast differentiation, Akt plays important roles in the induction of myoblast differentiation and muscle protein synthesis associated with myotube hypertrophy (24). Akt is a serine/threonine-protein kinase and is activated

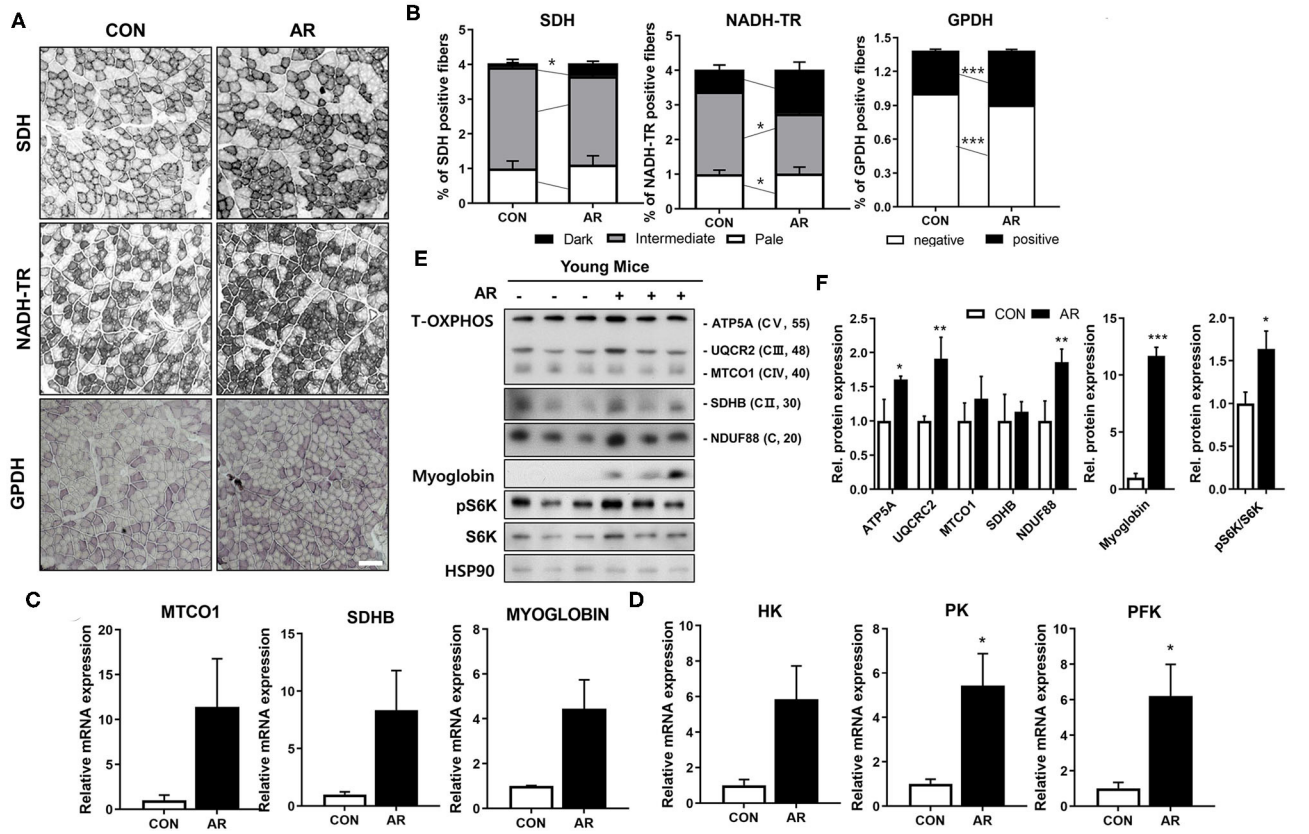

FIGURE 5 | AR increases muscle metabolism in young mice. **(A)** Histochemical staining for SDH, NADH-TR and GPDH enzymatic activities in TA muscles. Scale bar, 50 μm. **(B)** The staining intensities of SDH and NADH-TR are quantified as three different grades (dark, intermediate, and pale) and plotted as a percentile, n = 3. An unpaired two-tailed student's *t*-test. **(C)** qRT-PCR analysis for expression of mitochondrial genes (MTCO1, SDHB) and myoglobin in TA muscles. **(D)** qRT-PCR analysis for expression of glucose metabolism-related genes in TA muscles. An unpaired two-tailed student's *t*-test. **(E)** Immunoblotting analysis for the expression of Total-OXPHOS, myoglobin and S6K in Qu muscles. **(F)** Quantification of the levels of total-OxPHOS proteins from panel E, $n = 3$. An unpaired two-tailed student's *t*-test. The data represent the mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. AR, Aronia extract; TA, tibialis anterior; SDH, succinate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; NADH-TR, nicotinamide adenine dinucleotide tetrazolium reductase.

during myoblast differentiation. Overexpression of Akt promotes myogenic differentiation, whereas a dominant-negative form of Akt inhibits Akt activation and prevents myotube formation. In addition, ectopic expression of a constitutively active form of Akt can recover the inhibition of myoblast differentiation through inhibition of the PI3K (44). In addition, Akt plays a key role in PI3K/Akt/mTOR signaling and contributes to the regulation of energy metabolism and protein synthesis. The two major downstream target proteins of Akt/mTOR signaling regulating skeletal muscle hypertrophy are the p70S6K and the eIF4E-binding protein 1 (4EB-BP1), leading to upregulation of protein synthesis. The FoxO transcription factors regulate the activation of muscle-specific E3-ubiquitin ligases, MuRF1, and Atrogin-1 (22, 37). Especially, Akt/mTOR signaling enhances net protein accumulation by suppression of FoxO transcription factors and muscle-specific ubiquitin ligases implicated in protein catabolism (15, 16, 22). Akt induces the phosphorylation of FoxO transcription factors, resulting in inhibition of transcriptional functions of FoxOs through the exclusion of phosphorylated FoxO proteins from the nucleus. The Akt-mediated control of FoxO transcriptional functions is involved in the differentiation process of myoblasts (45). In addition to protein metabolic imbalance, the declined regenerative capacity of muscle stem cells is also tightly associated with the loss of muscle mass and function during aging or various muscle diseases (10). Our current results support that AR might promote the regenerative capacity of muscle stem cells and protein synthesis dependent on Akt, contributing to enhanced muscle mass and strength (**Figure 6**).

Peroxisome proliferator-activated receptor gamma coactivator-1 alpha plays a central role in a regulatory network governing the transcriptional control of mitochondrial biogenesis. PGC-1α evokes the expression of genes associated with exercise, such as genes involved in mitochondrial biogenesis, stimulation of fatty acid oxidation, angiogenesis, and resistance to muscle atrophy (46). The NADH and SDH participate in citric acid cycle of mitochondria. NADH-TR reaction is a histochemical reaction used for evaluation of the space between the myofibrils referred to as the inter-myofibrillary matrix. This reaction is catalyzed by enzymes in the mitochondria

