ADIPOSE TISSUE IN OBESITY AND METABOLIC DISEASE

EDITED BY: Simon Timothy Bond, Bhagirath Chaurasia, Prashant Rajbhandari, Ryan Ceddia and Sahar Keshvari

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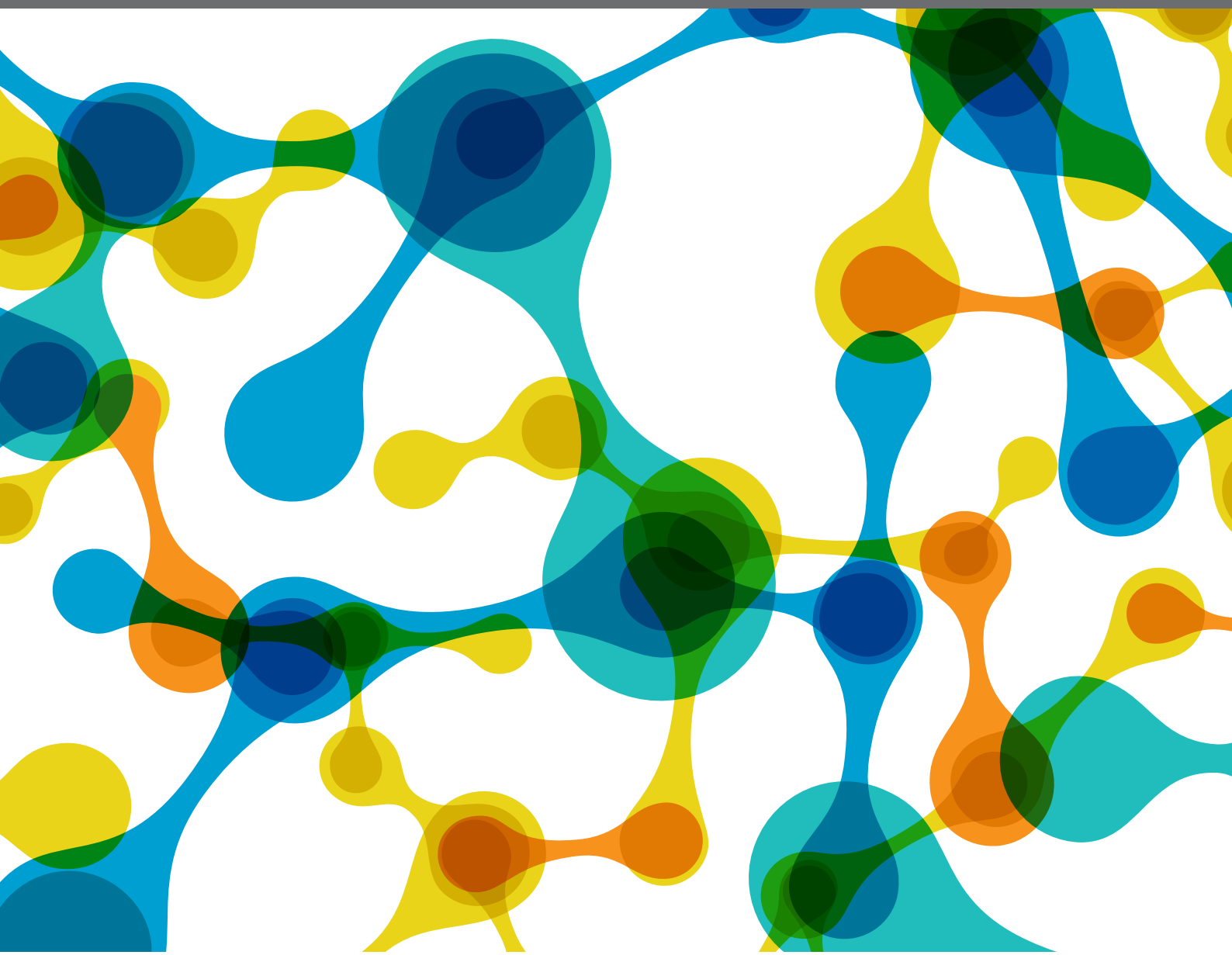

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ADIPOSE TISSUE IN OBESITY AND METABOLIC DISEASE

Topic Editors:
Simon Timothy Bond, Baker Heart and Diabetes Institute, Australia
Bhagirath Chaurasia, The University of Iowa, United States
Prashant Rajbhandari, Icahn School of Medicine at Mount Sinai, United States
Ryan Ceddia, Vanderbilt University Medical Center, United States
Sahar Keshvari, The University of Queensland, Australia

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EDITORIAL
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Editorial: Adipose Tissue in Obesity and Metabolic Disease

Sahar Keshvari[1], *Ryan P. Ceddia*[2], *Prashant Rajbhandari*[3], *Bhagirath Chaurasia*[4]* and *Simon T. Bond*[5,6,7]*

[1]Translational Research Institute, Mater Research Institute-The University of Queensland, Brisbane, QLD, Australia, [2]Division of Cardiovascular Medicine, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, United States, [3]Diabetes, Obesity, and Metabolism Institute, Icahn School of Medicine Mount Sinai, New York, NY, United States, [4]Division of Endocrinology, Department of Internal Medicine, Carver College of Medicine, Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, IA, United States, [5]Baker Heart and Diabetes Institute, Melbourne, VIC, Australia, [6]Baker Department of Cardiometabolic Health, The University of Melbourne, Melbourne, VIC, Australia, [7]Central Clinical School, Monash University, Melbourne, VIC, Australia

Keywords: adipose, adipose tissue, lipids, brown fat, obesity, metabolism

Editorial on the Research Topic

Adipose Tissue in Obesity and Metabolic Disease

Adipose tissue is a highly adaptive and critically important metabolic tissue, with intrinsic roles in both health and disease. It is now well established that adipose tissue plays a significant role in a diverse range of physiological processes, including metabolic homeostasis and disease progression. Obesity, a chronic condition defined by excessive adipose tissue expansion, has now become one of the greatest medical challenges of the modern world. Consequently, understanding the mechanisms that underpin adipose tissue in obesity and disease progression is becoming increasingly important, such that novel targets for the treatment of obesity and its complications may be identified. The aim of this Research Topic is to provide an overview of the important physiological roles that adipose tissue plays in both health and disease, by summarising current knowledge and providing novel insights into adipose tissue biology.

Excessive adipose tissue expansion is associated with declines in health and quality of life, however, the type of expansion (hyperplasia or hypertrophy) and the anatomical location of the adipose depot, are now known to be key factors that affect metabolic health. This is nicely summarised in the review by Nunn et al. where they describe the functional differences that occur in hypertrophic adipocytes and how this can contribute to metabolic complications. The authors detail how initiating adipogenesis (hyperplasia) as opposed to hypertrophy is metabolically beneficial and could be targeted therapeutically to treat metabolic disease. The review then evaluates known adipogenic regulators that could potentially be manipulated to induce adipogenesis. The concept of adipose tissue expansion is extended upon in a detailed review by Hilgendorf, which analyses the current literature regarding adipocyte primary cilia and their role in adipose tissue expansion and metabolic disease. The primary cilium is a cellular protrusion found on most mammalian cell types, including adipocytes, where it can regulate pro- and anti-adipogenic signalling pathways. Hilgendorf concludes by postulating that adipocyte primary cilium may function as a signalling hub through ciliary remodelling, enabling the primary cilium to sense and respond to extracellular signals, which can initiate adipogenesis. Garritson and Boudina further elucidate on mechanisms of adipose tissue expansion in a review that describes the effect of exercise on adipose tissue plasticity. It is well known that lifestyle interventions such as diet and exercise can reduce the risk for developing cardiometabolic disorders, but many of the underlying mechanisms remain unknown. Garritson and Boudina summarise the positive metabolic benefits of exercise on adipose tissue and highlight important questions that remain to be addressed.

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Edited and reviewed by:
Nada A. Abumrad,
Washington University in St. Louis, United States

***Correspondence:**
Bhagirath Chaurasia
bhagirath-chaurasia@uiowa.edu
Simon T. Bond
simon.bond@baker.edu.au

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There are three distinct forms of adipocytes; white (the body's main depot for storing energy), brown (accrue and burn lipid for energy) and beige (ability to switch between white and brown adipocyte roles). Since the discovery of brown fat in both humans and rodents, there has been significant advances in our understanding of its role in metabolic homeostasis, however, there are still existing knowledge gaps regarding brown fat biology. Although previous research has focused on glucose uptake into brown fat, Wade et al. provide a detailed update on the previously underappreciated role of lipid uptake into activated brown fat. Here, the authors highlight the importance of peripheral lipid storage for non-shivering thermogenesis and present an update on known mechanisms of lipid transport and uptake into brown fat. Researchers have long been looking for ways to artificially stimulate brown fat, such as the identification of novel browning mechanisms, to harness the positive metabolic effects of activated brown fat. Wang et al. original research article demonstrates a previously unknown CLK1-THRAP3-PPARγ axis that plays a role in adipose tissue browning and insulin sensitivity. The authors observe that CLK1 KO mice are resistant to high fat diet with preserved glucose tolerance and insulin sensitivity. They further demonstrate that CLK1 phosphorylates THRAP3 which promotes docking of PPARγ to inhibit PPARγ activity. Moreover, the original research article by Xiong et al. examines the role of the transcription factor E2F1 on mouse white adipose tissue browning and autophagy. In this article the authors demonstrate that global deletion of E2F1 leads to smaller adipocyte cell size and increased mitochondrial content which are associated with reduced expression of genes and proteins related to autophagy. Although activation of brown fat has been shown to be beneficial to metabolic health, the ability to therapeutically target brown adipose tissue has not yet translated to the clinic. Potentially the activation of brown fat in the setting of obesity provides many therapeutic challenges, including a reduction of brown fat in obesity and the difficulty in specifically targeting brown adipose tissue. Bond et al. review on adipose extracellular vesicles summarises the literature regarding the role of adipocyte extracellular vesicles in health and disease, where the authors postulate that extracellular vesicles secreted from activated brown fat may provide a new therapeutic avenue to mimic the metabolic benefits of brown fat activation.

Declines in insulin sensitivity are strongly correlated with obesity and dysfunctional adipose tissue. Original research by Van Meijel et al. investigates proteomic profile alterations in the abdominal subcutaneous adipose tissue from overweight/obese and insulin resistant male subjects caused by mild intermittent hypoxia. Using untargeted liquid chromatography-mass spectrometry the authors identify 123 proteins that are differentially expressed due to mild intermittent hypoxia exposure and find a correlation between adipose tissue insulin sensitivity and changes in TMOD3 expression. Approved treatments to improve insulin sensitivity include the thiazolidinedione class of drugs, which have had mixed success largely due to off target effects, where many of the beneficial and detrimental mechanism remain poorly understood. Original research by Palavicini et al. contributes additional knowledge on how the insulin-sensitiser pioglitazone exerts its cardiometabolic benefits in the setting of type 2 diabetes. Using shotgun lipidomics to measure changes in lipid species from human subcutaneous abdominal adipose tissue and vastus lateralis muscle from obese patients with type 2 diabetes following a chronic 6-month treatment with pioglitazone, the authors determine that the majority of the lipid composition changes occur in the glycerophospholipid pool. Glycerophospholipids enriched for the inflammatory pathway modulator arachidonic acid and its precursor linoleic acid were reduced by pioglitazone highlighting the importance of adipocyte membrane function in immunometabolic health. Adipose lipid metabolism, in particular ether lipids in obesity, is discussed further in a review by Schooneveldt et al., in which they delineate the functional and protective roles of ether lipids in the setting of obesity. The authors discuss how ether lipids have been linked to lipid droplet formation, regulating thermogenesis and mediating browning of white adipose tissue, as well as the therapeutic potential of ether lipid supplementation to alleviate obesity and its associated complications.

The overarching theme of this Research Topic is to better understand adipose tissue biology to uncover new therapeutic targets to treat obesity and metabolic disease. The potential of synthetic oligonucleotide technologies, as a means to target specific genes or proteins, to treat metabolic disease is explored in a review by Keating et al. Here the authors evaluate oligonucleotide-based therapies in the setting of obesity and fatty liver disease, as well as discussing the current limitations and challenges of oligonucleotide-based technologies in pre-clinical studies and their use as therapeutics.

In conclusion, this Research Topic on adipose tissue incorporates a Research Topic of reviews and novel data summarising our current understanding of adipose tissue biology and its role in maintaining metabolic homeostasis. Elucidating the physiological mechanisms of adipose tissue function, in both health and disease, could help to uncover novel therapeutic targets to treat obesity and metabolic disease.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Inhibition of a Novel CLK1-THRAP3-PPARγ Axis Improves Insulin Sensitivity

Zhenguo Wang[1,2†], Xiaojing Gao[2,3†], Qingrun Li[2], Hongwen Zhu[2], Xiangjie Zhao[1], Minerva Garcia-Barrio[1], Jifeng Zhang[1], Yanhong Guo[1], Y. Eugene Chen[1], Rong Zeng[2,4], Jia-Rui Wu[2,4*] and Lin Chang[1*]*

[1] Department of Internal Medicine, Cardiovascular Center, University of Michigan Medical Center, Ann Arbor, MI, United States, [2] CAS Key Laboratory of Systems Biology, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Hangzhou, China, [3] Key Laboratory of Systems Biology, CAS Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China, [4] School of Life Sciences and Technology, Shanghai Tech University, Shanghai, China

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Edited by:
Bhagirath Chaurasia,
The University of Iowa, United States

Reviewed by:
Henver Simionato Brunetta,
State University of Campinas, Brazil
Carmen De Miguel,
University of Alabama at Birmingham, United States

***Correspondence:**
Rong Zeng
zr@sibcb.ac.cn
Jia-Rui Wu
wujr@sibs.ac.cn
Lin Chang
lincha@umich.edu

[†] These authors have contributed equally to this work

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Increasing energy expenditure by promoting "browning" in adipose tissues is a promising strategy to prevent obesity and associated diabetes. To uncover potential targets of cold exposure, which induces energy expenditure, we performed phosphoproteomics profiling in brown adipose tissue of mice housed in mild cold environment at 16°C. We identified CDC2-like kinase 1 (CLK1) as one of the kinases that were significantly downregulated by mild cold exposure. In addition, genetic knockout of CLK1 or chemical inhibition in mice ameliorated diet-induced obesity and insulin resistance at 22°C. Through proteomics, we uncovered thyroid hormone receptor-associated protein 3 (THRAP3) as an interacting partner of CLK1, further confirmed by co-immunoprecipitation assays. We further demonstrated that CLK1 phosphorylates THRAP3 at Ser243, which is required for its regulatory interaction with phosphorylated peroxisome proliferator-activated receptor gamma (PPARγ), resulting in impaired adipose tissue browning and insulin sensitivity. These data suggest that CLK1 plays a critical role in controlling energy expenditure through the CLK1-THRAP3-PPARγ axis.

Keywords: insulin sensitivity, browning, CLK1, THRAP3, PPARγ

INTRODUCTION

Obesity features a massive expansion of white adipose tissue (WAT) in the visceral and subcutaneous regions due to excess energy storage in adipocytes (Choe et al., 2016). Obese people are at much higher risk for diabetes and cardiovascular complications (Akil and Ahmad, 2011). Therefore, preserving homeostatic energy storage within healthy levels in adipose tissues could be a major strategy to prevent obesity and related diabetes and cardiovascular complications (Sun et al., 2011). The finding that functional brown adipose tissue (BAT) exists in adult humans (Nedergaard et al., 2007, 2010) and its characteristics of high energy expenditure (Fruhbeck et al., 2009; Cypess and Kahn, 2010; Haas et al., 2012; Heeren and Munzberg, 2013) underlie BAT's emergence as a potential intrinsic target for prevention of obesity, diabetes and related cardiovascular diseases (CVDs). It is well-known that cold environmental temperature is the most

efficient strategy to activate thermogenesis and energy expenditure in BAT (Nedergaard et al., 2007), concurrent with a complex physiological response marked by the increase in food intake, oxygen consumption and heat generation (Robidoux et al., 2004). WAT undergoes a "browning" process in response to cold temperature (Fisher et al., 2012; Ohno et al., 2012; Lin et al., 2017), suggesting that cold stimulus mobilizes energy metabolism in both WAT and BAT. Stimulating "browning" in WAT could improve metabolic health by reducing the intrinsic adverse effects of WAT, and conferring beneficial effects on hyperlipidaemia and insulin resistance (Bartelt and Heeren, 2014). Due to the small amount of active BAT in adult humans, stimulating WAT "browning" is an attractive alternative strategy to promote metabolic health through increasing energy expenditure in the "browning" fat. Although environmental cold exposure strongly stimulates release of norepinephrine to acutely activate thermogenesis, environmental temperature is an important determinant of cardiac sympathetic and parasympathetic outflow, which in turn has a major impact on the cardiovascular system (Silvani et al., 2016). There is a significant negative correlation between the environmental temperature and the blood pressure and heart rate in older patients (Postolache et al., 1993). Consistently, seasonal morbidity and mortality due to CVDs is significantly increased in both the northern and southern hemispheres during the winter rather than in the summer (Manou-Stathopoulou et al., 2015). Therefore, it would be unrealistic to induce BAT activation by cold stimulation in humans. Some regulators of transcription such as PR/SET Domain 16 (PRDM16, a transcriptional coregulator that controls the development of brown adipocytes in BAT), and peroxisome proliferator-activated receptor gamma (PPARγ), hormones such as irisin and fibroblast growth factor 21 (FGF21) and chemical compounds such as thiazolidinediones (TZDs) were reported to induce WAT browning, increase energy expenditure, and protect mice from diet-induced obesity (Seale et al., 2011; Boström et al., 2012; Fisher et al., 2012; Ohno et al., 2012; Cohen et al., 2014). To date, none of those is applied clinically to induce WAT browning and prevent obesity and associated diseases.

Phosphorylation and dephosphorylation of proteins (including enzymes and receptors) is one of the crucial mechanisms to promote energy expenditure in response to cold stimuli (Ardito et al., 2017). The balanced action of protein kinases (for phosphorylation) and phosphoprotein phosphatases (for dephosphorylation) determines the overall protein phosphorylation state. Due to the importance of protein phosphorylation in biological process, tremendous efforts have been made to identify the various functions of protein kinase signal transduction pathways (Manning et al., 2002; Roskoski, 2015). Since cold exposure is a robust environmental stress, it will undoubtedly induce a series of phosphorylation and dephosphorylation events in thermogenic adipose tissues. Our goal is to explore novel protein kinase signaling pathways able to mimic moderate cold stimuli in adipose tissues with the objective of identifying potential targets for pharmacologic intervention. Therefore, we performed phosphoproteomics analysis in BAT from mice housed at 16°C. We identified that the kinase activity of CDC2-like kinase 1 (CLK1) in BAT is significantly reduced upon chronic mild cold exposure. It is well-established that CLK1, a dual-specificity tyrosine and serine/threonine protein kinase, plays critical roles in alternative splicing through phosphorylation of SR proteins. However, CLK1 function, regulation, and targets in a metabolic context are relatively unknown. In this study, we investigated the roles of CLK1 in metabolism under the hypothesis that inhibition of CLK1 may promote adipose tissue browning. We show here that inhibition of CLK1 improved insulin sensitivity by preventing phosphorylation of THRAP3 (thyroid hormone receptor-associated protein 3, a transcriptional cofactor) at Ser243. THRAP3 is an RNA-binding protein which regulates circadian clock-dependent alternative splicing of pre-mRNAs (Lande-Diner et al., 2013; Marcheva et al., 2020), androgen-independent prostate cancer cell growth (Ino et al., 2016), and adipocyte differentiation (Katano-Toki et al., 2013). It was reported that THRAP3 can directly interact with PPARγ when the latter is phosphorylated on Ser273 in adipocytes (Choi et al., 2014). Our data uncovered that phosphorylation on Ser243 of THRAP3 by CLK1 increased preferential docking of THRAP3 to PPARγ phosphorylated on Ser273 in adipocytes, which inhibits PPAR γ activity (Choi et al., 2014). Inhibition of CLK1 reduced THRAP3 phosphorylation and herein prevented docking to PPARγ, resulting in decreased PPARγ phosphorylation on Ser273, thus enhancing PPARγ activity to promote adipocyte browning, insulin signaling and glucose metabolism. These data strongly suggest that CLK1–THRAP3–PPARγ complexes could be a potent therapeutic target for obesity and associated type 2 diabetes.

MATERIALS AND METHODS

Detailed methods are described in the supplemental document.

Animal Study

C57BL/6J (stock number 000664) or diet-induced obese (DIO) male mice (stock number 380050) were purchased from The Jackson Laboratory. We generated *Clk1* knockout mice (CLK1 KO) in the C57BL/6J background using CRISPR/Cas9 technology, as described in detail in the supplemental document. All mice used for the studies were male and were housed in ventilated cages at either 22 or 16°C with 12h/12h light/dark cycle, with the dark phase starting at 6 pm. Mice had *ad libitum* access to standard chow diet (CD, D12450J, Research Diets, 20% proteins, 10% fat, 70% carbohydrate) or high-fat diet (HFD, D12492, Research Diets, 20% proteins, 60% fat, 20% carbohydrate) and water. The animal numbers used for each experiment are indicated in the corresponding figure legends. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Energy Expenditure Assay in Mice

Oxygen consumption (VO_2), carbon dioxide production (VCO_2), spontaneous motor activity and food intake were measured using the Comprehensive Laboratory Monitoring

System (CLAMS, Columbus Instruments), an integrated open-circuit calorimeter equipped with an optical beam activity monitoring device as we described before (Xiong et al., 2017). Total energy expenditure was calculated based on the values of VO_2, VCO_2, and the protein breakdown (Riachi et al., 2004).

Glucose and Insulin Tolerance Assay

For the glucose tolerance test (GTT), D-glucose (2 mg/g of body weight) was orally gavaged to 5-h fasted mice and glucose levels were monitored at 0, 30, 60, and 120 min subsequently using a Glucometer Elite (Bayer, Japan). For the Insulin-tolerance test (ITT), the experiments were performed on mice following a 5-h fast. Animals were injected intraperitoneally with 0.5 U/kg body weight of human insulin (Humulin, Eli Lilly Co., Indianapolis, IN, United States). Tail-blood samples were taken at 0, 30, 60, and 90 min after injection for measurement of blood glucose levels.

Mass Spectrometry for Phosphoproteomics

Male C57BL/6J wild-type mice (8-week-old) were fed a standard chow diet and housed at 16 or 22°C for 8 weeks. The BAT was collected and lysed in SDT buffer (4%SDS, 0.1 M DTT, 100 mM Tris-HCl, pH7.6) and homogenized using Precellys24 Homogenizer (Bertin Technologies). Phosphorylated peptides were separated by EASY-nLC 1000 C18 liquid chromatography (Thermo Fisher Scientific) and analyzed by Orbitrap Fusion (Thermo Fisher Scientific). For phosphoproteome analysis, raw mass spectrometry data were processed using the MaxQuant software version 1.5.2.8 and peak lists were analyzed against the mouse Uniprot database.

Statistical Analysis

The data were evaluated with two-tailed, unpaired Student's *t*-test or compared by One-way ANOVA with Dunnett's multiple comparisons test or Two-way ANOVA with Sidak's multiple comparisons test and were expressed as mean ± SEM or SD. A value of $p < 0.05$ was considered statistically significant. Although presented in the same graph for convenience, the independent expression of each given gene across the different tissues listed in the X axis was analyzed through pair-wise comparison by two-tailed, unpaired Student's *t*-test.

RESULTS

Phosphoproteomics Profiling of BAT Identifies Downregulation of CLK Kinase Activity in Response to Mild Cold Stimulation

Cold-induced thermogenesis is associated with the coordinate mobilization of glucose, lipid, and protein metabolism, which involves activation/inactivation of the corresponding enzymes in the corresponding metabolic pathways in multiple organs. As shown in **Figure 1A**, the body weight gain in mice fed a standard chow diet was significantly blunted when housed at 16°C (mild-cold environment) in comparison to mice housed at 22°C (standard room temperature). To identify changes in the signaling pathways in adipose tissues of mice housed at 16°C, a multiplex TMT based quantitative phosphoproteome strategy was applied as depicted in **Supplementary Figure 1**. After exposure of 8-week-old mice to 16 or 22°C in environmental chambers for 8 weeks, the BAT in the interscapular region was collected and analyzed by LC-MS/MS. Next, the differentially phosphorylated sites identified in proteins from BAT were analyzed to determine the functional kinases responsible for the cold-induced phosphorylation profiles. We identified 9,306 phosphorylation sites on 3,120 phosphorylated proteins, of which 6,810 phosphorylation sites on 2,775 phosphorylated proteins could be accurately localized without missing values (class I sites, localization probability >0.75, score diff >5, **Supplementary Table 1**) (Rigbolt et al., 2011). To compare phosphorylation changes in BAT in response to mild-cold exposure, hierarchical clustering was applied based on 662 sites to show 343 up-regulated and 319 down-regulated phosphorylation sites (adjusted p value < 0.05 of *t*-test, fold change >1.5). Those sites were further analyzed to predict the corresponding upstream kinases using NetworKIN 3.0 (Horn et al., 2014) and explore the overrepresented kinases using a Hypergeometric test. We identified both activated kinases, including PKCα, PKCβ, PAK1, PKAα, AMPKα2, and inhibited kinases, including CLK and CaMKIIα, upon mild-cold exposure (**Figure 1B**). Of relevance, PKA and AMPK signaling were reported to be activated in BAT under cold stimulation (Mulligan et al., 2007; Harms and Seale, 2013), consistent with our finding here. However, although the CLK represent one of the strongest inhibited kinase family upon mild-cold exposure, the contribution of their signaling pathway to thermogenesis and metabolism remains unknown. There are four genes in the CLK family in human (*CLK1*, *CLK2*, *CLK3*, and *CLK4*). Because of their high sequence conservation, we could not identify *a priori* which member in the CLK family was inhibited in BAT upon mild-cold exposure. Western blotting showed that the expression levels of the CLK family are higher in adipose tissues than those in other metabolic organs such as liver, heart, brain and skeletal muscle. CLK1 was mainly expressed in adipose tissues. Interestingly, expression of CLK1 in BAT, a thermogenic tissue, is lower than that in subcutaneous (sWAT) and gonadal WAT (gWAT). While CLK2 was widely expressed in different organs with higher abundance in adipose tissues. CLK3 has been reported predominantly expressed in the testis (Nayler et al., 1997). Our results show that adipose tissues and heart express high level of CLK3 as well. Beside adipose tissues, CLK4 is also highly expressed in liver, brain and testis, in which CLK1 is barely expressed (**Supplementary Figure 2**). By searching the gene expression profile in the GTEx Portal,[1] we found that CLK1 was the most enriched CLK family member in both human subcutaneous and omental adipose tissue (**Figure 1C**). We further demonstrated that the mRNA levels of *Clk1* were significantly higher than those of other *Clk* family members in both BAT and WAT of mice (**Figures 1D–F**). These data suggest that CLK1 is the predominant kinase in the CLK family in adipose tissues and

[1] https://www.gtexportal.org/home/gene/CLK1

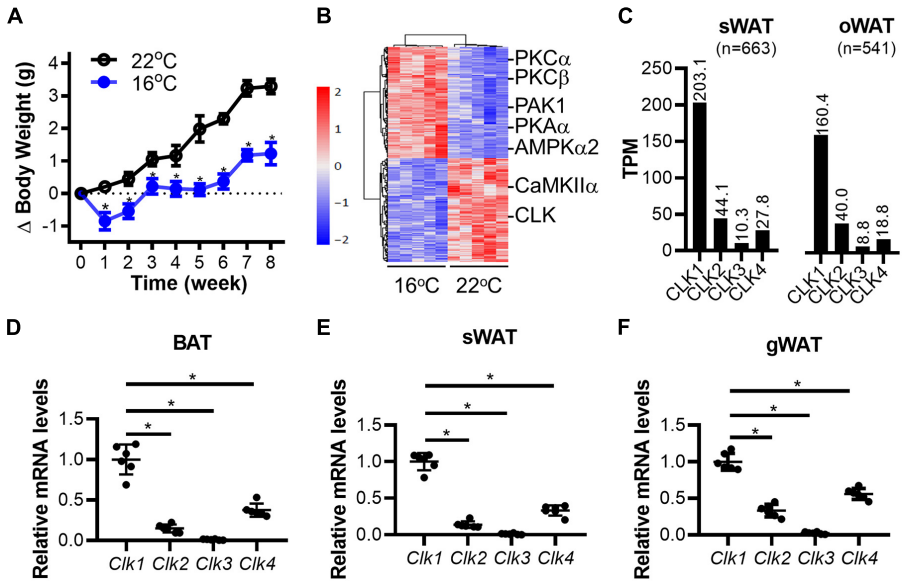

FIGURE 1 | Downregulation of CLK in brown adipose tissue in mice housed in a mild cold environment. **(A)** The body weight change of 8-week-old male mice fed a standard chow diet for 8 weeks in either 22 or 16°C environmental chambers. n = 6/group, data shown as mean ± SEM, $^*p < 0.05$ vs 22°C. (Two-way ANOVA with Sidak's multiple comparisons test). **(B)** At the end of the experiment described in panel **(A)**, the brown adipose tissue (BAT) in the interscapular region was collected and subjected to phosphoproteomics by LC-MS/MS. The hierarchical clustering and heatmap shows the sites differentially phosphorylated in response to mild-cold as determined in BAT. The color scale represents the relative abundance of sites. Further analysis identified CLK as one of the down-regulated family of kinases in interscapular BAT in mice housed at 16°C. n = 5/group. **(C)** Expression of the CLK family members in human subcutaneous (sWAT) and omental adipose tissues (oWAT). The data was adapted from the GTEx Portal (https://www.gtexportal.org/home/gene/CLK1). **(D–F)** The mRNA levels of the *Clk* family members (relative to 18S rRNA) in adipose tissues from interscapular (BAT), subcutaneous (sWAT) and gonadal (gWAT) regions of 8-week-old C57BL/6J mice on standard chow diet and housed at 22°C. The relative *Clk1* mRNA levels in adipose tissues were set as 1, n = 6/group, data shown as mean ± SD, $^*p < 0.05$ vs *Clk1*. One-way ANOVA with Sidak's multiple comparisons test.

might contribute to thermogenesis and metabolism in response to cold exposure.

CLK1 Is Highly Expressed in Brown-Like Adipose Tissues and Is Negatively Associated With Thermogenesis

To further validate the association of CLK1 expression in adipose tissues with obesity, C57BL/6J mice were housed at 22 or 16°C for 8 weeks and given a HFD. As shown in **Figure 2A**, the body weight gain was significantly reduced in mice housed at 16°C when compared to those housed at 22°C. Meanwhile, insulin and glucose tolerance were improved in the mice housed at 16°C (**Figures 2B,C**). Mild-cold exposure did not significantly alter uncoupling protein 1 (UCP1) abundance in BAT, but UCP1 was significantly increased in sWAT from obese mice. CLK1 protein abundance was comparable in BAT in obese mice housed at either 22 or 16°C. However, CLK1 was reduced in sWAT and gonadal WAT (gWAT) of mice housed at 16°C when compared to those at 22°C (**Figures 2D–F**), suggesting that CLK1 in obese sWAT and gWAT was negatively regulated in cold exposure. Even though CLK1 protein abundance was not significantly changed in BAT of obese mice, knockdown of *Clk1* in isolated mouse brown adipocytes increased mRNA levels of brown adipocyte markers such as *Ucp1*, *Cox8b*, *Elovl3*, and *Dio2* upon treatment with CL316,243, a β3-adrenergic receptor agonist (**Figure 2G**).