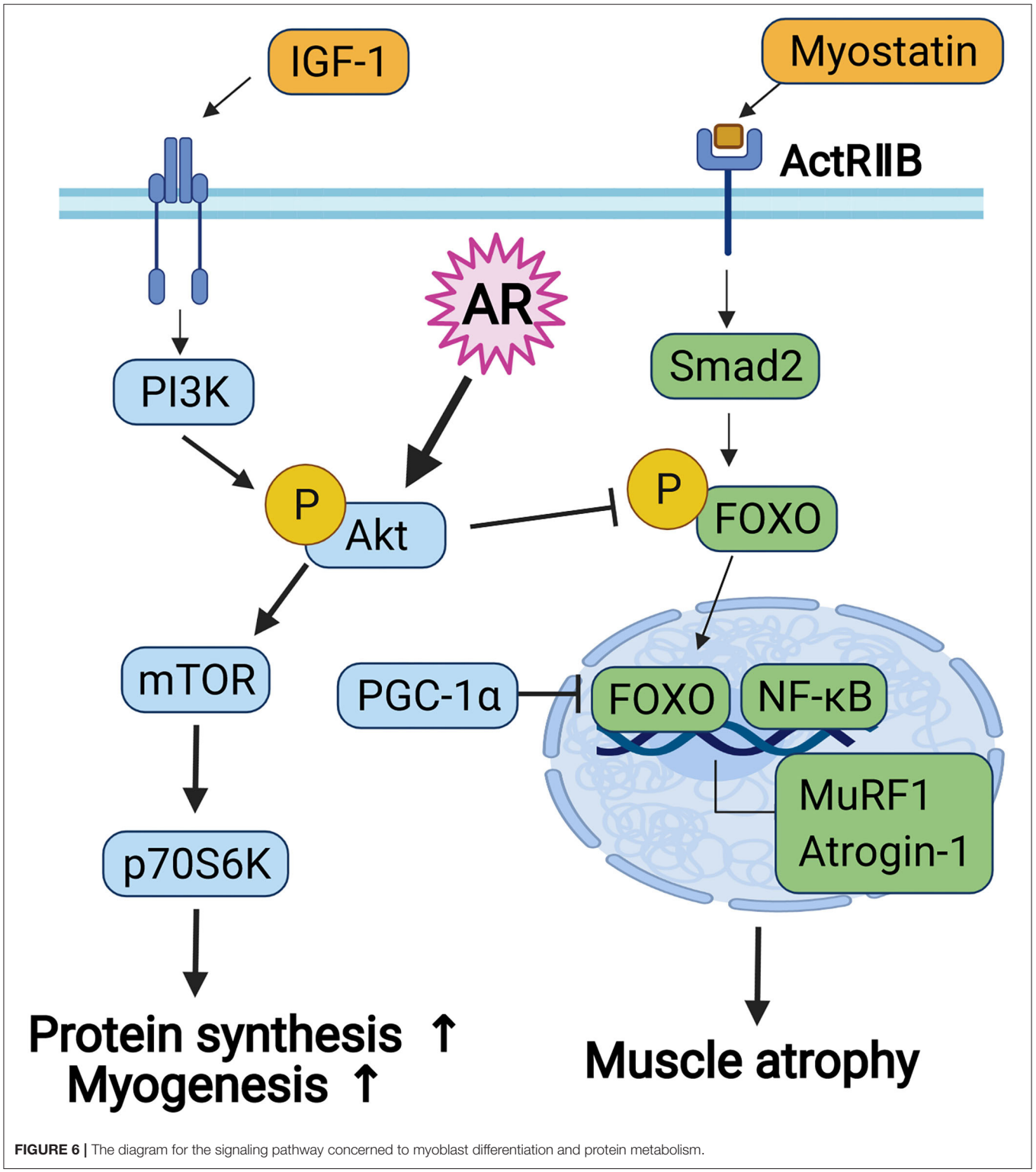

FIGURE 6 | The diagram for the signaling pathway concerned to myoblast differentiation and protein metabolism.

(NADH-dehydrogenase) or in the endoplasmic reticulum (NADH cytochrome b5 reductase). SDH is a reaction specific for mitochondria that are typically used to screen for mitochondrial myopathies. While NADH-TR reactivity implies activation of enzymes in the mitochondria or endoplasmic reticulum, SDH reactivity implies activation of enzymes specific to the mitochondria. Glucose 6-phosphate dehydrogenase (GPDH) is a marker for reductive biosynthesis; Mitochondrial GPDH is an integral component of the mitochondrial respiratory chain and functions as the rate-limiting step in the glycerophosphate

(GP) shuttle. Mitochondrial GPDH expression was significantly increased over the course of C2C12 myoblast differentiation, with an expression profile similar to that of Myogenin and MHC (47–49). Specifically, it regulates myogenic markers and myoblast differentiation by controlling mitochondrial biogenesis via CaMKKβ/AMPK. Mitochondrial GPDH$^{-/-}$ attenuates skeletal muscle regeneration *in vitro* and *in vivo*, while overexpression of mitochondrial GPDH ameliorates dystrophic pathology in mdx mice. Mitochondrial biogenesis in regulating myogenic differentiation pays attention as a potential therapeutic target for ameliorating muscle regeneration impairment and muscle pathology. In our results, AR induces the increase of both oxidative (MyhIIa) and glycolytic (MyhIIb) myofibers and enhances the expression of muscle metabolic genes, including mitochondrial components.

The fruit of aronia consists of a variety of ingredients, such as anthocyanin, flavonoids, ursolic acid, acetylursolic acid, and oleanolic acid (28, 50, 51). These components have been implicated in the increase of muscle mass, fast, and slow muscle fiber size and muscle strength (52–54). The treatment with ursolic acid, one of the main components of aronia, increases muscle mass in mice through enhancing muscle insulin/IGF-I signaling and inhibiting atrophy-associated muscle mRNA expression (52). Since Akt is the major downstream signaling component of IGF-1, this is in consistent with our current data. Although the functional aspect is not addressed, another study has proposed that the effect of aronia might be exerted through mTORC1 activation in response to resistance exercise without increasing muscle protein synthesis (50). Further studies are required to define the regulatory mechanisms by which aronia exerts the beneficial effect on muscle mass and strength. Our current study also demonstrates that aronia treatment upregulates the activities of both oxidative and glycolytic metabolic enzymes with increased expression of mitochondrial genes and glucose metabolism-related genes (**Figure 5**).

CONCLUSIONS

Our study demonstrates that AR promotes myogenic differentiation through Akt activation and protects the DEX-induced myotube atrophy through Akt activation that in turn represses the expression of muscle-specific ubiquitin ligases. In addition, AR treatment improves muscle mass and strength with increased expression of both glycolytic and oxidative myofibers with upregulated muscle-specific genes and muscle metabolic genes. Thus, given the current lack of therapies for skeletal muscle atrophy, AR might be a promising potential as a nutraceutical remedy to intervene in muscle weakness and atrophy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at Sungkyunkwan University School of Medicine (SUSM) and complied with the regulations of the Institutional Ethics Committee.

AUTHOR CONTRIBUTIONS

S-JL and G-UB conceptualized the project, designed the experiments, wrote the manuscript, and supervised the project. C-EY, H-KS, TAV, MWN, and SA performed the experiments. H-KL, KHK, and J-SK analyzed the results and performed the statistical analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Research Foundation Grant funded by the Korean Government (MSIP) (NRF-2019R1A2C2006233).

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Conflict of Interest: H-KS, TAV, and S-JL are employed by AniMusCure INC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Nutrition

ORIGINAL RESEARCH
published: 23 December 2021
doi: 10.3389/fnut.2021.779171

Impacts of Betaine Addition in Sow and Piglet's Diets on Growth Performance, Plasma Hormone, and Lipid Metabolism of Bama Mini-Pigs

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The present study evaluated the effects of betaine addition in sow and piglet's diets on growth performance, plasma hormone, and lipid metabolism of Bama mini-pigs. A total of 26 pregnant Bama mini-pigs and 104 weaned piglets were selected and divided into different dietary treatment groups (details in "Materials and Methods"). Blood and muscle samples were collected at 65-, 95-, and 125-day-old, respectively. The results showed that betaine addition in sow-offspring diets increased ($P < 0.05$) the body weight at 125-day-old, average daily gain from 35- to 65-day-old, and average daily feed intake at 35–65 and 35–95 days old of pigs compared with the control group. Betaine addition in sow-offspring diets increased ($P < 0.05$) the plasma gastrin level at 95-day-old, while betaine addition in sow diets decreased ($P < 0.05$) the plasma peptide YY and leptin levels at 65-day-old pigs. In the *longissimus dorsi* muscle of pigs, betaine addition in sow and sow-offspring diets increased ($P < 0.05$) the C12:0 content at 65-day-old while decreased at 95-day-old. Moreover, betaine addition in sow-offspring diets increased the C24:0 content and decreased the C18:1n9t content at 125-day-old ($P < 0.05$). In the *biceps femoris* muscle, the contents of C12:0 at 65-day-old and C20:4n6 at 125-day-old were decreased ($P < 0.05$) after the betaine addition in both sow and piglet's diets. In addition, betaine addition in sow diets decreased ($P < 0.05$) the C20:0 content at 125-day-old, while betaine addition in sow-offspring diets increased the C18:3n6 and decreased C24:0 contents at 65-day-old pigs ($P < 0.05$). In the *psoas major* muscle, betaine addition in sow and sow-offspring diets decreased ($P < 0.05$) the contents of C18:1n9t at 65-day-old and C20:1 at 95-day-old, while betaine addition in sow diets decreased ($P < 0.05$) the intramuscular fat content at 125-day-old. Moreover, betaine addition in sow-offspring diets was also associated with muscle lipid deposition and metabolisms by regulating the gene expressions related to fatty acid metabolism. These findings suggested that betaine addition in sow-offspring diets could improve the growth performance, whereas betaine addition in both sow and sow-offspring diets could enhance lipid quality by altering plasma hormone level and fatty acid composition and regulating the gene expressions related to fatty acid metabolism.