Next, we generated conventional *Clk1* knockout mice (CLK1 KO) (**Supplementary Figures 3A,B**) to investigate whether CLK1 contributes to metabolism *in vivo*. We further confirmed that *Clk1* in BAT and sWAT was negatively associated with expression of thermogenic genes at 22°C (**Supplementary Figures 3C,D**). The adipocyte browning markers were significantly increased in sWAT from CLK1 KO mice, and the mRNA abundance of genes associated with thermogenesis such as *Elovl3*, *Dio2*, and *Pgc1a* was increased in BAT from CLK1 KO mice when compared to those from littermate wild-type control mice. The body weight gain in CLK1 KO mice was significantly less than in littermate wild-type controls after HFD feeding for 8 weeks (**Figure 3A**). The oxygen consumption, carbon dioxide production and energy expenditure were significantly increased in the CLK1 KO mice after the 8 weeks' HFD feeding (**Figures 3B–D**). Since these metabolic indexes were comparable between the wild-type and CLK1 KO mice, either as absolute values per mouse or normalized per lean body mass (**Supplementary Figures 4A,B**), the increased metabolism in the CLK1 KO mice might be due to reduced fat mass. The histological analysis of adipose tissues indicated that *Clk1* knockout prevented BAT whitening and WAT hypertrophy in HFD, as evidenced by reduced lipid droplet sizes in both BAT and WAT (**Figure 3E**). Additionally, CLK1 KO mice showed improved glucose and insulin tolerance compared with wild-type control mice (**Figures 3F,G**). In agreement with the

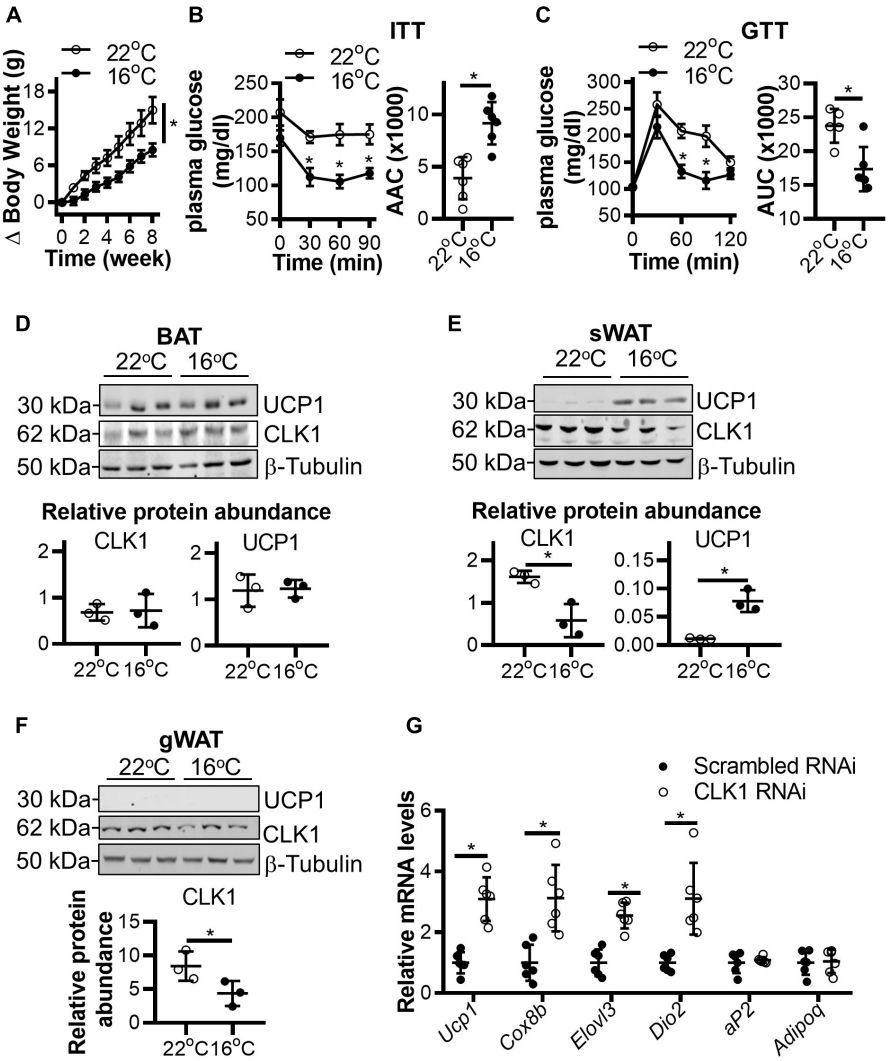

FIGURE 2 | CLK1 downregulation in adipose tissues is associated with insulin sensitivity in mice housed in a mild cold environment. **(A)** The body weight change of 8-week-old male mice being fed a high-fat diet (HFD) for 8 weeks while housed in either 22 or 16°C environmental chambers. n = 6/group, data shown as mean ± SEM, $^*p < 0.05$ vs 22°C. (Two-way ANOVA with Sidak's multiple comparisons test). **(B)** The insulin tolerance test (ITT) and **(C)** glucose tolerance test (GTT) in mice from panel **(A)** at the end of the 8-weeks of HFD feeding. Histograms show the corresponding areas above the curves (AAC) of ITT, and areas under the curves (AUC) of GTT. n = 6/group, data shown as mean ± SEM, $^*p < 0.05$ vs 22°C. (Two-way ANOVA with Sidak's multiple comparisons test). **(D–F)** The protein abundance of CLK1 and UCP1 in BAT **(D)**, sWAT **(E)**, and gWAT **(F)** in mice from panel **(A)** at the end point of the 8-week HFD feeding. The lower panels are the quantitative data of the corresponding Western blots shown in the upper panels. n = 3/group, data shown as mean ± SD, $^*p < 0.05$ vs 22°C. (Unpaired Student's t test). **(G)** The mRNA levels (relative to 18S rRNA) of browning markers in immortalized mouse brown adipocytes, originally isolated from mice on chow diet, and subjected to *Clk1* knockdown or siRNA control (10 nM) on day 5 of differentiation and treatment at differentiation day 7 with 10 mM CL316,243 for 24 h. n = 3, data shown as mean ± SD, $^*p < 0.05$ vs scrambled RNAi control. (Unpaired Student's t test).

lean phenotype in the CLK1 KO mice, the phosphorylation of hormone sensitive lipase (HSL) at Ser660 which promotes its lipolytic activity (Adebonojo et al., 1982), was significantly increased in all types of adipose tissue when compared to those in wild-type mice (**Figures 4A,B,D,E,G,H**). The phosphorylation of protein kinase B (Akt) at Ser473, which is considered as a surrogate of insulin activity (Krook et al., 1997), was increased in BAT and gWAT in the CLK1 KO mice as well (**Figures 4A,C,D,F,G,I**). These data suggest that *Clk1* knockout promotes thermogenesis and energy expenditure, resulting in a lean phenotype and underscoring the potential benefit of pharmacologic inhibition of CLK1 for the prevention of obesity and associated diabetes.

Identification of THRAP3 as a New Substrate of CLK1

We further applied a proteomics approach to uncover binding partners of CLK1. For this purpose, HA-tagged CLK1 was overexpressed in HEK293T cells and cell

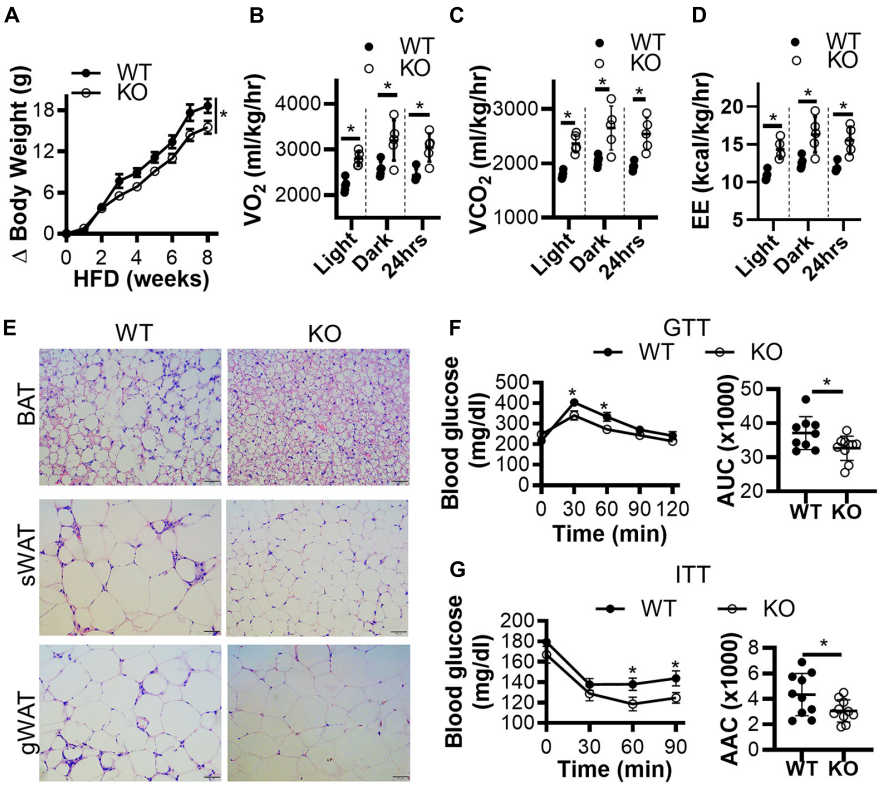

FIGURE 3 | Genetic knockout of CLK1 improves insulin sensitivity in mice. **(A)** The body weight gain of male CLK1 KO mice (KO) and wild-type littermate controls (WT) fed a high-fat diet (HFD) for 8 weeks, starting at 8 weeks of age and housed at regular room temperature at 22°C. n = 10 mice/group. Data shown as mean ± SEM. *p < 0.05 vs WT. (One-way ANOVA with Sidak's multiple comparisons test). **(B–D)** The whole-body energy expenditure of CLK1 KO mice in panel **(A)** were measured at the end of the 8 weeks of HFD feeding. The environmental temperature in the metabolic chambers was set at 22°C. The metabolic index was monitored for 24 h, and the results were averaged during the light-on period, and during the light-off period, respectively. n = 5 mice/group. Data shown as mean ± SD. *p < 0.05 vs WT. (Unpaired Student's t test). **(E)** H&E staining of BAT, sWAT, and gWAT at the end of the 8 weeks of HFD feeding in panel **(A)**, scale bar, 100 μm. **(F)** The glucose tolerance test (GTT) and **(G)** the insulin tolerance test (ITT); the histograms show the corresponding areas under the curves (AUC) for GTT, and areas above the curves (AAC) for ITT. n = 10 mice/group. Data shown as mean ± SEM. (One-way ANOVA with Sidak's multiple comparisons test).

lysates were immunoprecipitated with anti-HA monoclonal antibody-conjugated agarose beads. The resulting co-immunoprecipitants were then subjected to LC-MS/MS analysis. We identified 88 proteins that were efficiently pulled down by HA-tagged CLK1 (>1.5 fold than control empty vector), and THRAP3 was the most abundant protein among them (∼43 times higher than control, **Supplementary Table 2**). We further confirmed that CLK1 physically interacts with THRAP3 using co-immunoprecipitation (Co-IP) followed by western blot analysis (**Figure 5A**). As previously described (Vohhodina et al., 2017), THRAP3 has an SR-rich (serine/arginine) domain in the N-terminal region and a domain homologous to Bcl-2-associated transcription factor 1 (BCLAF1) in the C-terminus. To further determine the THRAP3 domain necessary for binding with CLK1, full-length and truncated versions of THRAP3 lacking the N-terminal, C-terminal or N- and C-terminal regions, respectively, were co-expressed with Flag-tagged CLK1 in HEK293T cells and subjected to Co-IP assays using anti-Flag monoclonal antibody-conjugated agarose beads followed by western blot analysis. The results indicated that the C-terminal domain of THRAP3 is required for the interaction with CLK1 (**Figure 5B**). When CLK1 and THRAP3 were co-expressed in HEK293T cells, the THRAP3 band shifted to a slightly heavier molecular weight by SDS-PAGE, while this mobility shift was abolished upon treatment with TG003, a potent CLK family inhibitor (Muraki et al., 2004), suggesting that CLK1 could phosphorylate THRAP3 (**Figure 5C**). The inhibitor also increased CLK1 mobility, indicative of autophosphorylation. Additionally, we also uncovered that phosphorylation on multiple sites in THRAP3, including S243, S253, and S379, were significantly reduced in BAT upon 16°C cold stimulation (**Supplementary Table 1**), which is consistent with the downregulation of the CLK1 kinase activity in response to mild cold stimulation. To further establish that CLK1 phosphorylates THRAP3, we co-expressed HA-tagged THRAP3 and Flag-tagged CLK1 in HEK293T cells. The immunoprecipitated THRAP3 was then analyzed for phosphorylation by mass spectrometry. As shown in **Figure 5D**, compared to control (empty vector), overexpression of CLK1 significantly increased THRAP3 phosphorylation at S243. Collectively, these data indicate that CLK1 binds to the C-terminal domain of THRAP3 and phosphorylates it at S243.

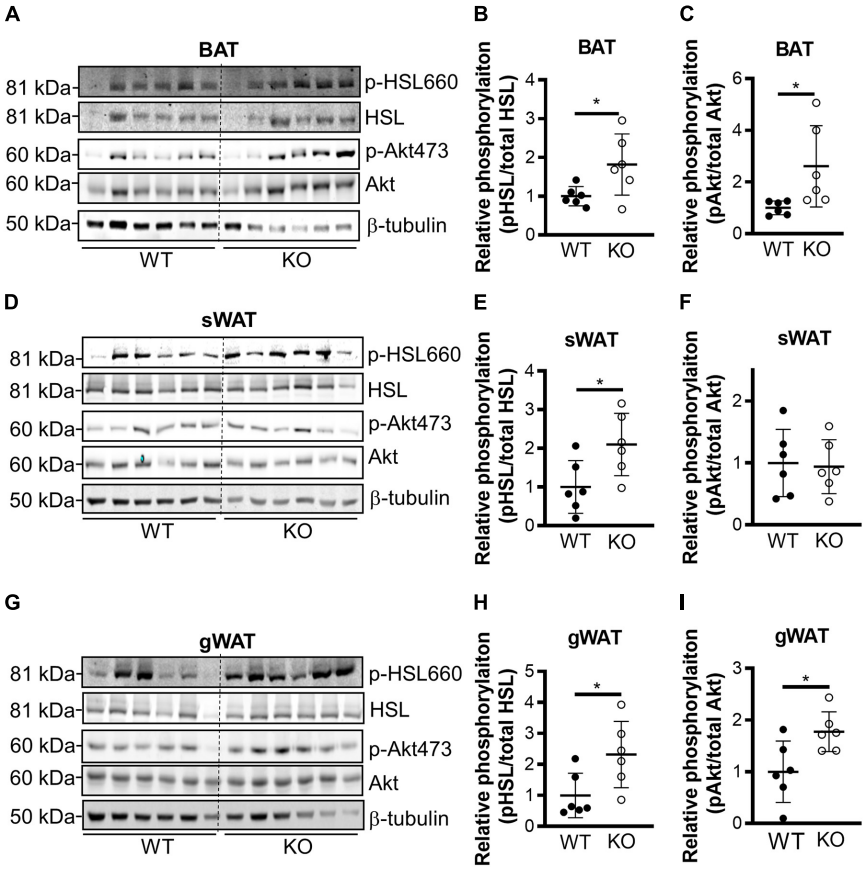

FIGURE 4 | Genetic knockout of CLK1 increases phosphorylation of HSL and Akt. Western blot analysis of the levels of phosphorylation of HSL on Ser660 and Akt on Ser473, with the corresponding densitometric quantification (as the ratio phospho/total protein) in the indicated adipose tissues from WT and KO mice at the end of 8 weeks' HFD feeding as in **Figure 3A**. BAT **(A–C)**, sWAT **(D–F)**, and gWAT **(G–I)**. $n =$ 6 mice/group. Data shown as mean ± SD, $^*p < 0.05$. (Unpaired Student's *t* test). The mean of WT was set as 1 for comparison.

CLK1-Phosphorylation on S243 in THRAP3 Promotes Its PPARγ-Binding Activity

It was reported that THRAP3 can directly interact with PPARγ when the latter is phosphorylated at S273 (Choi et al., 2014). We confirmed that wild-type THRAP3 interacts with PPARγ, and further demonstrated that THRAP3 S243D (mimicking constitutive phosphorylation) increases binding to PPARγ, while THRAP3 S243A (phosphorylation-deficient mutant) fails to increase the pulldown (**Figure 5E**). Furthermore, mutation of PPARγ S273 (S273A) significantly blocked PPARγ and THRAP3 interaction (**Figure 5F**). These data indicate that phosphorylation of S243 in THRAP3 and S273 in PPARγ are critical for their interaction. Indeed, the phosphorylation of S273 in PPARγ is reduced in all types of adipose tissues in the CLK1 KO mice (**Figures 6A–F**). Consistently, overexpression of CLK1 increased, while knockdown of CLK1 reduced phosphorylation of PPARγ at S273 in brown adipocytes (**Figure 6G**). These data imply that reduced phosphorylation of THRAP3 at 243 and of PPARγ at 273 might contribute to improved insulin sensitivity in the CLK1 KO mice.

Chemical Inhibition of CLK Improves Insulin Sensitivity in Obese Mice

To evaluate the potential translational application of these findings, we investigated the effects of CLK1 inhibition on whole body metabolism and insulin sensitivity. Wild-type C57BL/6J mice were fed an HFD for 24 weeks to induce obesity and diabetes while housed at 22°C. At 24 weeks, the mice were treated with TG003 (50 mg/kg). We found that after subcutaneous injection of a single dose of TG003 there was significantly increased oxygen consumption and carbon dioxide production without affecting the food intake and total activity (**Figures 7A–D** and **Supplementary Figures 5A,B**). Next, we continued treatment of the same obese mice with TG003 for an additional 4 weeks while keeping them on HFD. As shown in **Figure 7E**, the body weight of obese mice significantly declined during the TG003 treatment. Consistently, the sizes of lipid droplets in adipocytes in both BAT and gWAT were significantly smaller in the TG003-treated mice compared to the DMSO-treated mice (**Figure 7F**). Glucose and insulin tolerance tests showed that TG003 treatment of the obese mice significantly improved insulin sensitivity (**Figures 7G,H**), in association with the body weight decrease. Additionally, TG003

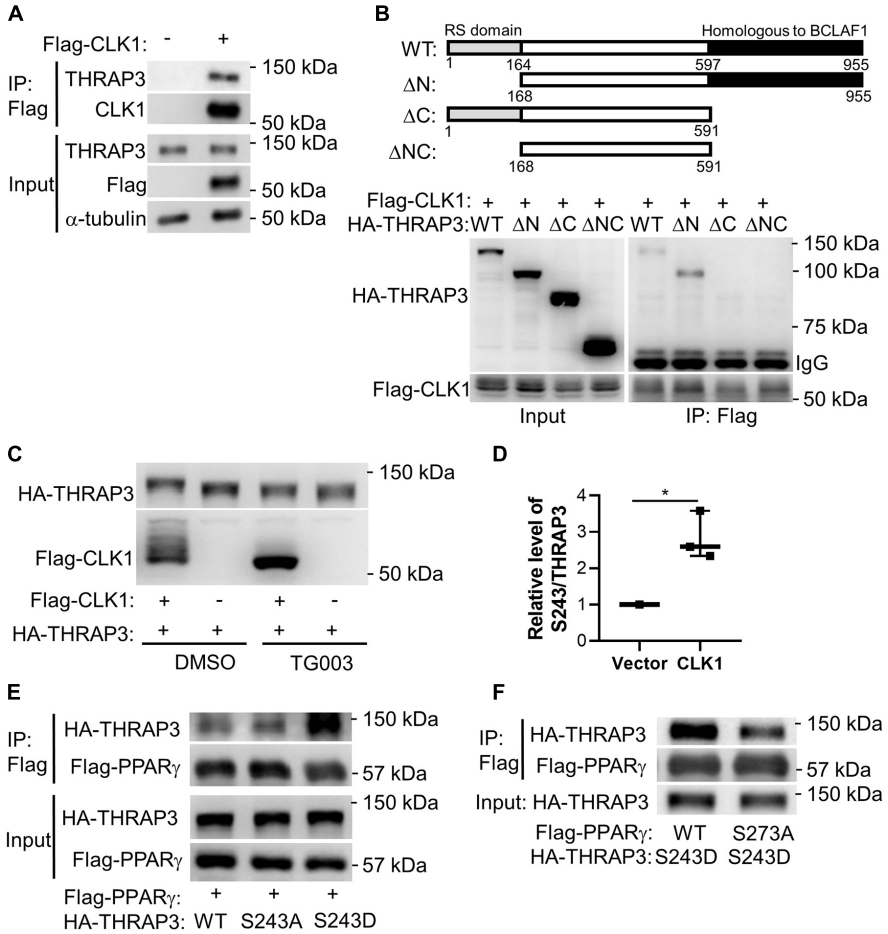

FIGURE 5 | Functional complexes of CLK1-THRAP3-PPARγ. **(A)** Flag-tagged CLK1 transfected into HEK293T cells was pulled down with an anti-Flag antibody after 48 h. THRAP3 co-immunoprecipitation (Co-IP) was verified by western blot. **(B)** Schematic illustration of full length and truncated mutants of THRAP3 **(top)**. Flag-tagged CLK1 and HA-tagged THRAP3 full length (WT) or truncated mutants (ΔN, ΔC, or ΔNC) were co-transfected into HEK293T cells and subjected to subsequent immunoprecipitation using anti-Flag antibody. Anti-HA antibody was used to detect THRAP3 bound to CLK1 in the Western blots **(bottom)**. **(C)** Co-transfection of Flag-tagged CLK1 and HA-tagged full length THRAP3 into HEK293T cells followed by treatment with the CLK1 inhibitor TG003 (10 μM) or vehicle control DMSO for 6 h. The cell lysates were collected and subjected to Western blotting. The mobility shift in both THRAP3 and CLK1 in the presence of the CLK1 inhibitor is indicative of increased THRAP3 phosphorylation and CLK1 autophosphorylation in the presence of overexpressed CLK1 without the inhibitor (heavier bands, lane 1). **(D)** Co-transfection of HA-tagged THRAP3 and Flag-tagged CLK1, or empty vector as control, into HEK293T cells for 24 h was followed by immunoprecipitation with anti-HA monoclonal antibody-conjugated agarose beads. The relative phosphorylation of THRAP3 at Ser243 in the immunoprecipitants was measured by Mass Spectrometry and was normalized relative to the vector control HEK293T cells set as 1. $n = 5$. $^*p < 0.05$ vs Vector. (Unpaired Student's t test). **(E)** HA-tagged phosphorylation-deficient (S243A) or phosphorylation-mimic (S243D) forms of THRAP3 were co-transfected with Flag-PPARγ into HEK293T cells. Following Flag-mediated Co-IP, their interactions were analyzed by western blotting. **(F)** HA-THRAP3 S243D mutants were co-expressed with the WT or S273A forms of Flag-PPARγ in HEK293T cells. The interactions were analyzed by Co-IP assays using anti-HA antibody followed by western blotting with anti-Flag antibody.

treatment significantly increased the expression of the browning markers *Ucp1*, *Cidea*, *Cox8b*, and *Elovl3* in sWAT and gWAT, and of *Cited* and *Elovl3* in BAT (**Figures 8A–C**). These data indicate that CLK1 inhibition induced WAT browning and could improve insulin sensitivity while reducing obesity in a WT obese mice model.

DISCUSSION

Cold exposure is the most potent stimulus to activate BAT or induce "browning" of WAT. The thermogenic function of BAT or the "browning" of fat is tightly controlled by norepinephrine released from the sympathetic nervous system (Cannon and Nedergaard, 2004). Although environmental cold exposure strongly stimulates the release of norepinephrine to acutely activate thermogenesis, environmental temperature is an important determinant of cardiac sympathetic and parasympathetic outflow, which in turn has a major impact on the cardiovascular system (Silvani et al., 2016). The heart rate and blood pressure of mice were reduced when the housing temperature was raised from 20 to 30°C, and increased when lowered from 30 to 20°C (Swoap et al., 2008). During mild/moderate cold exposure (12–17°C), the body core

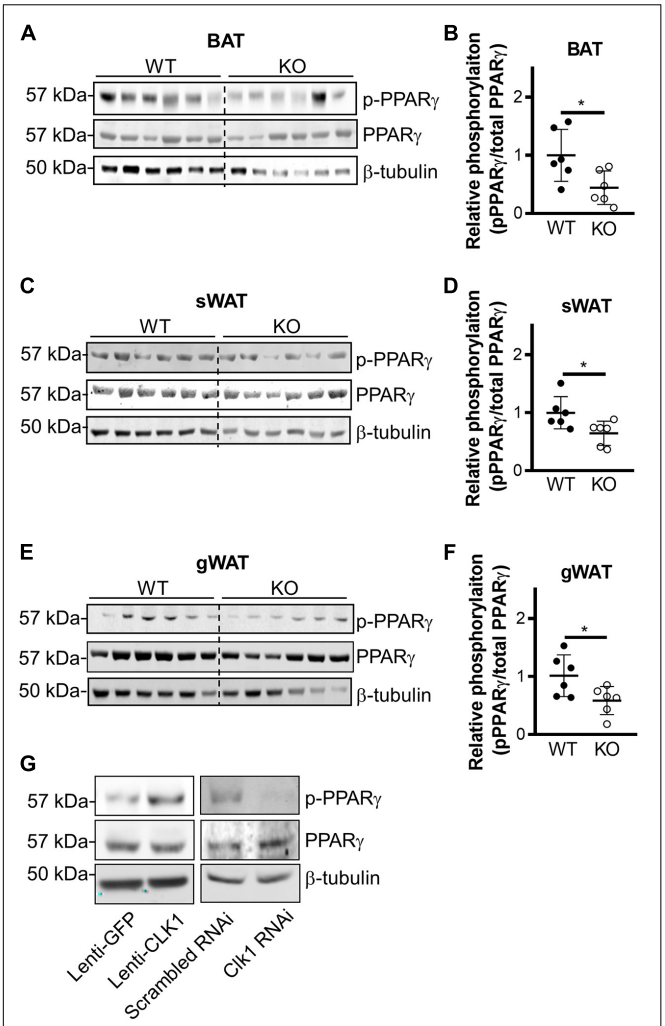

FIGURE 6 | CLK1 deficiency reduces phosphorylation of PPARγ on Ser273. **(A–F)** Male CLK1 KO (KO) or wildtype (WT) mice were fed a HFD for 8 weeks, starting at 8 weeks of age. Western blot (left panel) and quantitative densitometry data (right panel) showing phosphorylation of PPARγ in BAT **(A,B)**, sWAT **(C,D)**, and gWAT **(E,F)** in WT and KO mice at the end point of the 8-week's HFD feeding. Data shown as mean ± SD, $*p < 0.05$. (Unpaired Student's *t* test). **(G)** Western blot showing phosphorylation of PPARγ in immortalized mouse brown adipocytes with CLK1 overexpression (10 MOI of lentivirus, Lenti-GFP as control) or knockdown (10 nM of siRNA, scrambled siRNA as control).

temperature was significantly decreased and the blood pressure was increased in aging healthy subjects (Inoue et al., 1992) and in hypertensive patients as well (Hess et al., 2009). In fact, there is a significant negative correlation between the environmental temperature and the blood pressure and heart rate in older patients (Postolache et al., 1993). Consistently, seasonal morbidity and mortality due to CVDs is significantly increased in both the northern and southern hemispheres during the winter rather than in the summer (Manou-Stathopoulou et al., 2015). Even though we previously demonstrated that a mild cold environment at 16°C significantly induced adipose tissue browning and prevented atherosclerosis development in mice (Chang et al., 2012; Xiong et al., 2017), it is unrealistic and could be somewhat dangerous for humans to be exposed to cold environments. In this study, we defined inhibition of CLK1 as a potential strategy to induce WAT browning and prevent obesity and associated diabetes as an alternative to cold exposure.

The CLK family of evolutionarily conserved dual-specificity kinases consists of four isoforms (CLK1-4) found in most tissues and cell types. Despite high homology among the CLKs, each member may have a distinct biology in different tissues. CLK1 was identified as a kinase that regulates pre-mRNA splicing by catalyzing the phosphorylation of Serine and arginine-rich (SR) RNA binding proteins (Duncan et al., 1997; Dufresne et al., 1998). Hyper-phosphorylated SR proteins bind to pre-mRNA and stabilize the interactions of spliceosome components, promoting spliceosome assembly (Mermoud et al., 1994). CLK1 also directly phosphorylates and activates the mitogen-activated protein kinase signaling cascade, including ERK1/ERK2 and PTP-1B (Moeslein et al., 1999), and the splicing factor SPF45, a non-SR protein (Liu et al., 2013). CLK1 also activates the kinetochore protein kinase KKT2 *via* phosphorylation at the S508 residue, which is crucial for kinetochore localization and function during cell division (Saldivia et al., 2021). Additionally, CLK1 functions as a component of a wider signaling network. Akt serine/threonine kinase 2 (Akt2), belonging to a subfamily of serine/threonine kinases containing SH2-like (Src homology 2-like) domains, can phosphorylate CLK1 and promote CLK1-mediated phosphorylation of SR proteins (Jiang et al., 2009). Those CLK1-associated signaling pathways regulate cell migration and invasion are linked to tumor development and progression. Notably, SM08502, a CLK1 inhibitor, recently entered clinical trials for the treatment of advanced solid tumors (Tam et al., 2020). Additionally, pharmacological inhibition of CLK1 has been investigated pre-clinically for the treatment of Duchenne muscular dystrophy (Sako et al., 2017) and Alzheimer's disease (Jain et al., 2014; Hedou et al., 2016; Murar et al., 2017). Beyond these diseases, it was reported that lower temperature causes CLK1 activation *in vitro* in cultured HEK293 cells (Haltenhof et al., 2020). It is unknown if CLK1 activity is altered when the body temperature changes in mammals. By screening the cold-induced phosphoproteome, we found that CLK activity was significantly reduced in BAT from mice housed in a mild-cold environment.

The protein abundance of CLK1 upon mild cold exposure was markedly reduced in sWAT and gWAT, which may account for the reduced kinase activity of CLK1. Meanwhile, in BAT, CLK1 protein abundance was unchanged while the kinase activity was significantly reduced upon mild cold exposure, raising the possibility that post-translational modifications of CLK1 play a predominant role in the regulation of CLK1 kinase activity in BAT, which will be the object of future studies. As a dual-specificity protein kinase, CLK1 has been reported to auto-phosphorylate on tyrosine residues (Menegay et al., 2000). Similarly, although the BAT is the major thermogenic organ in rodents, the expression levels of UCP1 and other thermogenic genes in BAT were less responsive to cold stimuli when compared to those in beige adipose tissues. The mechanisms underlying this phenomenon remain unknown.