Keywords: Bama mini-pigs, betaine, growth performance, lipid metabolism, plasma hormone

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *18 September 2021*
Accepted: *15 November 2021*
Published: *23 December 2021*

Citation:
Cheng Y, Song M, Zhu Q, Azad MAK, Gao Q and Kong X (2021) Impacts of Betaine Addition in Sow and Piglet's Diets on Growth Performance, Plasma Hormone, and Lipid Metabolism of Bama Mini-Pigs. Front. Nutr. 8:779171. doi: 10.3389/fnut.2021.779171

INTRODUCTION

Intramuscular fat (IMF) plays an important role in various aspects of meat quality, and it is also critical for the nutritional value of meat (1). Higher content of IMF can increase the meat quality as it contributes to pork tenderness, flavor, and juiciness (2). Furthermore, muscle fatty acid profiles also play a key role in meat quality because of their high value for human health. Polyunsaturated fatty acids (PUFA), especially n-3 fatty acids, are considered as functional ingredients to prevent cardiovascular disease in humans (3). The n-3 PUFA deficiency and excessive content of n-6 PUFA are associated with the development of insulin resistance and metabolic disorders (4). Therefore, abnormal lipid metabolism could inhibit the growth and decrease the meat quality of animals. In addition, a recent study has reported that changes in fatty acid composition due to different feeding strategies are associated with the changes in the mRNA expression of genes related to fatty acid metabolism (5). For example, fatty acid synthase (*FAS*) and stearoyl coenzyme A desaturase (*SCD*) genes are associated with the regulation of fatty acid biosynthesis, while lipoprotein lipase (*LPL*) can regulate the fatty acid transportation from blood to fat cell (6, 7). Thus, the regulation of IMF content and fatty acid composition in muscles has been of great interest in recent years. Furthermore, antibiotics are commonly used in the feed industry to promote the growth of animals. Owing to the ban on in-feed antibiotics in the livestock industry, a number of alternative feed additives with similar effects to in-feed antibiotics in livestock production have attracted increased attention.

Betaine is a common term for trimethylglycine, a substrate for betaine-homocysteine methyltransferase (HMT) in liver and kidney (8). As a promising antioxidant agent, betaine can prevent lipid peroxidation and regulate lipid metabolism to improve growth performance and meat quality. Gheisari et al. (9) reported that the betaine addition (1 g/kg) to the methionine-deficient diet could significantly improve antioxidant defense and meat quality by decreasing lipid peroxidation in breast muscles of broilers. In addition, dietary betaine could improve the meat quality of lambs, and rumen-protected betaine exhibited better effects than that of the unprotected-betaine (10). However, there are limited studies about maternal betaine addition on offspring's growth performance and lipid metabolism.

Bama mini-pig is a high-quality local pig breed in China because of its characteristics of delicious meat, high roughage tolerance, and good adaptability. However, the lower dietary nutrient level and extensive management methods have resulted in lower growth, feed conversion, and lean meat rate of this mini-pig. Our previous studies showed that 3.50 kg/t betaine addition to sow and sow-offspring diets could increase the carcass weight, carcass yield, lean meat rate, meat color, shear force, and crude protein content of muscles at different stages by altering the plasma biochemical parameters and amino acid composition, and regulating the expression level of genes related to myosin heavy-chain (MyHC) isoform and myogenic regulatory factors (MRFs) in skeletal muscle (11). Moreover, dietary betaine hydrochloride addition (3.50 kg/t) to sows from gestation to lactation could improve suckling piglet's health by enhancing intestinal morphology and immunity and altering intestinal microbiota (12). Therefore, we hypothesized that dietary betaine might influence the growth performance and meat quality of pigs by regulating the lipid content, IMF content, and mRNA expression of genes related to fatty acid metabolism in skeletal muscle. Thus, the current study was conducted to test our hypothesis that the addition of dietary betaine to sow and sow-offspring would improve the offspring's growth performance and thereby influence the lipid metabolism. Furthermore, to explain the mechanism of dietary betaine addition on growth performance and lipid metabolism of Bama mini-pigs by evaluating plasma hormones and the fatty acid metabolism-related genes.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

Twenty-six pregnant Bama mini-pigs with similar body weight (BW) were selected and randomly divided into control group (sows fed a basal diet; $n = 12$) and betaine group (sows fed a basal diet supplemented with 3.50 kg/t betaine hydrochloride; $n = 14$). The sows were housed individually in gestation crates (2.2×0.6 m) from mating to day 104 of gestation. On day 105 of gestation, the sows were transferred to individual farrowing crates (2.2×1.8 m) with a heated floor pad for offspring piglets with freely accessible water. After weaning, at 35-day-old, a total of 104 piglets with an average BW of litters were selected from the two groups (48 piglets from the control group and 56 piglets from the betaine group) and divided into three dietary treatments as follows: a) control group, piglets from the control group fed a basal diet ($n = 48$); b) sow betaine group, piglets from the betaine group fed a basal diet ($n = 28$); and c) sow-offspring betaine group, piglets from the betaine group fed a basal diet with 2.50 kg/t betaine hydrochloride ($n = 28$). Four piglets were housed in one pen, and the rearing compartments were temperature-controlled (23–26°C) and had forced ventilation. Betaine hydrochloride (purity $\geq$ 95%) was purchased from Sunwin Biotech Shandong Co., Ltd (Shandong, China). The betaine was mixed with the basal diets before feeding the sows and piglets. The supplementing dose of betaine was determined considering the tolerance of sows and piglets and based on previous studies (13, 14).

During the trial, the sows were fed pregnant diets from day 3 after mating to day 104 of pregnancy and fed lactating diets from day 105 of pregnancy to weaning. The piglets were fed pre-nursery diets from 35- to 95-day-old and fed late-nursery diets from 96- to 125-day-old. The nutrient levels of basal diets for sows and piglets met the Chinese local swine nutrient requirements (NY/T 65-2004), and the premixes met the National Research Council (NRC, 2012) diet requirements (15, 16). The composition and nutrient levels of basal diets for sows and piglets are presented in **Supplementary Tables 1**, **2**, respectively. The sows were fed twice daily (at 08:00 and 17:00) and changed with their body condition. The piglets were fed four times daily (at 08:00, 12:00, 15:00, and 19:00), and the dietary stage was based on the physiological stage of piglets. All animals had *ad libitum* access to drinking water throughout the trial.

Sample Collection

The animals were weighed at 65-, 95-, and 125-day-old after a 12 h fasting, respectively. A total of 26 pigs (including 12, 7, and 7 pigs from the control group, sow betaine group, and sow-offspring betaine group, respectively) were selected at each stage (65-, 95-, and 125-day-old) and sacrificed under commercial conditions *via* electrical stunning (120 V, 200 Hz) and exsanguination (17). Blood samples (5 mL) were collected from the anterior vena cava, anticoagulated with heparin, and centrifuged at 2 000 × *g* for 10 min to obtain plasma. After slaughter, *longissimus dorsi* (LD), *biceps femoris* (BF), and *psoas major* (PM) muscles were sampled. One part of the muscle samples was stored at −20°C for measuring IMF content and fatty acid composition, and another part was stored at −80°C for analyzing the expression level of genes related to fatty acid metabolism.

Growth Performance

During the trial, the feed intake of each pen was recorded daily, and the BW (12 h fasting) was taken at 65-, 95-, and 125-day-old. The average daily gain (ADG), average daily feed intake (ADFI), and feed/gain ratio (F:G) were calculated.