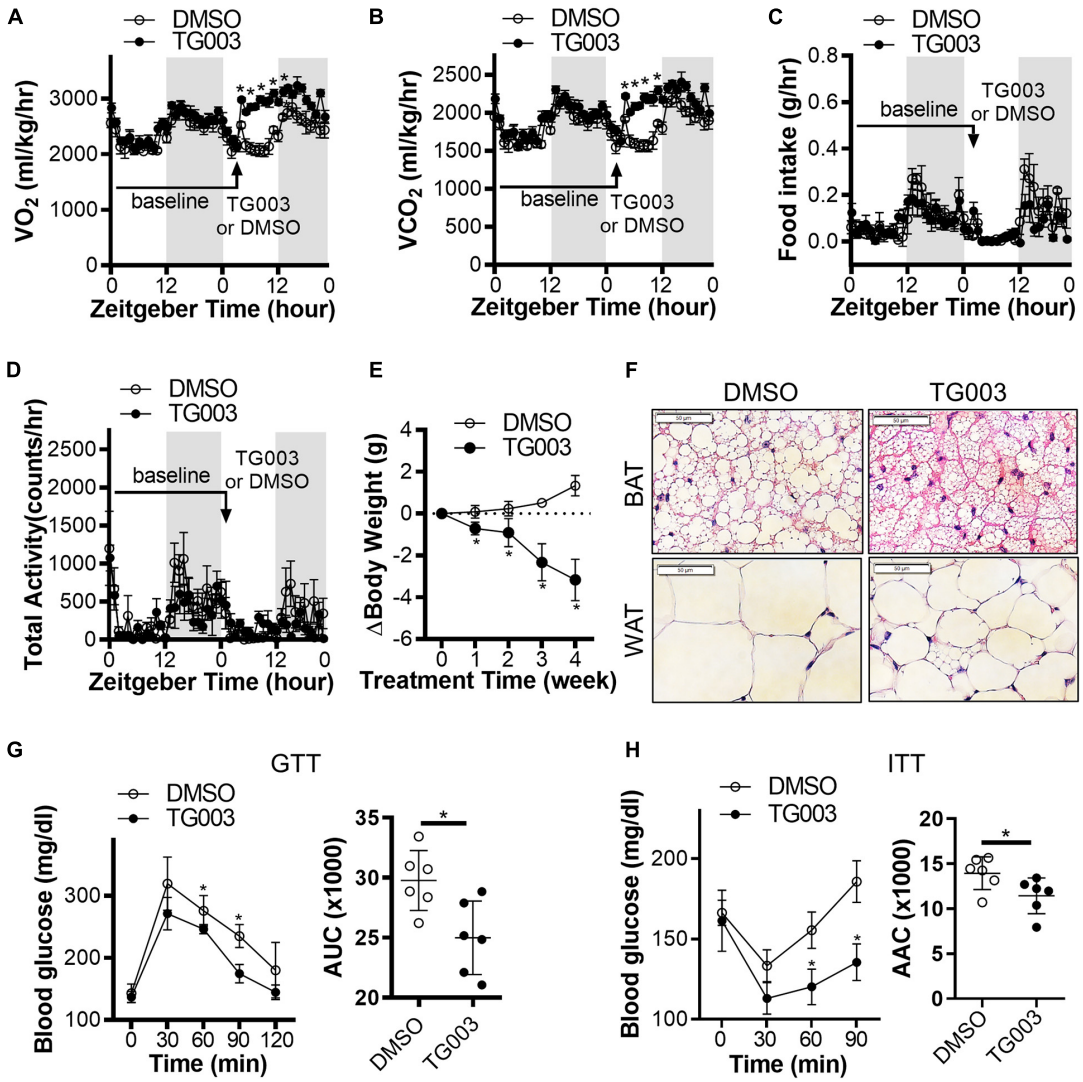

FIGURE 7 | Inhibition of CLK improves insulin sensitivity in obese mice. **(A–D)** Diet induced obesity (DIO) in male C57BL/6J mice was induced by high-fat diet (HFD) for 24 weeks starting at 6 weeks of age, at 22°C. Energy expenditure in obese mice was measured at baseline for 24 h after 24 weeks of HFD feeding. After subcutaneous injection one bolus of CLK inhibitor TG003 (50 μg/kg) or vehicle control DMSO (black arrow), the energy expenditure was measured for another 24 h. Gray area indicates the light-off period. $n = 5$ mice/group. Data shown as mean ± SEM. $^*p < 0.05$ vs DMSO. (One-way ANOVA with Sidak's multiple comparisons test). **(E)** After the energy expenditure measurements in panels **(A–D)**, the DIO mice continued the HFD feeding while being treated with TG003 (50 μg/kg) or DMSO (subcutaneous injection, once a day) for an additional 4 weeks. The body weight change of mice was calculated by subtracting the body weight on the first day of TG003 or DMSO treatment from that of each week of TG003 or DMSO treatment. $n = 6$ mice/group. Data shown as mean ± SEM. $^*p < 0.05$ vs DMSO. (One-way ANOVA with Sidak's multiple comparisons test). **(F)** H&E staining of the interscapular brown adipose tissue (BAT) and subcutaneous white adipose tissue (WAT), scale bar, 50 μm. **(G,H)** The oral glucose tolerance test **(G)** and insulin tolerance test **(H)**, at the end point of HFD feeding and TG003 or DMSO treatment as described in panel **(E)**. Histograms show the corresponding areas under the curves (AUC) of GTT and areas above the curves (AAC) of ITT. $n = 6$ mice/group. Data shown as mean ± SD. $^*p < 0.05$ vs DMSO. [One-way ANOVA with Sidak's multiple comparisons test for the curves in panels **(G,H)** and unpaired Student's *t* test for histograms in panels **(G,H)**].

We further showed that CLK1 deficient mice were resistant to HFD-induced obesity under regular room temperature conditions. Unlike the phenotype of the *Clk1* knockout mice reported here, overexpression of CLK2 in the mediobasal thalamus can partially reverse the HFD-induced obese phenotype in mice (Quaresma et al., 2017), and mice lacking *Clk2* in adipose tissue exhibited exacerbated obesity (Hatting et al., 2017). The underlying mechanisms of the reversed phenotype on obesity between CLK1 and CLK2 are unknown and will be further explored in future studies. This phenomenon was also observed in a study of HIV-1 replication. CLK1 had opposite effects to those of CLK2 on viral replication. CLK1 enhanced Gag production while CLK2 inhibited the virus, while CLK3 and CLK4 had no significant effect on viral replication (Wong et al., 2011). Phosphorylated cAMP-response element binding protein (CREB) is a transcriptional activator of UCP1. CLK2

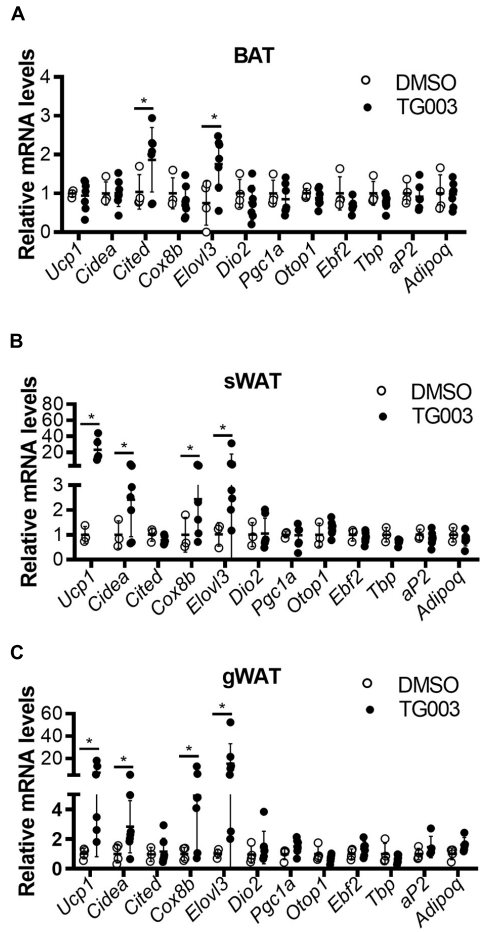

FIGURE 8 | TG003 treatment increases expression of markers of browning in adipose tissues. **(A–C)** mRNA levels of browning marker genes in BAT **(A)**, sWAT **(B)**, and gWAT **(C)** at the end point of HFD feeding combined with TG003 or DMSO treatment, as described in **Figure 7E**. $n = 6$ mice/group. Data shown as mean ± SD. $^*p < 0.05$ vs DMSO. (Unpaired Student's *t* test for each gene).

decreases CREB dephosphorylation in a protein phosphatase 2-dependent manner. Deletion of *Clk2* in adipose tissues reduced energy expenditure in mice as well as UCP1 abundance in brown adipocytes due to enhanced CREB dephosphorylation (Hatting et al., 2017). Contrasting the lower energy expenditure in CLK2 knockout mice, the CLK1 deficient mice showed enhanced energy expenditure per body weight. However, the energy expenditure normalized by lean body mass or without normalization was comparable between wildtype and *Clk1* knockout mice, suggesting that less fat mass or maintenance of functional fat contributed to the enhanced energy expenditure in the CLK1 deficient mice. Furthermore, we found that Akt phosphorylation levels were strongly elevated in BAT of CLK1 KO mice, presumably involving a feedback mechanism. But how Akt regulates CLK1 kinase activity and the potential feedback of CLK1 on Akt kinase activity will require further investigation. It was reported that inhibition of CLK1 blocked adipocyte differentiation *in vitro*. 3T3-L1 cells carrying mutations of putative Akt phosphorylation sites in CLK1 failed to form lipid droplets during differentiation (Li et al., 2013). Our study showed that the lipid droplets of adipocytes were smaller in WAT and BAT of CLK1 KO mice than those in WT mice, which might be also due to the reduced differentiation capability of CLK1 deficient preadipocytes.

We additionally found that the lack or inhibition of CLK1 improves glucose tolerance and preserves insulin sensitivity upon HFD feeding. This phenotype might be solely due to reduced fat mass since it is well-known that dysfunctional fat accumulation causes insulin resistance.

We further uncovered a novel pathway defined by a CLK1-THRAP3-PPARγ axis which might impair insulin sensitivity under obese conditions. THRAP3 is a transcriptional cofactor containing an SR domain and has been identified as a component of the spliceosome which is required for pre-mRNA splicing and it activates splicing *in vivo* (Lee et al., 2010). Loss of THRAP3 results in sensitivity to DNA damaging agents, genomic instability, and defective DNA repair, which are in themselves promising targets for chemotherapy of cancers (Vohhodina et al., 2017). Additionally, THRAP3 acts as a cofactor of sex-determining region Y-box 9 (SOX9) and negatively regulates the transcriptional activity of the SOX9 complex during chondrogenesis (Sono et al., 2018). However, the roles of THRAP3 in energy metabolism are largely unknown. THRAP3 binds the helicase motifs in helicase with zinc finger 2 (HELZ2). HELZ2 and THRAP3 synergistically augmented transcriptional activation mediated by PPARγ in differentiated 3T3-L1 cells (Katano-Toki et al., 2013), suggesting that THRAP3 and HELZ2 interaction contributes to adipocyte differentiation through activation of PPARγ-mediated gene expression. THRAP3 has also been shown to directly interact with PPARγ to control diabetic gene programming (Choi et al., 2014). In this study, we showed that CLK1 could phosphorylate THRAP3 at Ser243, and promotes its docking to PPARγ when it is phosphorylated at Ser273 and resulting in inhibition of PPARγ activity (Choi et al., 2014). Consistent with the reduced CLK1 kinase activity in BAT upon mild cold exposure, the phosphorylation levels of THRAP3 at Ser243 were also markedly decreased. Thus, inhibition of CLK1 could block THRAP3 phosphorylation and increase PPARγ activity. Moreover, mild cold exposure altered other phosphorylation sites on THRAP3 in BAT (**Supplementary Table 1**), implying that THRAP3 may actively participate in the regulation of thermogenesis. Further studies will focus on the interaction of the different phosphorylated forms of THRAP3 with PPARγ and other key transcription factors, and the functional consequences of those interactions for thermogenesis, adipocyte homeostasis and systemic glucose metabolism.

Peroxisome proliferator-activated receptor gamma is one of the most effective targets to improve insulin signaling. PPARγ ligands such as thiazolidinediones were widely used for control of type 2 diabetes in the clinic. Nevertheless, thiazolidinediones showed multiple side effects such as fluid retention, obesity, and congestive heart failure, which led to the withdrawal of rosiglitazone from the market. However, it is still unknown whether those side effects are only PPARγ-dependent

or -independent, as well. Post-transcriptional modification of Ser273 on PPARγ is critical for its anti-diabetic roles. Inhibition of phosphorylation of PPARγ on Ser273 could be a key therapeutic mechanism for full and partial agonists or non-agonist drugs targeting PPARγ. It has been established that PPARγ phosphorylation at Ser273 promotes insulin resistance in obese and diabetic mice, and classical PPARγ ligands such as TZDs inhibit Ser273 phosphorylation to improve insulin sensitivity (Choi et al., 2010). Interestingly, inhibition of PPARγ Ser273 phosphorylation by non-agonist ligands was also anti-diabetic (Kamenecka et al., 2010; Choi et al., 2011; Li et al., 2011; Khim et al., 2020). ERK directly phosphorylated Ser273 of PPARγ. Accordingly, inhibition of ERK significantly improved insulin resistance in diabetic mice (Banks et al., 2015). The relationship between ERK and CLK1 is unknown. Whether this underlies the inhibition of PPARγ Ser273 phosphorylation will be addressed in follow up studies. It was suggested that interfering with docking of THRAP3 on PPARγ could be a strategy to screen for compounds for diabetes treatment (Choi et al., 2014). In this study, we found that CLK1 binds THRAP3 and phosphorylates it on Ser243, thus promoting THRAP3 interaction with PPARγ when the latter is phosphorylated on Ser273 (Choi et al., 2014), suggesting that a CLK1-THRAP3-PPARγ axis regulates insulin sensitivity. Additionally, our results indicate that the mRNA levels of adipocyte browning markers are negatively correlated with CLK1 in adipose tissue of obese mice, with *Clk1* knockout significantly increasing adipocyte browning.

In summary, the present study provides evidence that knockout or inhibition of CLK1 will prevent obesity and improve insulin resistance. Our findings support further exploration of pharmacologic inhibition of CLK1 as a potential new treatment for obesity associated diabetes, beyond oncology, Duchenne muscular dystrophy and Alzheimer's disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ProteomeXchange Consortium (accession no: PXD027958) via the iProX partner repository (Ma et al., 2019).

ETHICS STATEMENT

The animal study was reviewed and approved by University of Michigan.

AUTHOR CONTRIBUTIONS

ZW, XG, QL, HZ, XZ, and LC conducted the experiments. RZ, J-RW, and LC designed the experiments. ZW, MG-B, and LC wrote the manuscript. JZ, MG-B, YG, RZ, and YEC contributed to the data interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.699578/full#supplementary-material

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REVIEW
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Primary Cilia Are Critical Regulators of White Adipose Tissue Expansion

*Keren I. Hilgendorf**

Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT, United States

The primary cilium is a microtubule-based cellular protrusion found on most mammalian cell types in diverse tissues. It functions as a cellular antenna to sense and transduce a broad range of signals, including odorants, light, mechanical stimuli, and chemical ligands. This diversity in signals requires cilia to display a context and cell type-specific repertoire of receptors. Recently, primary cilia have emerged as critical regulators of metabolism. The importance of primary cilia in metabolic disease is highlighted by the clinical features of human genetic disorders with dysfunctional ciliary signaling, which include obesity and diabetes. This review summarizes the current literature on the role of primary cilia in metabolic disease, focusing on the importance of primary cilia in directing white adipose tissue expansion during obesity.

Keywords: primary cilia, diabetes, adipogenesis, signaling, obesity

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The University of Iowa, United States

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Nina-Naomi Kreis,
University Hospital Frankfurt, Germany
Sayee Anakk,
University of Illinois at Urbana-Champaign, United States

***Correspondence:**
Keren I. Hilgendorf
keren.hilgendorf@biochem.utah.edu

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INTRODUCTION

White adipose tissue expands in response to caloric imbalance both by generating more adipocytes via *de novo* adipogenesis (hyperplasia) and by storing more fat in existing adipocytes (hypertrophy) (Haczeyni et al., 2018). The relative contribution of these two mechanisms of expansion has a profound effect on metabolic health, and this is independent of body mass index (BMI) (Ghaben and Scherer, 2019; Vishvanath and Gupta, 2019). Specifically, patients with metabolic disorders tend to have hypertrophic adipocytes, which in turn is linked to tissue hypoxia, inflammation, and fibrosis. In contrast, white adipose tissue containing smaller adipocytes, even if present in greater number, is associated with a healthy metabolic profile (Ye et al., 2021). Notably, mechanisms that promote adipogenesis have been shown to drive healthy white adipose tissue expansion in response to excess nutrients (Shao et al., 2018). Identifying ligands and signaling pathways that can trigger adipogenesis thus represent a therapeutic avenue to limit the pathogenic consequences of obesity.

The primary cilium is a sensory organelle that is highly conserved throughout eukaryotic evolution and found on most mammalian cells (Goetz and Anderson, 2010). All cells in the human body are either ciliated or derived from a ciliated stem cell (Yanardag and Pugacheva, 2021). Cilia are critical to human development and physiology by organizing signal transduction pathways such as hedgehog signaling in vertebrate cells (Kopinke et al., 2021). Consistent with the near ubiquitous presence and function of cilia in cells throughout the human body, a heterogeneous group of human genetic disorders with dysfunctional primary cilia (ciliopathies) manifest with a broad range of clinical features, including polydactyly, cysts in the kidney and liver, retinal degeneration, learning disabilities, obesity, and diabetes (Reiter and Leroux, 2017).

Understanding the function of primary cilia in diverse tissues can provide powerful insight into metabolic disease: Primary cilia on POMC (proopiomelanocortin) and AGRP (agouti-related protein) neurons in the arcuate nucleus of the hypothalamus are critical in the regulation of food intake and satiety sensing, and ciliary dysfunction in these neurons results in obesity due to excessive eating (hyperphagia) (Engle et al., 2020). In peripheral tissues, primary cilia on pancreatic islet cells regulate glucose homeostasis, primary cilia on cholangiocytes in the bile duct regulate bicarbonate secretion, and primary cilia on pre-adipocytes regulate adipogenesis (Gerdes et al., 2014; Mansini et al., 2018; Hilgendorf et al., 2019; Volta et al., 2019; Hughes et al., 2020; Wu et al., 2021). This review focuses on recent advances in our understanding of how the primary cilium can organize both pro- and anti-adipogenic signaling pathways during adipose tissue expansion in obesity.

THE PRIMARY CILIUM—THE CELL'S ANTENNA

Structure and Molecular Composition of Primary Cilia

There are two broad classes of cilia. Motile cilia/flagella are found on specialized cells. This includes sperm, where the flagellum enables motility, and ependymal cells, where multiple motile cilia generate directional flow of cerebrospinal fluid (Mitchison and Valente, 2017). In contrast, most other mammalian cells contain a single, immotile primary cilium that is 2–10 μm in length (Nachury and Mick, 2019). This primary cilium is comprised of a microtubule-based axoneme and a surrounding ciliary membrane (**Figure 1**). The axoneme consists of nine outer microtubule doublets arranged in a 9 + 0 fashion and is nucleated by the basal body (Fisch and Dupuis-Williams, 2011). The basal body is composed of the mother centriole and pericentriolar material, and it is anchored to the cell membrane by distal appendages. Since centrosomes are required for the formation of both the mitotic spindle and the primary cilium, the biogenesis of the primary cilium is cell cycle-dependent, with ciliary disassembly occurring prior to mitosis and reassembly occurring during the G_1 or G_0 phase of the cell cycle (Ford et al., 2018; Ho et al., 2020). Within the primary cilium, the intraflagellar transport (IFT) machinery couples to the microtubule motor proteins kinesin-2 or dynein-2 to transport proteins along the axoneme in an anterograde or retrograde direction, respectively.

The ciliary membrane is contiguous with the plasma membrane. To generate a unique and compartmentalized signaling organelle, the transition zone at the base of the cilium functions as a diffusion barrier (Malicki and Johnson, 2017). The ciliary membrane has a unique lipid composition enriched for the phosphoinositide PI(4)P, rather than the $PI(4,5)P_2$ typically found in the plasma membrane, and this is generated via the ciliary localization of the ciliopathy-associated phosphoinositide 5-phosphatase INPP5E (Chavez et al., 2015; Garcia-Gonzalo et al., 2015; Jensen et al., 2015). Similarly, a high ciliary concentration of calcium channels generates an approximately

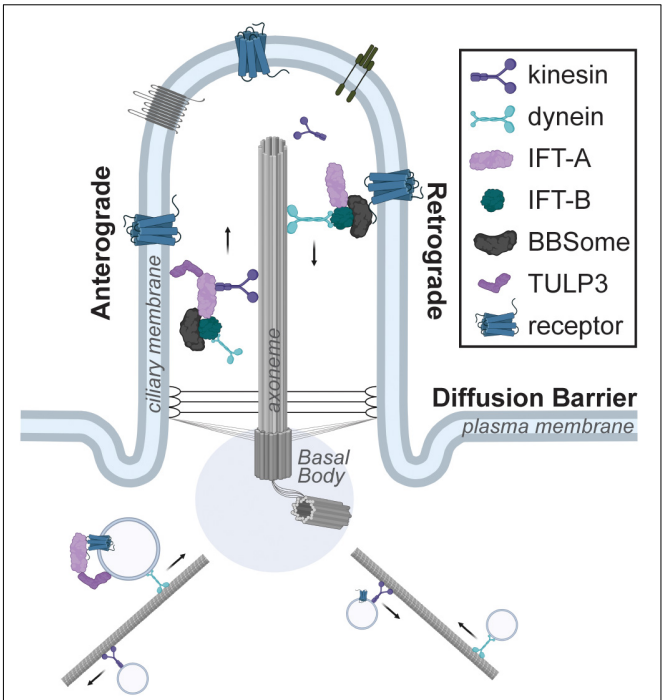

FIGURE 1 | Schematic of the primary cilium. The cilium is composed of a microtubule-based axoneme and the surrounding ciliary membrane, which is highly enriched for receptors. The intraflagellar transport system couples to kinesin or dynein motors for anterograde or retrograde transport within the cilium. Trafficking of ciliary cargo such as receptors into or out of the primary cilium is tightly regulated and requires adaptor proteins such as TULP3 or the BBSome complex, respectively.

sevenfold higher resting calcium concentration in the cilium compared to the cytoplasm (DeCaen et al., 2013; Delling et al., 2013). The localized concentration of cyclic AMP (cAMP) may also be elevated in the primary cilium compared to the cytoplasm in some cell types (Moore et al., 2016; Jiang et al., 2019; Truong et al., 2021). Finally, the ciliary membrane is highly enriched for receptors and its molecular composition varies according to the cell type and cellular signaling status (Hilgendorf et al., 2016). Numerous mechanisms regulate trafficking of ciliary cargo into and out of the primary cilium (Nachury et al., 2007; Mukhopadhyay et al., 2010; Wright et al., 2011). Most clinical features of ciliopathies are due to genetic mutations deregulating ciliary entry and exit, cargo trafficking, and signaling.

Ciliary Signal Transduction Pathways

Numerous receptors localize to the primary cilium, including several receptors critical for energy homeostasis and metabolic health (**Table 1**). Depending on the cell and tissue context, these signaling pathways organized by the primary cilium can have both positive and negative effects on energy homeostasis. Intriguingly, some receptors can localize to and signal from both the primary cilium and the plasma membrane. We do not fully understand why ciliary localization is required for some receptors and in some cellular contexts to signal. One possible explanations is that the increased surface to volume ratio of the

TABLE 1 | List of receptors relevant to energy homeostasis and capable of localizing to the primary cilium.

Receptor	References for ciliary localization	Cell and tissue contexts described for ciliary localization *(overexpressed* and endogenous)	Ligand(s)	Function related to energy homeostasis
DRD1	Marley and von Zastrow, 2010; Domire et al., 2011	*NIH3T3, IMCD3*, several regions of brain including primary striatal neurons and amygdala	Dopamine	Feeding
EP4/PTGER4	Jin et al., 2014; Wu et al., 2021	RPE-1, IMCD3, Min6, aTC9, pancreatic beta and alpha cells	Prostaglandin E2	Glucagon and insulin secretion
FFAR4/GPR120	Hilgendorf et al., 2019; Wu et al., 2021	3T3-L1, white adipose tissue, Min6, αTC9, pancreatic beta and alpha cells	Omega-3 fatty acids	Adipogenesis; glucagon and insulin secretion
GALR2	Loktev and Jackson, 2013	*RPE-1*	Galanin	Feeding
GALR3	Loktev and Jackson, 2013	*RPE-1*, cultured hypothalamic neurons, hypothalamus section	Galanin	feeding
GPR83	Loktev and Jackson, 2013	*RPE-1*, several regions of brain, e.g., olfactory tubercle, nucleus accumbens	Orphan	Feeding
HTR6	Hamon et al., 1999; Brailov et al., 2000	Several regions of brain including nucleus accumbens and olfactory tubercles	Serotonin	Feeding
IGF-1R	Zhu et al., 2009; Dalbay et al., 2015	3T3-L1, MSCs	IGF-1, insulin	Multiple including adipogenesis
Insulin Receptor (IR isoform A only)	Gerdes et al., 2014	Min6, pancreatic beta cells	Insulin	Beta cell insulin secretion (IR-A is not expressed in mature adipocytes
KISSR1	Koemeter-Cox et al., 2014	*IMCD3*, medial hypothalamus	Kisspeptin	Physical activity, energy expenditure
MC4R	Siljee et al., 2018	*MEF*, *RPE-1*, *IMCD3*, subset of hypothalamic neurons	Melanocyte stimulating hormone	Feeding
MCHR1	Berbari et al., 2008	*IMCD3*, nucleus accumbens	Melanin-concentrating hormone	Feeding
NMUR1	Omori et al., 2015	*NIH3T3*	Neuromedin U	Feeding
NPY2R	Loktev and Jackson, 2013; Omori et al., 2015	*NIH3T3*, *RPE-1*, cultured hypothalamic neurons, hypothalamus section (arcuate nucleus)	Neuropeptides NPY, peptide YY	Feeding
NPY5R	Loktev and Jackson, 2013	RPE-1, cultured hypothalamic neurons, hypothalamus section	Neuropeptides NPY, peptide YY	Feeding
PRLHR	Omori et al., 2015	*NIH3T3*, third ventricle mouse brain	Prolactin-releasing hormone	Feeding
PTCH1	Rohatgi et al., 2007	*MEFs*, NIH3T3, mouse embryo mesoderm cells	Hedgehog	Multiple including MSC commitment
QRFPR	Loktev and Jackson, 2013	*RPE-1*, cultured hypothalamic neurons, hypothalamus section	Neuropeptide QRFP	Feeding
SMO	Corbit et al., 2005	*MDCK*; MEFs, IMCD3, nodal cells	Oxysterols	Multiple including MSC commitment
SSTR3	Handel et al., 1999; Iwanaga et al., 2011	Several regions of brain including hypothalamus, amygdala and cerebellum; pancreatic islets; anterior pituitary	Somatostatin	Feeding
TGR5	Keitel et al., 2010	Isolated cholangiocytes, liver sections	Bile acid	Bile composition
P2YR12	Masyuk et al., 2008	Rat liver section (cholangiocytes)	ADP	Bile composition

primary cilium and the unique ciliary signaling environment may increase the sensitivity of a signaling pathway to a ligand. The distinct composition of ciliary proteins and metabolites may also allow receptors to activate different downstream effector proteins in the cilium vs. the plasma membrane. Finally, the primary cilium may enable the sensing and integration of multiple simultaneous signals into common ciliary second messengers or effector proteins to elicit a binary cellular response (Hilgendorf et al., 2016; Malicki and Johnson, 2017; Nachury and Mick, 2019).

Most ciliary receptors identified to-date are G protein-coupled receptors (GPCRs) (Wachten and Mick, 2021). Trafficking of GPCRs into and out of the primary cilium is tightly regulated. The TUBBY family of proteins is required for trafficking GPCRs to the primary cilium (Mukhopadhyay et al., 2010; Sun et al., 2012). Expression of TUBBY family members is tissue-specific, with TULP3 being expressed most broadly (Badgandi et al., 2017). Another multi-protein complex, the BBSome, is required for β-arrestin2 mediated removal of GPCRs out of the primary cilium (Lechtreck et al., 2009; Ye et al., 2018). Since genes mutated in Bardet-Biedl syndrome encode the BBSome, the clinical features of this ciliopathy are likely a result of aberrant ciliary receptor composition (Nachury et al., 2007). Together,

these two protein families are integral to the generation of cell type-specific repertoires of ciliary GPCRs appropriate for a particular biological context (**Figure 1**). However, we do not yet understand how this cell and context-specific trafficking of GPCRs is achieved.

CILIOPATHIES AND METABOLIC DISEASE

Genetic disorders affecting the biogenesis and maintenance of the cilium, known collectively as ciliopathies, serve as powerful tools to discover new drivers of human disease (Wheway et al., 2018). A subset of ciliopathies is associated with obesity: Alström syndrome (ALMS, OMIM #203800) and Bardet-Biedl syndrome (BBS, OMIM #209900). ALMS is caused by mutations in the *ALMS1* gene (Collin et al., 2002; Hearn et al., 2002). ALMS1 localizes to the centrosome, including the basal body in ciliated cells (Knorz et al., 2010). While the molecular function of ALMS1 has not been fully elucidated, ALMS1 is thought to regulate ciliary signaling and transport of ciliary cargo (Li et al., 2007; Jagger et al., 2011). BBS is caused by mutations in one of 22 genes and the majority of BBS genes encode proteins that together form or are associated with a large complex called the BBSome (Nachury et al., 2007). The BBSome is required for transporting a subset of GPCRs out of the primary cilium. Despite the similarities of BBS and ALMS with regard to obesity and ciliary dysfunction, the two disorders present notable differences with regard to metabolic health: ALMS patients are moderately obese with extreme insulin resistance and increased incidence of early onset type 2 diabetes mellitus compared to patients matched for pubertal stage and body composition (Minton et al., 2006). In contrast, even though BBS patients are morbidly obese and have greater visceral adiposity, patients have better glucose tolerance than BMI-matched control subjects (Beales et al., 1999; Feuillan et al., 2011; Marion et al., 2012). This disparity illustrates the complexity of ciliary signaling pathways, with primary cilia regulating numerous cellular processes in diverse tissues. Since the main focus of this review is to discuss the role of cilia in adipose tissue, the reader is referred to a more detailed review for the role of cilia in other contexts including in hypothalamic neurons to regulate satiety sensing (Engle et al., 2020).

Alström Syndrome

Patients with Alström syndrome and ALMS1 mutant mice are obese, but with increased insulin resistance that is disproportionate to body weight, adiposity, and fat distribution (Gathercole et al., 2016; Han et al., 2018). Obesity develops early during childhood, along with hypertriglyceridemia, hyperinsulinemia, extreme insulin resistance, and the development of type 2 diabetes mellitus with a mean age of onset at 16 years (Marshall et al., 2011). The BMI tends to normalize in older individuals, but insulin resistance continues to increase (Minton et al., 2006). Childhood hyperphagia and lower levels of physical activity may contribute to the development of obesity. However, while all ALMS mouse models recapitulate the clinical features of the disease, including obesity and insulin resistance, this is not driven by hyperphagia in most of these mouse models (Marshall et al., 2011). This suggests that ALMS1 functions in peripheral organs to regulate metabolic health, rather than in the central nervous system.

Patients with ALMS1 have increased adipocyte hypertrophy in subcutaneous white adipose tissue when compared to BMI-matched control subjects (Geberhiwot et al., 2021). Molecularly, depletion of ALMS1 in pre-adipocytes inhibits adipogenesis and depletion of ALMS1 in mature adipocytes impairs glucose uptake via an insulin signaling-independent mechanism (Huang-Doran and Semple, 2010; Favaretto et al., 2014). ALMS1 mutant mice present with hyperglycemia, insulin resistance and hyperleptinemia prior to the development of obesity (Geberhiwot et al., 2021). Together, this argues that mutations in ALMS1 cause adipose tissue dysfunction, which in turn drives insulin resistance, type 2 diabetes mellitus, and obesity in ALMS patients. In support of this model, Geberhiwot et al. (2021) recently generated a mouse model re-expressing ALMS1 in the adipose tissue of mutant mice using an *Adipo*-CRE allele. Remarkably, ALMS1 expression in adipose tissue completely rescued obesity, adipocyte hypertrophy, glucose tolerance, and insulin sensitivity. Of note, both fetal pre-adipocytes and mature adipocytes express adiponectin and hence *wild-type* ALMS1 in this mouse model (Hong et al., 2015). Further investigation using a CRE recombinase that is specific to mature adipocytes may ascertain the relative contribution of ALMS1 function in adipogenesis vs. adipocyte glucose uptake. Taken together, these data show that ALMS1 mutation causes adipose tissue dysfunction, leading to the development of obesity, extreme insulin resistance, and early-onset type 2 diabetes mellitus in ALMS patients.

Bardet-Biedl Syndrome

Mouse models of BBS are obese due to hyperphagia, decreased locomotor activity, and hyperleptinemia (Rahmouni et al., 2008). This reproduces the clinical features of human patients, including evidence that BBS patients have lower levels of physical activity when compared to BMI-matched control subjects (Grace et al., 2003). In mouse knockout models, leptin resistance precedes the development of obesity, suggesting that dysfunctional satiety sensing leads to obesity in BBS (Rahmouni et al., 2008). Notably, pair-feeding (matching the amount of food consumed) normalizes the body weight of BBS knockout mice. Yet despite the normalized body weight, BBS knockout mice still present with increased adiposity (Rahmouni et al., 2008). This suggests that the BBSome has additional roles outside the central nervous system, including in adipose tissue.

Molecularly, depletion of BBS12 in pre-adipocytes promotes adipogenesis (Marion et al., 2009). Visceral adipocytes in *Bbs12* knockout mice are heterogeneous in size and visceral adipose tissue expansion in *Bbs12* knockout mice is driven by both hyperplasia and adipocyte hypertrophy (Marion et al., 2012). As described above, excessive adipocyte hypertrophy is linked to the development of type 2 diabetes mellitus, while hyperplasia in adipose tissue is generally considered metabolically healthy (Ghaben and Scherer, 2019; Vishvanath and Gupta, 2019). Consistent with this model, *Bbs12* knockout mice have improved,

rather than diminished insulin and glucose tolerance compared to lean littermates despite increased body weight and adiposity (Marion et al., 2012). Similarly, *Bbs12* knockout mice on a high fat diet are more obese, but have lower blood glucose levels and white adipose tissue inflammation compared to control littermates. Together, this argues that the BBSome can regulate both food intake and adipogenesis, such that loss-of-function mutations in BBS genes cause hyperphagia-driven obesity as well as metabolically healthy, hyperplastic white adipose tissue expansion.

Thus, while both ALMS and BBS patients are obese, this is driven by distinct functions of the primary cilium, either exclusively in pre-adipocytes (ALMS) or in multiple tissues (BBS), resulting in dramatic differences regarding metabolic health. Of note, some studies have described higher, rather than lower incidence of metabolic disease in BBS patients when normalized to BMI-matched control patients, and this disparity may be explained by the noted misdiagnoses of ALMS patients as BBS patients in cases where categorization was purely based on clinical features (Aliferis et al., 2012).