Plasma Hormone Analysis

The levels of plasma hormones, including cholecystokinin (CCK), gastrin (Gas), growth hormone (GH), insulin-like growth factor (IGF), leptin (LEP), pancreatic polypeptide (PP), peptide YY (PYY), and somatostatin (SS) were measured using commercially available enzyme-linked immunosorbent assay kits (Jiangsu Yutong Biotechnology Co., Ltd., Jiangsu, China) on a Multiscan Spectrum Spectrophotometer (Infinite M200 Pro; Tecan, Männedorf, Switzerland) in accordance with the manufacturers' instructions.

Intramuscular Fat and Fatty Acid Analysis

The muscle samples were cut into thin slices, dried in a vacuum freeze dryer at (10 ± 5) Pa and –(45 ± 5)°C for 48 h, and then ground into powder. The IMF was measured according to GB/T 9695.7-2008 (technical manual for testing the total ether extract contents in the meat). The composition of medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) in muscles were measured by using gas chromatography as previously described by Li et al. (18).

Analysis of the Expression Levels of Genes Related to Fatty Acid Metabolism in Muscles

Total RNA was isolated from LD, BF, and PM muscles using TRIzol (Invitrogen, Shanghai, China). β-actin and target genes based on related cDNA sequences of pigs in the GeenBank database in NCBI are presented in **Supplementary Table 3**, and the gene-specific primers were synthesized by Invitrogen Biotech Co., Ltd. (Shanghai, China). The reverse transcription-polymerase chain reaction (RT-PCR) assays were conducted using the SYBR Premix Ex Taq^{TM} Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The RNA quality detection, including the RT-PCR and cDNA synthesis, was used the same methods as described in the previous study (19). The RT-PCR conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s, and finally extension at 72°C for 30 s. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (20).

Statistical Analysis

The data analyses were performed with SPSS 22.0 software (IBM Corporation, Armonk, NY, USA) using a one-way analysis of variance (ANOVA). The means of different groups were compared by Duncan tests. All data are presented as means ± SE unless otherwise indicated. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Growth Performance

The growth performance of pigs is presented in **Table 1**. The BW in the sow-offspring betaine group was increased ($P < 0.05$) at 125-day-old compared with the control group and at 95-day-old compared with the sow betaine group. The ADG from 35- to 65-day-old and ADFI from 35- to 65- and from 35- to 95-day-old were increased ($P < 0.05$) in the sow-offspring betaine group compared with the other two groups. Moreover, the ADG was increased ($P < 0.05$) in the sow-offspring betaine group from 35- to 95-day-old compared with the sow betaine group. However, dietary betaine addition in sow and sow-offspring diets did not affect ($P > 0.05$) the F:G of piglets.

Plasma Hormone Level

The plasma hormone levels of pigs at different stages are presented in **Table 2**. Betaine addition in sow-offspring diets increased ($P < 0.05$) the plasma Gas level of 95-day-old pigs compared with the other two groups. Compared with the control group, betaine addition in sow diets decreased ($P < 0.05$) the plasma PYY and LEP levels of 65-day-old pigs. There were no significant differences ($P > 0.05$) in the plasma hormone levels of CCK, GH, IGF, PP, and SS at different stages among the three groups.

Intramuscular Fat Content

The IMF content in the LD, BF, and PM muscles is presented in **Tables 3–5**, respectively. The sow betaine group had a lower ($P < 0.05$) IMF content in the PM muscle of 125-day-old pigs compared with the other two groups. There was no significant difference in the IMF content in the LD and BF muscles among the three groups ($P > 0.05$).

Medium- and Long-Chain Fatty Acid Contents in Muscles

The MCFA and LCFA contents in the LD muscle are presented in **Table 3**. Compared with the control group, betaine addition in sow-offspring diets increased ($P < 0.05$) the content of C24:0 and decreased ($P < 0.05$) the content of C18:1n9t in the 125-day-old pigs. Moreover, betaine addition in sow and sow-offspring diets increased ($P < 0.05$) the content of C12:0 in the 65-day-old pigs, while decreased ($P < 0.05$) the content of C12:0 in the

TABLE 1 | Effect of dietary betaine addition on growth performance of Bama mini-pigs.

Items	Day-old	Control group	Sow betaine group	Sow-offspring betaine group
BW (kg)	35	4.94 ± 0.17	4.99 ± 0.36	4.70 ± 0.17
	65	9.11 ± 0.25	9.77 ± 0.31	9.72 ± 0.12
	95	14.64 ± 1.07^{ab}	12.24 ± 0.80^{b}	17.06 ± 1.07^{a}
	125	22.66 ± 2.69^{b}	27.00 ± 1.79^{ab}	32.46 ± 2.55^{a}
ADG (kg)	35–65	0.15 ± 0.01^{b}	0.16 ± 0.01^{b}	0.19 ± 0.01^{a}
	35–95	0.16 ± 0.01^{ab}	0.13 ± 0.01^{b}	0.20 ± 0.02^{a}
	35–125	0.21 ± 0.03	0.22 ± 0.02	0.29 ± 0.03
ADFI (kg)	35–65	0.41 ± 0.01^{b}	0.39 ± 0.02^{b}	0.47 ± 0.02^{a}
	35–95	0.51 ± 0.02^{b}	0.53 ± 0.04^{b}	0.66 ± 0.02^{a}
	35–125	0.66 ± 0.05	0.69 ± 0.05	0.82 ± 0.03
F:G	35–65	2.84 ± 0.13	2.51 ± 0.12	2.53 ± 0.16
	35–95	3.66 ± 0.34	4.28 ± 0.25	3.49 ± 0.31
	35–125	3.67 ± 0.61	3.10 ± 0.09	2.89 ± 0.25

Data are presented as means ± SE. Values in the same row without a common superscript letter are different ($P < 0.05$). Control group, n = 12, 11, and 8; sow betaine group, n = 6, 7, and 6; sow-offspring betaine group, n = 7, 7, and 6; at 65-, 95-, and 125-day-old, respectively. ADG, average daily gain; ADFI, average daily feed intake; BW, body weight; F:G, feed/gain ratio.

TABLE 2 | Effect of dietary betaine addition on plasma hormone levels of Bama mini-pigs.

Items	Day-old	Control group	Sow betaine group	Sow-offspring betaine group
CCK (pg/mL)	65	940.73 ± 83.74	627.35 ± 39.32	846.33 ± 53.10
	95	498.05 ± 25.09	503.02 ± 29.26	491.08 ± 16.63
	125	607.61 ± 71.89	630.29 ± 71.47	492.72 ± 47.82
Gas (ng/mL)	65	0.81 ± 0.08	0.60 ± 0.07	0.66 ± 0.04
	95	0.84 ± 0.06^{b}	0.89 ± 0.08^{b}	1.30 ± 0.15^{a}
	125	1.04 ± 0.13	0.87 ± 0.03	0.82 ± 0.05
GH (ng/mL)	65	45.77 ± 3.21	33.60 ± 6.45	32.43 ± 3.86
	95	29.91 ± 2.26	27.36 ± 1.88	29.27 ± 2.24
	125	29.85 ± 2.69	33.49 ± 3.91	25.26 ± 0.87
IGF (ng/mL)	65	228.87 ± 16.06	167.31 ± 31.57	162.16 ± 19.30
	95	146.49 ± 10.90	151.52 ± 7.65	141.63 ± 6.59
	125	190.08 ± 21.78	189.96 ± 19.03	179.96 ± 12.59
LEP (ng/mL)	65	30.44 ± 2.08^{a}	22.26 ± 0.83^{b}	25.99 ± 2.22^{ab}
	95	19.29 ± 1.06	20.68 ± 1.52	22.58 ± 2.05
	125	22.41 ± 2.16	19.45 ± 0.71	20.77 ± 1.56
PP (ng/mL)	65	4.14 ± 0.38	3.01 ± 0.55	2.96 ± 0.22
	95	2.99 ± 0.23	2.89 ± 0.16	3.00 ± 0.14
	125	2.94 ± 0.27	2.45 ± 0.11	2.44 ± 0.09
PYY (pmol/L)	65	20.37 ± 1.58^{a}	14.54 ± 0.71^{b}	16.62 ± 1.30^{ab}
	95	15.74 ± 1.03	17.98 ± 1.54	18.14 ± 1.73
	125	18.21 ± 2.37	16.61 ± 0.90	17.07 ± 1.27
SS (pg/mL)	65	171.46 ± 14.73	136.07 ± 25.40	124.67 ± 9.12
	95	160.66 ± 15.48	154.64 ± 12.12	167.31 ± 14.43
	125	112.21 ± 6.60	163.88 ± 21.68	168.48 ± 32.71

Data are presented as means ± SE. Values in the same row without a common superscript letter are different ($P < 0.05$). Control group, n = 11, 11, and 8; sow betaine group, n = 6, 7, and 6; sow-offspring betaine group, n = 7, 7, and 5; at 65-, 95-, and 125-day-old, respectively. CCK, cholecystokinin; Gas, gastrin; GH, growth hormone; IGF, insulin-like growth factor; LEP, leptin; PP, pancreatic polypeptide; PYY, peptide-YY; SS, somatostatin.