PRIMARY CILIA DIRECT HOW WHITE ADIPOSE TISSUE EXPANDS

All characterized stem and progenitor cells in the human body are ciliated (Yanardag and Pugacheva, 2021). This includes the embryonic stem cell, mesenchymal stem cells (MSCs), and all committed progenitor cells in the mesenchymal lineages giving rise to adipose tissue, muscle, bone, and cartilage. Thus, the primary cilium can be used as a marker of stem cells *in vivo*. Moreover, numerous studies have shown that stem cell ciliation is also critical for differentiation (Tummala et al., 2010; Kopinke et al., 2017; Lyu and Zhou, 2017). Since differentiation is regulated by tissue-specific ligands, this suggests that stem and progenitor cells in different tissues express context-specific, ciliary receptors to sense ligands and regulate stem cell fate.

The Primary Cilium Is a Biomarker for Pre-adipocytes in White Adipose Tissue

MSCs and committed pre-adipocytes, pre-osteoblasts, pre-chondrocytes, and muscle stem cells are ciliated (Marion et al., 2009; Jaafar Marican et al., 2016; Yuan and Yang, 2016). Specifically, we recently showed that approximately 80% of isolated murine pre-adipocytes (Lin^- $CD34^+$ $CD29^+$ $SCA1^+$) are ciliated (Hilgendorf et al., 2019). Using whole-mount imaging on a transgenic mouse model with fluorescently marked cilia, we also showed that approximately 30% of all perivascular cells in both subcutaneous (inguinal) and visceral (epididymal, perirenal, and mesenteric) white adipose tissue are ciliated *in vivo*. Similarly, we confirmed that PDGFRα-lineage traced perivascular cells in murine white adipose tissue are ciliated *in vivo*. Finally, we showed that ciliated perivascular pre-adipocytes are activated to reenter the cell cycle in response to high fat diet as determined by BrdU incorporation. Thus, we propose that the primary cilium can be used as a biomarker to visualize the location, abundance, and adipogenic responsiveness of pre-adipocytes in white adipose tissue. Notably, approximately 75% of isolated human pre-adipocytes are also uniformly ciliated (Marion et al., 2009; Forcioli-Conti et al., 2015).

The 3T3-L1 pre-adipocyte cell line as well as isolated primary human and murine pre-adipocytes *in vitro* become uniformly ciliated upon reaching confluency and becoming growth-arrested (Zhu et al., 2009). Mature, lipid-laden adipocytes are not ciliated (Marion et al., 2009; Forcioli-Conti et al., 2015; Hilgendorf et al., 2019). Proliferating pre-adipocytes are also not ciliated, consistent with the cell cycle-dependent biogenesis of primary cilia. Growth-arrest is required for pre-adipocytes to differentiate *in vitro* (Ailhaud et al., 1989). This suggests that the pre-adipocyte primary cilium functions to regulate initiation of adipogenesis.

Dynamic Remodeling of Ciliary Receptors During Adipogenesis Enables Multi-Functionality of the Primary Cilium

Genetic manipulations of MSCs and pre-adipocytes have revealed that the primary cilium can both inhibit and promote adipogenesis. Specifically, depletion of BBS12, FBF1, or PKD1 promotes adipogenesis, while depletion of ALMS1, KIF3A, or IFT88 inhibits adipogenesis (Marion et al., 2009, 2012; Zhu et al., 2009; Huang-Doran and Semple, 2010; Qiu et al., 2010; Zhang et al., 2021). Notably, we recently showed that genetic ablation of ciliation specifically in pre-adipocytes of adult male and female mice, via the depletion of *Ift88* using a tamoxifen-inducible *Pdgfrα*-CreERT, completely prevented *in vivo* adipogenesis (Hilgendorf et al., 2019). We propose that the primary cilium functions to inhibit MSC commitment to the adipogenic lineage, but is required for adipogenesis *in vitro* and *in vivo*. The ciliary signaling pathways discovered to-date that mediate these pro- and anti-adipogenic functions are discussed below (**Figure 2**).

Cell biological findings support the hypothesis that the primary cilium is dynamic, showing that the morphology of the primary cilium changes during MSC commitment and during adipogenesis. Specifically, the relative prevalence of ciliation and the length of the primary cilium increases in isolated human MSCs exposed to adipogenic differentiation media (Dalbay et al., 2015; Forcioli-Conti et al., 2015). In contrast, exposure to osteogenic differentiation media results in a decrease in percent ciliation but increase in ciliary length, while exposure to chondrogenic differentiation media causes a decrease in percent ciliation and decrease in ciliary length (Dalbay et al., 2015). Further, ciliary length is maximal on Day 3 of MSC adipogenesis, before decreasing in length once again as the differentiating pre-adipocyte starts to accumulate lipid (Forcioli-Conti et al., 2015). Finally, the mature, lipid-laden adipocyte no longer has a primary cilium. The transient increase in ciliary length during adipogenesis is dependent on the IFT machinery and mediated by the presence of the glucocorticoid dexamethasone in the adipogenic differentiation media (Dalbay et al., 2015; Forcioli-Conti et al., 2015). Ciliary length is controlled by trafficking and recruitment of ciliary cargo (Kuhns et al., 2019).

We postulate that the molecular composition of the primary cilium, including ciliary receptors, dynamically changes during MSC commitment and adipogenesis. This ciliary remodeling

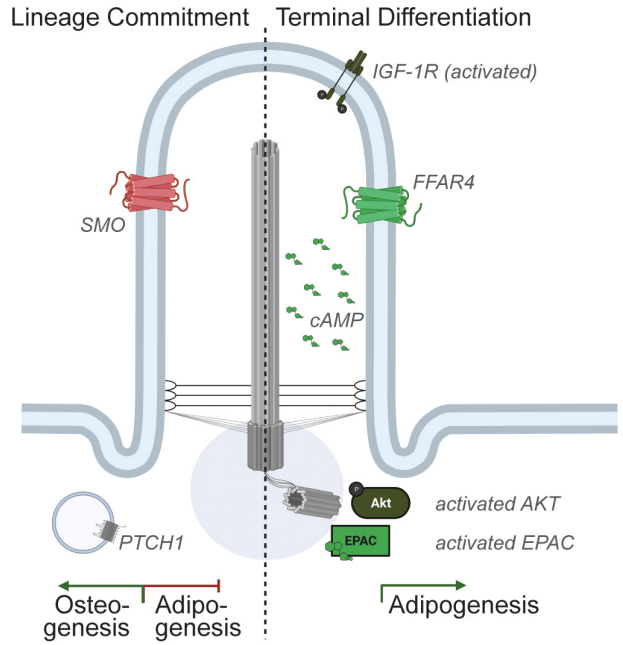

FIGURE 2 | Ciliary signaling in pre-adipocytes. Multiple receptors localize to the pre-adipocyte primary cilium and activation of ciliary signaling pathways can either promote or inhibit adipogenesis. Specifically, Hedgehog signaling has been shown to inhibit MSC commitment to adipogenesis. In contrast, the ciliary IGF-1 receptor and ciliary FFAR4/GPR120 are expressed in committed pre-adipocytes, and activation of these ciliary signaling pathways promotes adipogenesis.

would enable the primary cilium to sense and appropriately respond to extracellular signals. Of note, both ciliary and non-ciliary signaling can induce dynamic changes to the length of the primary cilium in both epithelial and mesenchymal cells, including PKA and MAPK-mediated ciliary lengthening (Besschetnova et al., 2010; Abdul-Majeed et al., 2012). This suggests that ciliary remodeling may be part of a positive feedback loop, such that the MSC primary cilium becomes more sensitive to adipogenic signals upon receiving sufficient signals to commit to the adipogenic lineage. Further ciliary remodeling may also occur during terminal differentiation.

Ciliary Signaling Pathways Inhibiting Adipogenesis

The hedgehog signaling pathway is essential for both embryonic development and for adult tissue homeostasis, and the primary cilium is required for hedgehog signaling in vertebrate cells (Huangfu et al., 2003; Briscoe and Therond, 2013). Activation of the hedgehog signaling pathway inhibits adipogenesis of the multipotent MSC cell lines C3H/10T1/2 and ST2 via upregulation of osteogenic markers (Spinella-Jaegle et al., 2001; Suh et al., 2006). Thus, ciliary hedgehog signaling can regulate MSC commitment toward osteogenesis. Loss of primary cilia in this context results in de-repression and hence potentiation of adipogenesis.

Genetic alterations that change the ciliary receptor composition can promote adipogenesis. Specifically, depletion of BBS12 promotes adipogenesis in human primary MSCs and causes adipocyte hyperplasia in *Bbs12* knockout mice (Marion et al., 2009, 2012). Since the BBSome is required for ciliary removal of receptors, we postulate that loss of BBSome function results in ciliary remodeling in MSCs, which in turn results in increased adipogenic commitment and/or increased recruitment of MSCs to white adipose tissue. Similarly, mouse models deficient for *Fbf1*, which can regulate ciliary entry and exit by ensuring proper BBSome assembly, are obese with improved glucose and insulin tolerance, in part due to increased white adipose tissue hyperplasia and beiging (Zhang et al., 2021). Mouse models deficient for *Pkd1*, which encodes the protein polycystin-1 and is commonly mutated in autosomal dominant polycystic kidney disease, have skeletal abnormalities, and isolated MSCs and primary osteoblasts have a decreased osteogenic but increased adipogenic potential (Qiu et al., 2010). Depletion of the axonemal kinesin-2 subunit KIF3A, required for trafficking of ciliary cargo and hence for any ciliary remodeling, rescues the abnormal skeletal development in the $Pkd1^{-/-}$ $Kif3a^{-/-}$ double mutant and leads to decreased adipogenesis. Together, this argues that mutations that lead to ciliary receptor remodeling affect MSC commitment to the adipogenic vs. osteogenic lineage.

Interestingly, while hedgehog signaling inhibits adipogenesis in white adipose tissue, it does not inhibit adipogenesis in brown fat (Pospisilik et al., 2010; Nosavanh et al., 2015). Instead, non-canonical hedgehog signaling promotes insulin-independent glucose uptake in brown adipose tissue (Teperino et al., 2012). This suggests that the ciliary receptor repertoire of progenitor cells differs in white compared to brown adipose tissue.

Ciliary Signaling Pathways Promoting Adipogenesis

While the primary cilium can inhibit MSC commitment and hence adipogenesis, numerous studies in which the primary cilium was genetically ablated in committed pre-adipocytes unequivocally showed that primary cilia on pre-adipocytes are required for adipogenesis. Specifically, removal of the primary cilium via depletion of *Ift88* or *Kif3a*, genes required for the biogenesis and maintenance of primary cilia, in 3T3-L1 preadipocytes or *in vivo* in PDGFRα-lineage perivascular cells, dramatically inhibits adipogenesis (Zhu et al., 2009; Hilgendorf et al., 2019). Consistent with these data, ALMS1 depletion in 3T3-L1 pre-adipocytes inhibits adipogenesis and ALMS patients and mouse models present with adipocyte hypertrophy (Huang-Doran and Semple, 2010; Geberhiwot et al., 2021). Thus, pre-adipocyte primary cilia express ciliary receptors that can promote adipogenesis. To-date, two pro-adipogenic ciliary receptors have been identified.

Zhu et al. (2009) showed that the IGF-1 receptor localizes both to the primary cilium and the plasma membrane in growth-arrested 3T3-L1 pre-adipocytes. Intriguingly, ciliary IGF-1R was more sensitive than IGF-1R localized to the plasma membrane, with insulin stimulation activating the receptor (as

determined by tyrosine phosphorylation) both faster and at lower concentrations of insulin. Downstream, insulin stimulation resulted in the accumulation of activated IRS1 and AKT at the basal body. Thus, the primary cilium may increase the sensitivity of pre-adipocytes to insulin, lowering the threshold at which insulin triggers adipogenesis *in vivo*. Another recent study showed that IGF-1R and AKT can also accumulate in caveolin-containing lipid rafts at the ciliary base upon insulin stimulation, and that this is cilia-dependent (Yamakawa et al., 2021). We do not yet understand why ciliary IGF-1R is more sensitive to insulin stimulation than IGF-1R localized to the plasma membrane.

Further, Dalbay et al. (2015) showed that IGF-1R also localizes to the primary cilium of MSCs induced to undergo adipogenesis. Ciliary recruitment of IGF-1R is IFT-dependent and concurrent with the transient elongation of the primary cilium in differentiating MSCs on Day 3 of MSC adipogenesis. At this same time-point, pre-adipocytes become less responsive to anti-adipogenic Sonic Hedgehog signaling (Forcioli-Conti et al., 2015). Together, this argues that the primary cilium is remodeled upon commitment to express ciliary IGF-1R, which increases the adipogenic potential of the committed pre-adipocyte. What regulates the recruitment of IGF-1R from the plasma membrane to the primary cilium upon lineage commitment remains to be elucidated.

Recently, we identified the presence of an additional pro-adipogenic, ciliary receptor in pre-adipocytes (Hilgendorf et al., 2019). Specifically, we discovered that depletion of TULP3 potently inhibited adipogenesis. TULP3 is an adaptor protein that is required for trafficking of GPCRs to the primary cilium. Subsequent screening for ciliary localization of GPCRs expressed in pre-adipocytes identified that the omega-3 fatty acid receptor FFAR4/GPR120 is ciliary in 3T3-L1 pre-adipocytes, primary isolated mouse and human pre-adipocytes, and pre-adipocytes *in vivo*. Activation of ciliary FFAR4 by the omega-3 fatty acid DHA (docosahexaenoic acid) triggers adipogenesis by activating ciliary cAMP, which in turn activates the guanine-nucleotide exchange factor EPAC. Further downstream, DHA promotes activation of the regulator of chromatin architecture CTCF and looping of enhancers to promoter sites of several adipogenic genes including *Cebpα*, inducing their transcription. Non-FFAR4 ligands, such as saturated and mono-unsaturated fatty acids, do not trigger adipogenesis. Notably, the importance of cAMP and EPAC to the initiation of adipogenesis is well established, as illustrated by the inclusion of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in the standard adipogenic cocktail (Petersen et al., 2008). The primary cilium and the discovery of ciliary FFAR4 now link a physiological ligand, dietary omega-3 fatty acids, to cAMP elevation in pre-adipocytes to promote adipogenesis.

Intriguingly, FFAR4, like IGF-1R, can localize to both the primary cilium and the plasma membrane. In mature adipocytes, which are not ciliated, activation of FFAR4 in the plasma membrane by omega-3 fatty acids promotes glucose uptake in a $G_{\alpha q}$-dependent manner (Oh et al., 2010). This suggests that white adipose tissue can utilize the same ligand-receptor pair and respond to nutritional fluxes in dietary fatty acids by both activating pre-adipocytes (via ciliary FFAR4 and cAMP) and increasing glucose uptake in mature adipocytes (via plasma membrane-localized FFAR4 coupled to $G_{\alpha q}$). We speculate that ciliary vs. plasma membrane localization of FFAR4 enables compartmentalized, differential G_{α} coupling. What regulates trafficking of FFAR4 to the primary cilium in pre-adipocytes remains to be elucidated.

Genetic ablation of cilia inhibits adipogenesis *in vitro* and *in vivo*, showing that one of more ciliary signaling pathways are required for adipogenesis. As described above, both ciliary IGF-1R and ciliary FFAR4 can promote adipogenesis and we do not yet understand their relative importance to this process. Moreover, additional ciliary signaling pathways may exist in pre-adipocytes, including anti-adipogenic ones. Recently, Cook et al. (2021) showed that the melanin concentrating hormone receptor MCHR1 is not only expressed in the primary cilium of hypothalamic neurons, but also in 3T3-L1 pre-adipocytes, and that MCH inhibits adipogenesis. Together, this suggests that the primary cilium may function like a decision center for the committed pre-adipocyte, sensing and integrating both pro- and anti-adipogenic signals to regulate adipogenesis and white adipose tissue expansion in obesity.

CONCLUSION AND FUTURE CHALLENGES

The primary cilium is a signaling hub for the cell. Ciliary signaling mechanisms are critical for energy homeostasis, and this is illustrated by the clinical features of two ciliopathies, Bardet-Biedl syndrome and Alström syndrome. However, primary cilia in different cell types and tissue contexts express a distinct repertoire of receptors. We are only starting to decipher which signaling pathways are organized by the primary cilium in which cells. We do not yet know the functional significance and molecular mechanisms underlying the regulated trafficking of ciliary receptors and signaling components to the primary cilium in certain cells, but not in all ciliated cells.