TABLE 3 | Effect of dietary betaine addition on medium- and long-chain fatty acid contents in *longissimus dorsi* muscle of Bama mini-pigs (fresh weight basis; %).

Items	Control group	Sow betaine group	Sow-offspring betaine group
65-day-old			
Intramuscular fat	2.55 ± 0.18	2.62 ± 0.26	2.67 ± 0.22
C12:0	0.04 ± 0.01^{b}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}
C14:0	0.49 ± 0.10	0.45 ± 0.06	0.41 ± 0.04
C15:0	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.02
C16:0	8.20 ± 0.79	7.58 ± 0.42	7.53 ± 0.35
C16:1	1.16 ± 0.16	1.10 ± 0.23	1.10 ± 0.26
C17:0	0.12 ± 0.07	0.12 ± 0.03	0.11 ± 0.02
C18:0	4.27 ± 0.36	4.04 ± 0.36	4.15 ± 0.39
C18:1n9c	8.87 ± 1.21	8.38 ± 0.51	8.28 ± 0.88
C18:1n9t	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C18:2n6c	4.17 ± 0.49	4.03 ± 0.50	4.48 ± 0.68
C18:3n3	0.17 ± 0.02	0.17 ± 0.02	0.16 ± 0.01
C18:3n6	0.02 ± 0.01^{ab}	0.03 ± 0.02^{a}	0.01 ± 0.02^{b}
C20:0	0.06 ± 0.02	0.06 ± 0.01	0.05 ± 0.01
C20:1	0.24 ± 0.08	0.19 ± 0.02	0.19 ± 0.03
C20:2	0.16 ± 0.03	0.14 ± 0.01	0.15 ± 0.03
C20:3n6	0.10 ± 0.03	0.09 ± 0.01	0.12 ± 0.01
C20:4n6	0.81 ± 0.39	0.71 ± 0.09	1.06 ± 0.24
C22:6n3	0.04 ± 0.02	0.04 ± 0.01	0.02 ± 0.03
C24:0	0.05 ± 0.02	0.05 ± 0.03	0.07 ± 0.05
95-day-old			
Intramuscular fat	2.40 ± 0.31	2.83 ± 0.38	3.07 ± 0.27
C12:0	0.04 ± 0.01^{a}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}
C14:0	0.49 ± 0.08	0.41 ± 0.19	0.47 ± 0.22
C15:0	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
C16:0	8.26 ± 0.78	8.22 ± 0.56	8.49 ± 0.57
C16:1	0.71 ± 0.07	0.81 ± 0.13	0.76 ± 0.14
C17:0	0.10 ± 0.04	0.11 ± 0.03	0.09 ± 0.02
C18:0	5.13 ± 0.64	5.00 ± 0.29	5.08 ± 0.85
C18:1n9c	8.81 ± 1.10	8.91 ± 0.97	9.45 ± 1.47
C18:1n9t	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C18:2n6c	3.29 ± 0.86	3.29 ± 0.72	2.98 ± 0.45
C18:3n3	0.13 ± 0.01	0.13 ± 0.02	0.12 ± 0.02
C20:0	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.01
C20:1	0.30 ± 0.08	0.25 ± 0.05	0.35 ± 0.09
C20:2	0.15 ± 0.02	0.13 ± 0.02	0.15 ± 0.02
C20:3n6	0.09 ± 0.04	0.09 ± 0.03	0.07 ± 0.02
C20:4n6	0.66 ± 0.53	0.60 ± 0.19	0.46 ± 0.11
C24:0	0.02 ± 0.03	0.03 ± 0.02	0.03 ± 0.02
125-day-old			
Intramuscular fat	2.48 ± 0.33	2.49 ± 0.49	2.57 ± 0.21
C12:0	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
C14:0	0.51 ± 0.12	0.47 ± 0.10	0.44 ± 0.10
C16:0	8.22 ± 0.99	8.22 ± 1.25	7.54 ± 0.57
C16:1	0.82 ± 0.14	0.80 ± 0.15	0.76 ± 0.10
C17:0	0.09 ± 0.03	0.10 ± 0.05	0.07 ± 0.02
C18:0	4.73 ± 0.54	5.01 ± 1.23	4.44 ± 0.25
C18:1n9c	9.79 ± 1.00	10.01 ± 1.71	8.83 ± 0.58
C18:1n9t	0.043 ± 0.01^{a}	0.039 ± 0.01^{a}	0.029 ± 0.00^{b}
C18:2n6c	2.58 ± 0.43	2.69 ± 0.19	2.63 ± 0.37
C20:0	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.01
C20:1	0.10 ± 0.02	0.10 ± 0.02	0.09 ± 0.00
C20:2	0.11 ± 0.02	0.10 ± 0.03	0.08 ± 0.01
C20:3n6	0.08 ± 0.03	0.09 ± 0.02	0.10 ± 0.02
C20:4n6	0.54 ± 0.25	0.67 ± 0.22	0.79 ± 0.22
C24:0	0.05 ± 0.02^{b}	0.08 ± 0.03^{ab}	0.09 ± 0.02^{a}

Data are presented as means ± SE. Values in the same row without a common superscript letter are different ($P < 0.05$). Control group, n = 12, 11, and 8; sow betaine group, n = 6, 7, and 6; sow-offspring betaine group, n = 7, 7, and 6; at 65-, 95-, and 125-day-old, respectively.

95-day-old pigs compared with the pigs in the control group. Compared with the sow betaine group, betaine addition in sow-offspring diets decreased ($P < 0.05$) the content of C18:3n6 in the 65-day-old pigs.

The MCFA and LCFA contents in the BF muscle are presented in **Table 4**. Compared with the control group, betaine addition in sow-offspring diets increased ($P < 0.05$) the content of C18:3n6 and decreased ($P < 0.05$) the content of C24:0 in the 65-day-old pigs, while betaine addition in sow diets decreased ($P < 0.05$) the content of C20:0 in the 125-day-old pigs. In addition, betaine addition in sow and sow-offspring diets decreased ($P < 0.05$) the contents of C12:0 in the 65-day-old pigs and C20:4n6 in the 125-day-old pigs, when compared with the control group.

The MCFA and LCFA contents in the PM muscle are presented in **Table 5**. Compared with the control group, betaine addition in sow and sow-offspring diets decreased ($P < 0.05$) the contents of C18:1n9t in the 65-day-old pigs and C20:1 in the 95-day-old pigs, while betaine addition in sow-offspring diets decreased ($P < 0.05$) the content of C18:2n6c in the 95-day-old pigs.

The Expression Levels of Genes Related to Fatty Acid Metabolism in Muscles

The expression levels of genes related to fatty acid metabolism in the LD, BF, and PM muscles of pigs are shown in **Figure 1**. In the LD muscle, dietary betaine addition in sow-offspring diets down-regulated ($P < 0.05$) the mRNA expression levels of *FAS* and sterol regulatory element-binding protein 1 (*SREBP1*) in the 65-day-old pigs compared with the sow betaine group, as well as the mRNA expression level of *SREBP1* in the 125-day-old pigs compared with the pigs in the control and sow betaine groups. However, dietary betaine addition in sow and sow-offspring diets did not affect ($P > 0.05$) the gene expression levels of the LD muscle at 95-day-old pigs. In the BF muscle, dietary betaine addition in sow-offspring diets down-regulated ($P < 0.05$) the mRNA expression level of *FAS* compared with the pigs in the control and sow betaine groups, while up-regulated ($P < 0.05$) the mRNA expression level of *LPL* compared with the sow betaine group in the 95-day-old pigs. In addition, dietary

TABLE 4 | Effect of dietary betaine addition on medium- and long-chain fatty acid contents in *biceps femoris* muscle of Bama mini-pigs (fresh weight basis; %).

Items	Control group	Sow betaine group	Sow-offspring betaine group
65-day-old			
Intramuscular fat	1.16 ± 0.07	1.24 ± 0.18	1.54 ± 0.26
C12:0	0.05 ± 0.01^{a}	0.04 ± 0.01^{b}	0.03 ± 0.01^{b}
C14:0	0.36 ± 0.06	0.33 ± 0.04	0.32 ± 0.04
C15:0	0.02 ± 0.02	0.03 ± 0.02	0.02 ± 0.01
C16:0	6.71 ± 0.48	6.50 ± 0.30	6.68 ± 0.38
C16:1	1.03 ± 0.14	0.96 ± 0.10	1.07 ± 0.28
C17:0	0.11 ± 0.04	0.11 ± 0.03	0.10 ± 0.02
C18:0	3.69 ± 0.28	3.66 ± 0.23	3.64 ± 0.41
C18:1n9c	7.76 ± 0.66	7.49 ± 1.12	8.06 ± 0.91
C18:1n9t	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.02
C18:2n6c	4.82 ± 0.50	4.64 ± 0.37	4.50 ± 0.70
C18:3n3	0.15 ± 0.01	0.14 ± 0.02	0.14 ± 0.01
C18:3n6	0.01 ± 0.02^{b}	0.02 ± 0.02^{ab}	0.03 ± 0.01^{a}
C20:0	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.01
C20:1	0.18 ± 0.04	0.16 ± 0.04	0.18 ± 0.03
C20:2	0.15 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
C20:3n6	0.14 ± 0.02	0.13 ± 0.03	0.12 ± 0.02
C20:4n6	1.41 ± 0.38	1.32 ± 0.39	1.25 ± 0.36
C22:6n3	0.07 ± 0.02	0.06 ± 0.03	0.05 ± 0.01
C23:0	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.02
C24:0	0.10 ± 0.04^{a}	0.08 ± 0.02^{ab}	0.06 ± 0.03^{b}
95-day-old			
Intramuscular fat	1.08 ± 0.20	1.02 ± 0.17	1.28 ± 0.18
C14:0	0.37 ± 0.05	0.33 ± 0.04	0.39 ± 0.06
C15:0	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
C16:0	6.75 ± 0.40	6.57 ± 0.37	6.97 ± 0.41
C16:1	0.76 ± 0.10	0.75 ± 0.13	0.80 ± 0.15
C17:0	0.08 ± 0.02	0.09 ± 0.02	0.07 ± 0.01
C18:0	4.18 ± 0.45	4.10 ± 0.15	4.08 ± 0.54
C18:1n9c	7.86 ± 0.97	7.71 ± 1.17	8.41 ± 0.73
C18:1n9t	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C18:2n6c	4.26 ± 0.74	4.08 ± 0.79	3.55 ± 0.53
C18:3n3	0.11 ± 0.01	0.11 ± 0.02	0.10 ± 0.01
C20:0	0.04 ± 0.02	0.04 ± 0.00	0.05 ± 0.01
C20:1	0.22 ± 0.06	0.19 ± 0.04	0.25 ± 0.04
C20:2	0.14 ± 0.03	0.13 ± 0.02	0.13 ± 0.02
C20:3n6	0.16 ± 0.07	0.14 ± 0.04	0.12 ± 0.03
C20:4n6	1.59 ± 0.80	1.38 ± 0.36	1.10 ± 0.17
C22:6n3	0.02 ± 0.03	0.04 ± 0.02	0.01 ± 0.02
C24:0	0.09 ± 0.03	0.08 ± 0.02	0.07 ± 0.02
125-day-old			
Intramuscular fat	1.87 ± 0.17	1.88 ± 0.10	1.92 ± 0.23
C14:0	0.41 ± 0.06	0.39 ± 0.05	0.37 ± 0.20
C16:0	7.50 ± 1.06	7.21 ± 0.42	5.17 ± 4.08
C16:1	0.85 ± 0.12	0.82 ± 0.14	0.84 ± 0.07
C17:0	0.09 ± 0.03	0.10 ± 0.05	0.08 ± 0.03
C18:0	4.31 ± 0.67	4.17 ± 0.71	3.57 ± 1.81
C18:1n9c	9.55 ± 1.22	9.76 ± 0.60	10.36 ± 1.04
C18:1n9t	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C18:2n6c	3.17 ± 0.63	2.86 ± 0.24	3.11 ± 0.63
C20:0	0.05 ± 0.01^{a}	0.05 ± 0.01^{b}	0.06 ± 0.01^{a}
C20:1	0.10 ± 0.02	0.09 ± 0.01	0.14 ± 0.08
C20:2	0.10 ± 0.02	0.10 ± 0.02	0.11 ± 0.02
C20:3n6	0.11 ± 0.03	0.10 ± 0.02	0.11 ± 0.02
C20:4n6	0.99 ± 0.34^{a}	0.15 ± 0.36^{b}	0.39 ± 0.61^{b}
C22:6n3	0.02 ± 0.02	0.02 ± 0.02	0.03 ± 0.08
C24:0	0.09 ± 0.03	0.08 ± 0.01	0.05 ± 0.04

Data are presented as means ± SE. Values in the same row without a common superscript letter are different (P < 0.05). Control group, n = 12, 11, and 8; sow betaine group, n = 6, 7, and 6; sow-offspring betaine group, n = 7, 7, and 6; at 65-, 95-, and 125-day-old, respectively.

betaine addition in sow diets up-regulated ($P < 0.05$) the mRNA expression level of *LPL* in the 125-day-old pigs compared with the control and sow-offspring betaine groups. Moreover, dietary betaine addition had no impacts ($P > 0.05$) on the mRNA gene expression levels of the BF muscle at 65-day-old pigs. In the PM muscle, compared with the control group, dietary betaine addition in sow and sow-offspring diets up-regulated ($P < 0.05$) the mRNA expression levels of the *FAS* and *SREBP1* in the 65-day-old pigs, whereas down-regulated ($P < 0.05$) the mRNA expression level of *SCD* in the 125-day-old pigs. In addition, dietary betaine addition in sow-offspring diets down-regulated ($P < 0.05$) the mRNA expression level of *SREBP1* in the 125-day-old pigs compared with the pigs in the control group. Furthermore, dietary betaine addition in sow-offspring diets down-regulated ($P < 0.05$) the mRNA expression level of *FAS* in the 95-day-old pigs compared with the control and sow betaine groups.

DISCUSSION

As an osmotic regulator, dietary betaine has been widely studied as a methyl donor on protein synthesis and nitrogen metabolism. Moreover, there are lots of creditability evidence showing that dietary betaine can influence growth performance and meat quality through its different functional properties. Previous studies have found that dietary betaine addition during the growing and finishing stages improved the growth performance and meat quality of animals (21, 22). In addition, maternal nutrition also influences the offspring's growth later in life (23). Therefore, the present study evaluated the effects of betaine addition in sow and sow-offspring diets on the growth performance and meat quality-associated parameters of pigs at different stages. The results showed that the betaine addition in sow and sow-offspring diets influenced the growth performance, plasma hormone, and lipid metabolism of Bama mini-pigs.

Betaine is widely used in animal production because of its positive effects on animal performance. In the present study,

TABLE 5 | Effect of dietary betaine addition on medium- and long-chain fatty acid contents in *psoas major* muscle of Bama mini-pigs (fresh weight basis; %).

Items	Control group	Sow betaine group	Sow-offspring betaine group
65-day-old			
Intramuscular fat	1.40 ± 0.16	1.62 ± 0.10	1.13 ± 0.19
C12:0	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C14:0	0.40 ± 0.12	0.40 ± 0.12	0.30 ± 0.06
C15:0	0.03 ± 0.02	0.03 ± 0.02	0.02 ± 0.02
C16:0	7.50 ± 0.75	7.23 ± 0.89	6.63 ± 0.54
C16:1	0.93 ± 0.17	0.91 ± 0.18	0.77 ± 0.21
C17:0	0.13 ± 0.06	0.13 ± 0.04	0.11 ± 0.02
C18:0	4.39 ± 0.44	4.18 ± 0.42	4.09 ± 0.33
C18:1n9c	7.22 ± 1.08	7.20 ± 1.17	6.06 ± 0.54
C18:1n9t	0.04 ± 0.01^{a}	0.02 ± 0.01^{b}	0.02 ± 0.02^{b}
C18:2n6c	5.24 ± 1.11	4.80 ± 0.89	5.54 ± 0.64
C18:3n3	0.15 ± 0.01	0.16 ± 0.02	0.14 ± 0.01
C18:3n6	0.03 ± 0.02	0.01 ± 0.01	0.02 ± 0.02
C20:0	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.02
C20:1	0.18 ± 0.05	0.17 ± 0.04	0.14 ± 0.03
C20:2	0.15 ± 0.02	0.14 ± 0.01	0.13 ± 0.03
C20:3n6	0.14 ± 0.05	0.12 ± 0.06	0.14 ± 0.02
C20:4n6	1.38 ± 0.75	1.09 ± 0.80	1.62 ± 0.40
C22:6n3	0.05 ± 0.03	0.04 ± 0.04	0.07 ± 0.02
C23:0	0.07 ± 0.04	0.04 ± 0.03	0.07 ± 0.02
C24:0	0.08 ± 0.04	0.08 ± 0.05	0.10 ± 0.02
95-day-old			
Intramuscular fat	1.17 ± 0.12	1.15 ± 0.13	1.32 ± 0.18
C14:0	0.32 ± 0.05	0.31 ± 0.05	0.35 ± 0.05
C15:0	0.027 ± 0.01	0.032 ± 0.01	0.020 ± 0.01
C16:0	6.58 ± 0.32	6.81 ± 0.48	6.76 ± 0.48
C16:1	0.52 ± 0.06	0.58 ± 0.12	0.56 ± 0.09
C17:0	0.09 ± 0.03	0.11 ± 0.02	0.08 ± 0.01
C18:0	4.52 ± 0.52	4.53 ± 0.30	4.42 ± 0.51
C18:1n9c	6.17 ± 0.39	6.47 ± 0.72	6.91 ± 1.05
C18:1n9t	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C18:2n6c	5.13 ± 0.77^{a}	5.00 ± 0.69^{a}	4.11 ± 0.62^{b}
C20:0	0.04 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
C20:1	0.18 ± 0.06^{a}	0.12 ± 0.01^{b}	0.10 ± 0.01^{b}
C20:2	0.15 ± 0.04	0.13 ± 0.02	0.13 ± 0.02
C20:3n6	0.17 ± 0.07	0.16 ± 0.04	0.13 ± 0.05
C20:4n6	1.77 ± 0.82	1.49 ± 0.20	1.17 ± 0.30
C22:6n3	0.03 ± 0.03	0.03 ± 0.02	0.01 ± 0.02
C24:0	0.09 ± 0.03	0.10 ± 0.01	0.09 ± 0.02
125-day-old			
Intramuscular fat	1.64 ± 0.13^{a}	1.23 ± 0.04^{b}	1.55 ± 0.08^{a}
C14:0	0.38 ± 0.17	0.39 ± 0.10	0.42 ± 0.11
C15:1	2.65 ± 0.20	2.53 ± 0.22	2.70 ± 0.41
C16:1	0.67 ± 0.11	0.61 ± 0.11	0.63 ± 0.11
C17:0	0.12 ± 0.05	0.13 ± 0.07	0.11 ± 0.04
C18:0	5.05 ± 0.35	5.05 ± 0.65	4.09 ± 2.06
C18:1n9c	8.73 ± 0.69	8.54 ± 0.64	8.87 ± 1.06
C18:1n9t	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.01
C18:2n6c	4.93 ± 0.58	4.95 ± 0.18	4.90 ± 0.63
C18:3n3	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
C18:3n6	0.02 ± 0.02	0.03 ± 0.03	0.03 ± 0.02
C20:0	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
C20:1	0.26 ± 0.06	0.25 ± 0.08	0.27 ± 0.10
C20:2	0.13 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
C20:3n6	0.15 ± 0.03	0.16 ± 0.03	0.16 ± 0.02
C20:4n6	1.33 ± 0.33	1.43 ± 0.19	1.28 ± 0.21
C24:0	0.11 ± 0.06	0.15 ± 0.04	0.14 ± 0.03

Data are presented as means ± SE. Values in the same row without a common superscript letter are different ($P < 0.05$). Control group, n = 12, 11, and 8; sow betaine group, n = 6, 7, and 6; sow-offspring betaine group, n = 7, 7, and 6; at 65-, 95-, and 125-day-old, respectively.

betaine addition in sow-offspring diets increased the BW of 125-day-old pigs, as well as the ADG and ADFI at 35–65 and 65–95 day-old. Therefore, the results indicate that betaine addition in sow-offspring diets can improve the growth of the piglets by enhancing the feed intake at an earlier age. Huang et al. (24) also found that dietary addition of 1.25 kg/t betaine for 42 days could increase the ADG of finishing pigs by 5.50%, but not the ADFI and F:G. In a sub-optimal methionine diet (15 g Met/kg CP), dietary betaine addition (0.80 kg/t) could improve the BW gain of broilers at 21-day-old, as well as the breast yield and F:G of broilers at 42-day-old (25). Furthermore, juvenile freshwater prawn *Macrobrachium rosenbergii* fed with 5 g/kg glycine betaine presented higher BW, food intake, and F:G (26). Therefore, these findings suggested that dietary betaine addition might increase the protein synthesis and decomposition through its methyl donor properties, thus increasing the growth and development of pigs.

Plasma hormone levels can reflect the physiological and health status of pigs. Growth hormone can regulate the growth and development of animals directly or indirectly by stimulating IGF-1 (27). Huang et al. (28) reported that dietary betaine addition could stimulate the release of GH and promote the growth of finishing pigs. However, the present study showed that betaine addition in sow and sow-offspring diets did not affect the plasma GH level, which might be related to the measured time of the plasma GH level. Kraetzl et al. (29) reported that the GH secretion is pulsatile, and its level is related to the measured time in pigs, that GH levels were higher during the night (20:00–08:00) than during the day (08:00–20:00). Therefore, the inconsistencies of these results might be related to the measuring time, as the present study only measured the GH level during the daytime. Gastrin and growth hormone-releasing factors could jointly promote GH secretion, regulate protein and sugar metabolism, and thus promote the growth of animals (30). As a type of gastrointestinal-derived hormone, PYY can inhibit feed intake, delay gastric emptying, increase the absorption of electrolytes in the ileum, and reduce the secretion of starch (31). The LEP is a protein hormone that can suppress the appetite and play a

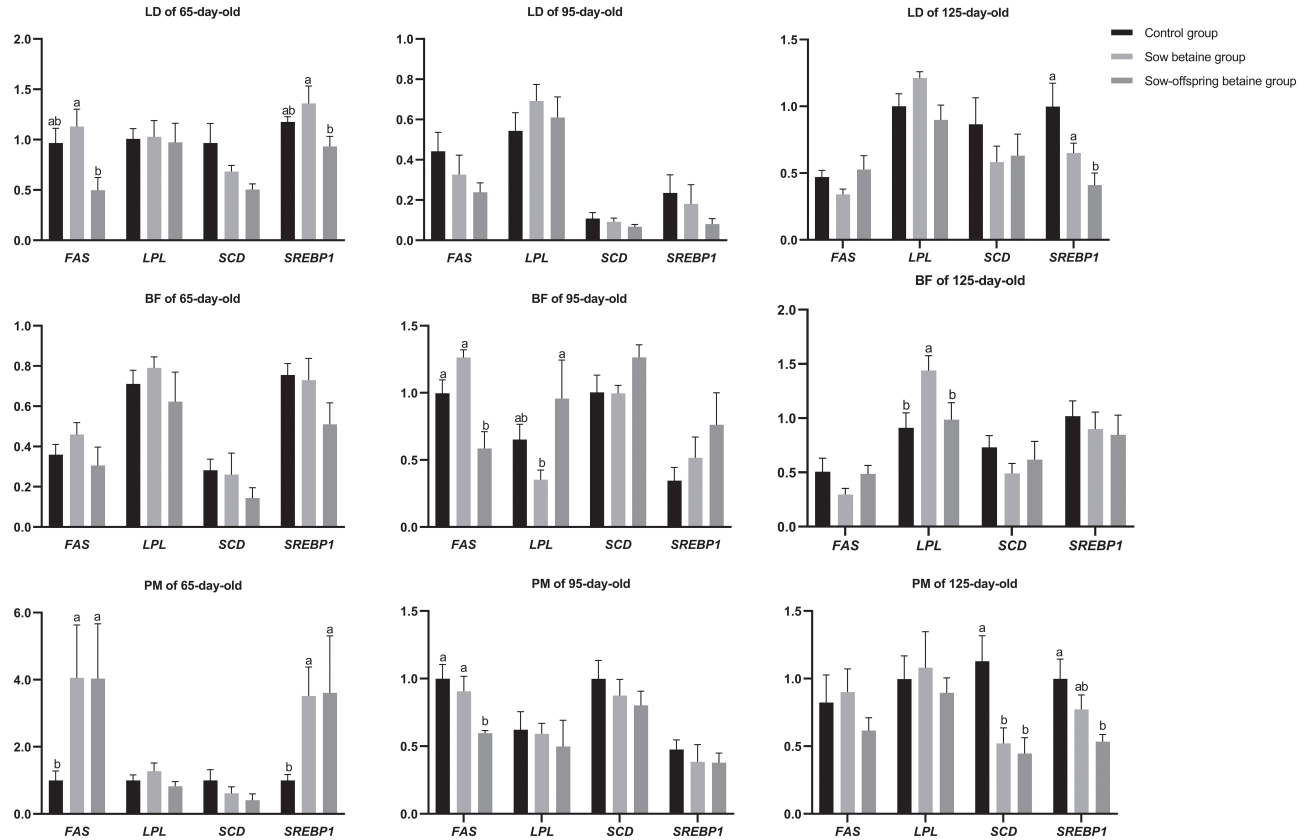

FIGURE 1 | Effect of dietary betaine addition on the expression levels of genes related to lipid metabolism in muscles of Bama mini-pigs. Results are presented as the means ± SE. Bars marked with different lowercase letters are significantly different at $P < 0.05$. Control group, $n = 12$, 11, and 8; sow betaine group, $n = 6$, 7, and 6; sow-offspring betaine group, $n = 7$, 7, and 6; at 65-, 95-, and 125-day-old, respectively. *FAS*, fatty acid synthase; *LPL*, lipoprotein lipase; *SCD*, stearoyl coenzyme A desaturase; *SREBP1*, sterol regulatory element binding protein 1. BF, *biceps femoris* muscle; LD, *longissimus dorsi* muscle; PM, *psoas major* muscle.

major role in regulating body fat, BW, and energy balance in animals (32). In the present study, betaine addition in sow diets decreased plasma PYY and LEP levels of 65-day-old pigs, while betaine addition in sow-offspring diets increased the Gas level of 95-day-old pigs, which are beneficial to promote the feed intake and growth of pigs.

The IMF mainly reflects muscle tenderness and flavor, and is a key factor to the formation of muscle taste. Existing studies showed that the IMF content between 2 and 3% is the best (33). In the present study, the IMF content was significantly decreased in the PM muscles of 125-day-old pigs but not in LD and BF muscles. However, Martins et al. (34) reported that 1.00 kg/t betaine addition in sow diets for 20 weeks could increase the IMF content in LD and BF muscles of Alentejano pigs. Huang et al. (35) found that 1.20 kg/t betaine addition in corn-soybean meal diets for 42 days increased the IMF content in LD muscle of finishing pigs. Therefore, these inconsistencies of IMF content might be related to the age of pigs. Moreover, the results suggested that dietary betaine mainly increased the IMF deposition in the earlier age of pigs, while could inhibit the IMF deposition in the later age.

The composition of fatty acid in the muscle plays an important role in the formation of pork flavor. High levels of unsaturated lipids may contribute to desirable flavors, like oxidized off-flavors (36). In the present study, dietary betaine addition tends to decrease the contents of PUFA, such as C18:1n9t, C18:3n6, C18:2n6c, and C20:4n6. These findings are in agreement with those of Madeira et al. (37), who reported that dietary betaine addition mainly decreases the percentage of monounsaturated fatty acid (including C16:1cis-9 and C18:1cis-11) proportions in the muscles of pigs. These findings might be resulted by the methyl donor properties of betaine. The principal physiologic role of betaine is as a methyl donor, which means transmethylation of betaine participates in the carnitine and phosphatidylcholine synthesis and fatty acid oxidation. Therefore, dietary betaine addition in sow diets may enhance the oxidation of PUFA and then reduce fatty acid and IMF contents (38, 39).

The *SREBP1* is a membrane-bound protein that can regulate the most enzymes involved in fatty acid synthesis, including acetyl-CoA carboxylase (*ACC*), *FAS*, and *SCD* (40, 41). The *LPL* is an important lipid regulatory enzyme involved in hydrolyzing triglycerides of plasma lipoproteins and supplying free fatty acids for storage in adipocytes or oxidation in other tissues (42). Moreover, *FAS* is a multifunctional protein, which main functional role is to catalyze the synthesis of palmitate from the

acetyl-CoA and malonyl-CoA, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), into long-chain saturated fatty acid (43). In the present study, betaine addition in sow-offspring diets down-regulated the level of the *FAS* in the LD muscle of 65-day-old pigs, as well as in the BF and PM muscle of 95-day-old pigs, while dietary betaine addition in sow and sow-offspring diets up-regulated the level of *FAS* in the PM muscle of 65-day-old pigs. Moreover, betaine addition in sow diets up-regulated the level of *LPL* in the BF muscle of 125-day-old pigs while down-regulated in the BF muscle of 95-day-old pigs. Therefore, these findings suggest that dietary betaine can regulate fatty acid synthesis and fat deposition by enhancing lipogenesis and reducing lipolysis. Meanwhile, dietary betaine addition in sow and sow-offspring diets up-regulated the expression level of *SREBP1* in the PM muscle of 65-day-old pigs, while dietary betaine addition in sow-offspring diets down-regulated the *SREBP1* level in the LD and PM muscles of 125-day-old pigs. Thus, the present study showed that dietary betaine could improve the synthesis of fatty acids in the earlier age, while inhibiting the process in the later age, which is also consistent with the changes of the IMF content in the muscles of pigs.

CONCLUSION

In summary, betaine addition in sow-offspring diets could improve the growth performance of piglets by increasing body weight, average daily gain, and average daily feed intake, decreasing the contents of several fatty acids, and inhibiting the fat deposition in muscles. Betaine addition in both sow and sow-offspring diets could influence the plasma hormones related to ingestion. Moreover, betaine addition in sow-offspring diets had more distinct effects than the addition in sow diets. These findings provide a reference for regulating lipid metabolism and promoting the growth of pigs in practical production by dietary betaine addition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The present study was conducted following Chinese guidelines for animal welfare and experimental protocols and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China (ISA-2018-071).

AUTHOR CONTRIBUTIONS

YC, MS, QZ, and MA performed sampling and nutrient measurements, analyzed data, interpreted the data, and drafted the manuscript. MS and QG conducted animal feeding and sampling. XK contributed to experimental concepts and design, provided scientific direction, and together with MA finalized the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31772613), Special Funds for Construction of Innovative Provinces in Hunan Province (2019RS3022), and Production and Research Talent Support Project of the CAS Wang Kuancheng Initiative Talent Program.

ACKNOWLEDGMENTS

The authors thank the staff and postgraduate students from the Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process for collecting samples and technicians from the CAS Key Laboratory of Agro-ecological Processes in Subtropical Region for providing technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.779171/full#supplementary-material

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