Mouse genetics show that the primary cilium inhibits commitment to the adipogenic lineage, but is required for adipogenesis of the committed pre-adipocyte. Further, the primary cilium of committed pre-adipocytes can organize both pro-and anti-adipogenic signaling pathways. We postulate that the primary cilium undergoes dynamic remodeling during lineage commitment and adipogenesis. Future investigation using proximity proteomics may unravel how the protein composition of the primary cilium varies according to cell type and differentiation status.

It is intriguing to consider that ciliary receptor composition or ciliary receptor sensitivity differ in distinct contexts associated with differential adipogenic potential, such as pre-adipocytes located in subcutaneous vs. visceral white adipose tissue, in men vs. women, young vs. old, and lean vs. obese individuals. Interestingly, Ritter et al. (2018) showed that human adipose-derived MSCs isolated from lean vs. obese female patients differ in both the prevalence of ciliation and ciliary length. Specifically, both subcutaneous and visceral pre-adipocytes from obese subjects had fewer and shorter primary cilia. This ciliation

defect correlated with decreased adipogenic potential, decreased motility, and increased secretion of inflammatory cytokines. This suggests that ciliary shortening may be a pathogenic consequence of obesity. We do not yet know if changes to ciliary length alter the expression of ciliary receptors and thus may affect the ciliary, adipogenic signaling pathways described above. Future investigations will establish the temporal relationship between the ciliation defect and the adipogenesis defect, whether the ciliation defect of obese adipose-derived MSCs contributes to pathology, and if cilia are restored upon weight loss. Interestingly, addition of inhibitors against cilia-destabilizing kinases rescues ciliary length of obese adipose-derived mesenchymal stem cell and increases both their migratory and adipogenic capacity (Ritter et al., 2019).

Together, these studies suggest targeting of ciliary signaling pathways as a therapeutic strategy to increase the adipogenic potential of pre-adipocytes, including in obese white adipose tissue to promote hyperplastic white adipose tissue expansion and potentially limit the incidence and severity of metabolic disease. Further investigation is necessary to identify potential targets to trigger pro-adipogenic, ciliary signaling pathways directly. We postulate that the primary cilium may function as a decision center, integrating multiple signals to elicit the binary response of triggering adipogenesis. Molecularly, this may be achieved by reaching a threshold amount of second messengers such as calcium or cAMP in the primary cilium to activate adipogenic kinases and exchange factors, and further investigation using live-imaging with ciliary calcium or cAMP sensors is necessary to establish this paradigm. We propose that downstream signaling nodes commonly activated by multiple pro-adipogenic, ciliary signaling pathways may be particularly enticing therapeutic targets to increase the adipogenic potential of pre-adipocytes.

AUTHOR CONTRIBUTIONS

KH wrote the manuscript.

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The Effects of Exercise on White and Brown Adipose Tissue Cellularity, Metabolic Activity and Remodeling

*Jacob D. Garritson and Sihem Boudina**

Department of Nutrition and Integrative Physiology, College of Health, University of Utah, Salt Lake City, UT, United States

Emerging evidence suggests a significant functional role of adipose tissue in maintaining whole-body metabolic health. It is well established that obesity leads to compositional and morphological changes in adipose tissue that can contribute to the development of cardiometabolic disorders. Thus, the function and size of adipocytes as well as perfusion and inflammation can significantly impact health outcomes independent of body mass index. Lifestyle interventions such as exercise can improve metabolic homeostasis and reduce the risk for developing cardiometabolic disorders. Adipose tissue displays remarkable plasticity in response to external stimuli such as dietary intervention and exercise. Here we review systemic and local effects of exercise that modulate white and brown adipose tissue cellularity, metabolic function and remodeling in humans and animals.

Keywords: exercise, training, white adipose tissue, brown adipose tissue, inflammation, progenitors

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***Correspondence:**
Sihem Boudina
sboudina@u2m2.utah.edu

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INTRODUCTION

Obesity continues to be a growing public health concern and has detrimental economic impacts on healthcare systems worldwide. In general, an increase in body mass index (BMI) is associated with increased rates of development and mortality from cardiovascular disease and various cancers (Berrington De Gonzalez et al., 2010). While this relationship is well established, emerging evidence suggests that the location, composition and function of adipose depots may play a role in overall metabolic health independent of BMI. For example, it is believed that *de novo* recruitment and differentiation of preadipocytes following chronic overnutrition results in smaller, metabolically healthy adipocytes when compared to hypertrophic growth of existing adipocytes. Moreover, smaller adipocytes have been shown to be protective against metabolic decline (Lundgren et al., 2007; McLaughlin et al., 2007) and adipocyte size may be used as a predictor for the development of insulin resistance in obese individuals (Lönn et al., 2010; Yang et al., 2012; Verboven et al., 2018). Adipose tissue has traditionally been thought of simply as an energy storage depot, however, we are beginning to appreciate the exceptional plasticity of adipose tissue in response to environmental stimuli and the functional role that it plays in metabolic homeostasis.

Adipose tissue is a complex organ composed of different cells and niches that control key processes including adipogenesis, adipokine secretion and inflammatory responses. While mature

Abbreviations: BMI, body mass index; APC, adipose progenitor cell; SVF, stromal vascular fraction; SUB, subcutaneous; VIS, visceral; WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; Mdm2, murine double minute-2; VEGFA, vascular endothelial growth factor A; HFD, high fat diet; FGF21, fibroblast growth factor 21; PGC-1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

adipocytes are the main functional cells regulating lipid uptake and release, they only constitute a fraction (about 20% in humans) of the cells contained in this tissue (Lee et al., 2013). Other cells such as endothelial cells, immune cells, adipose progenitor cells (APCs), preadipocytes, fibroblasts and neural cells can be found with varying proportions in the stromal vascular fraction (SVF) in each fat depot. Here we will examine the effect of exercise on white and brown adipose tissue cellularity, metabolic function and remodeling in the context of obesity. We will contrast findings in humans and rodents and discuss areas of future investigation.

EFFECTS OF EXERCISE ON ADIPOSE TISSUE CELLULARITY

Effects of exercise on adipose tissue cellularity in rats have been observed as early as the 1970's. This early work highlights the potential of exercise to favorably modulate adipose tissue cellularity, with exercising groups consistently presenting with smaller adipocytes in white adipose tissue (Oscai et al., 1972; Askew et al., 1975; Askew and Hecker, 1976). These effects appear to be consistent across subcutaneous (SUB) and visceral (VIS) fat depots. For example, treadmill exercise in weight-reduced Wistar rats resulted in smaller adipocytes in both VIS and SUB fat (Higgins et al., 2011). Additionally, exercise in rats during weight regain increased cell number and decreased cell size in both SUB and VIS fat pads (Giles et al., 2016). While exercise appears to positively affect adipose tissue cellularity in both VIS and SUB fat pads, these effects may be mediated through different mechanisms. Sertie et al. (2019) found that cessation of exercise resulted in increased size of SUB and VIS fat pads. In VIS fat this increase was due primarily to fat cell hypertrophy, whereas, in SUB fat they observed both fat cell hypertrophy, hyperplasia, and reduced apoptosis (Sertie et al., 2019). Stimulation of lipogenesis and disruption of angiogenesis may contribute to the increase in size and dysfunction of adipocytes with obesity. Exercise is known to stimulate lipolysis (Woo and Kang, 2016), decrease lipogenic gene expression in adipose tissue (Giles et al., 2016), and promote angiogenesis through an increased expression of murine double minute-2 (Loustau et al., 2020). These three mechanisms may explain how exercise promotes smaller, metabolically healthy adipocytes by limiting hypertrophic growth and hypoxia in adipose tissue.

In comparison to the work in animal models described above, evidence for an effect of exercise on adipose tissue cellularity in humans is lacking. This is likely due to limitations in obtaining adipose tissue biopsies, especially in VIS fat pads. Additionally, investigators are finding that the physiology of adipose depots in mice may not directly translate to human adipose tissue (Severinsen et al., 2020). In comparison to the consistent observations in animal models described above, a recent study found that there was no effect of exercise on SUB adipocyte morphology in obese men (Stinkens et al., 2018). While the effects of exercise on adipose tissue cellularity have not been studied extensively in humans, it is well established that structured exercise is effective in reducing fat mass (Sabag et al., 2017; Wedell-Neergaard et al., 2019). It is less clear, however, if similar changes in lipolysis, lipogenesis, and angiogenesis occur in human fat tissue. For example, one study found that adipose tissue triglyceride lipase activity was increased in lean and obese individuals with exercise (Petridou et al., 2017), while others have found no effect of exercise intervention on adipose tissue lipolysis (Stinkens et al., 2018). It should be noted that exercise may only acutely affect WAT lipolysis. Petridou et al. (2017) observed increased lipolysis in lean and obese men for 10–30 min following moderate intensity cycling, which returned to baseline 30 min post-exercise. Conversely, Stinkens et al. (2018) found no change in WAT lipolysis when samples were measured 72 h after the final exercise bout. Additionally, one study found that there was no change in angiogenic genes with weight loss and exercise in human adipose tissue (Cullberg et al., 2013). Evidence in animal models strongly argues that exercise promotes favorable WAT morphology, but evidence for this in humans is lacking. The discrepancy could be caused by heterogeneity of the subjects, their metabolic status and differences in exercise interventions.

Adipose tissue expands primarily through hypertrophic growth of existing adipocytes or *de novo* recruitment and differentiation (adipogenesis) of APCs. Recruitment and differentiation of preadipocytes is believed to be protective against metabolic decline by limiting cell hypertrophy, hypoxia, and adverse tissue remodeling. It has recently been demonstrated that functionally distinct populations of APCs exist in adipose tissue of mice and humans (Marcelin et al., 2017; Hepler et al., 2018; Buffolo et al., 2019; Raajendiran et al., 2019). These distinct populations are identified based on the expression level of the surface markers CD34 and CD9, respectively, with pro-adipogenic APCs described as $CD34^{low}$ and $CD9^{low}$ and anti-adipogenic/pro-fibrotic APCs described as $CD34^{hi}$ and $CD9^{hi}$. It has been demonstrated that the population of pro-fibrotic and pro-inflammatory APCs increases with obesity and promote fibrosis, inflammation, and exacerbate metabolic dysfunction (Marcelin et al., 2017; Hepler et al., 2018; Buffolo et al., 2019). Considering the positive effects of exercise on metabolic health and adipose tissue cellularity, exercise may have the potential to positively regulate APC function. Information on this topic is limited, however, a recent study found that acute exercise in obese adults resulted in a decrease in pro-fibrotic $CD34^{hi}$ APCs and no change in $CD34^{low}$ APCs in abdominal SUB fat in humans (Ludzki et al., 2020). This suggests that exercise may positively regulate the APC pool by limiting the amount of anti-adipogenic, pro-fibrotic APCs. Additional studies are needed to examine whether this effect is limited to SUB fat or can also occur in VIS fat considering the differences in APC populations between these depots.

In addition to modulating white adipose tissue (WAT), treadmill exercise in high fat diet (HFD) mice resulted in an increase in preadipocytes in brown adipose tissue (BAT), with increased adipogenic capacity *in vitro* and increased expression of UCP1 (Xu et al., 2011). Further, a recent study found that exercise-induced secreted factors from skeletal muscle may alter APC function (Zeve et al., 2016). The authors identified R-spondin 3 (Rspo3) from skeletal muscle of trained mice as a potential mechanism that decreases differentiation of white

preadipocytes. The decrease in differentiation of APCs with exercise may seem counter-intuitive, however, this could be explained by improved health of existing adipocytes and reduced apoptosis and adipocyte turnover. More work is needed to uncover the influence of exercise on APCs and adipogenesis, however, this early work suggests that exercise has the potential to positively regulate the APC pool. What remains to be determined is if exercise modulates APC proliferation and differentiation in a depot-specific manner.

REMODELING OF ADIPOSE TISSUE WITH EXERCISE

There are three primary types of adipose tissue that have been very well characterized: white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipose tissue. WAT has long been thought of as an energy storage depot with the primary function of storing excess lipids. It is now well established that WAT functions as an endocrine organ, secreting various adipokines that affect whole body metabolic homeostasis and normal adipose tissue function is disrupted with obesity (Trayhurn and Beattie, 2001). BAT is important for regulation of body temperature by generating heat through non-shivering thermogenesis. This type of adipose tissue is characterized by its multilocular morphology, high density of mitochondria, and increased expression of uncoupling protein 1 (UCP1). Beige adipose tissue is inducible and is recruited in response to beta adrenergic stimulation. Diet-induced obesity reduces the relative amount of BAT and aerobic exercise has been shown to reverse this effect and improve metabolic health (Fu et al., 2021). We are gaining a better appreciation of the complexity of adipose tissue physiology and the external factors that can affect it, such as exercise. Adipose tissue possesses a tremendous flexibility as it continuously remodels by changing its mass and its composition in response to internal and external stimuli. Here, we will discuss the effect of exercise on white and brown adipose tissue remodeling, with a special focus on angiogenesis, fibrosis and immune infiltration. These effects have been summarized in **Figure 1**.

Angiogenic Remodeling

It has been consistently reported that diet-induced obesity results in a significant decrease in angiogenesis and capillary density in white adipose tissue (Kolahdouzi et al., 2019; Loustau et al., 2020). This angiostatic effect results in an increase in hypoxia in adipose tissue that is linked to increased inflammation and insulin resistance (Ye, 2009). There has been early work in humans that observed an acute increase in circulating endothelial progenitor cells with exercise (Rehman et al., 2004; Walther et al., 2009). While this does not directly show an increase in angiogenesis in adipose tissue with exercise, these observations suggest that repeated bouts of acute exercise may increase the potential for angiogenesis and vascular repair (**Figure 1**). The effect of exercise on adipose angiogenesis appears to depend on insulin sensitivity in human. Thus, Walton et al. (2015) showed that aerobic exercise training (3 days/week for 12 weeks) failed to increase vessel density in SUB of insulin resistant subjects whereas insulin sensitive subjects showed an increase. It is worth noting that this training protocol did not decrease body weight or improve insulin sensitivity, which may have influenced the pro-angiogenic effect of exercise in this tissue.

In animal models, one study found that exercise training in obese rats resulted in improved vessel density in both SUB and VIS fat when compared to sedentary obese animals (Kolahdouzi et al., 2019). Interestingly, this effect appears to be more pronounced with higher intensity exercise. For example, aerobic-interval training was more effective at improving vessel density in obese rats compared to lower intensity continuous training (Kolahdouzi et al., 2019). In agreement with these results, a recent clinical trial in men and women with insulin resistance found that both moderate intensity continuous training and sprint interval training improved vessel density in SUB adipose tissue (Honkala et al., 2020). Mechanistically, it has recently been suggested that murine double minute-2 (MDM2) may play a role in exercise-mediated increase in angiogenesis in WAT. *Mdm2* expression increases in both SUB and VIS fat in trained mice and knockdown of *Mdm2 in vitro* resulted in a decrease in capillary growth following adrenergic stimulation (Loustau et al., 2020). In comparison to WAT, less is known about the effects of exercise on angiogenesis in BAT. One study found that exercise in obese mice resulted in an increased expression of vascular endothelial growth factor A (VEGFA) and increased BAT mass relative to sedentary groups (Fu et al., 2021). In contrast, another study found that angiogenic gene expression increased in WAT, but was unchanged in BAT (Lee, 2018). While it is clear that exercise improves adipose tissue function, more work is needed to determine the role of increased angiogenesis in this observation, particularly in BAT.

Fibrotic Remodeling

Obesity is known to cause increased fibrosis in adipose tissue which has been linked to metabolic dysfunction by limiting the healthy expansion of adipose tissue (Khan et al., 2009). Minimal work has been done investigating a direct link between exercise and fibrosis in adipose tissue. One study found that treadmill training in mice fed a HFD decreased fibrosis in VIS adipose tissue measured by decreased Picro Sirius red staining and decreased fibrotic gene expression (Kawanishi et al., 2013b). Additionally, another study investigating fibrosis in obese mice following exercise intervention found that exercise attenuated collagen deposition and fibrotic gene expression in VIS adipose tissue (Li et al., 2021). In comparison, aerobic exercise in obese adults did not affect any markers of fibrosis in SUB adipose tissue (Van Pelt et al., 2017). This discrepancy could be due to the fact that exercise in the human study was self-reported and utilized a different fat depot making it difficult to draw conclusions between species.

Immune Remodeling

Infiltration of inflammatory immune cells in adipose tissue has be extensively studied and is known to contribute to obesity-related inflammation and insulin resistance (Liu and Nikolajczyk, 2019). Exercise is known to be immunomodulatory and thus has the potential to alleviate obesity-induced inflammation in

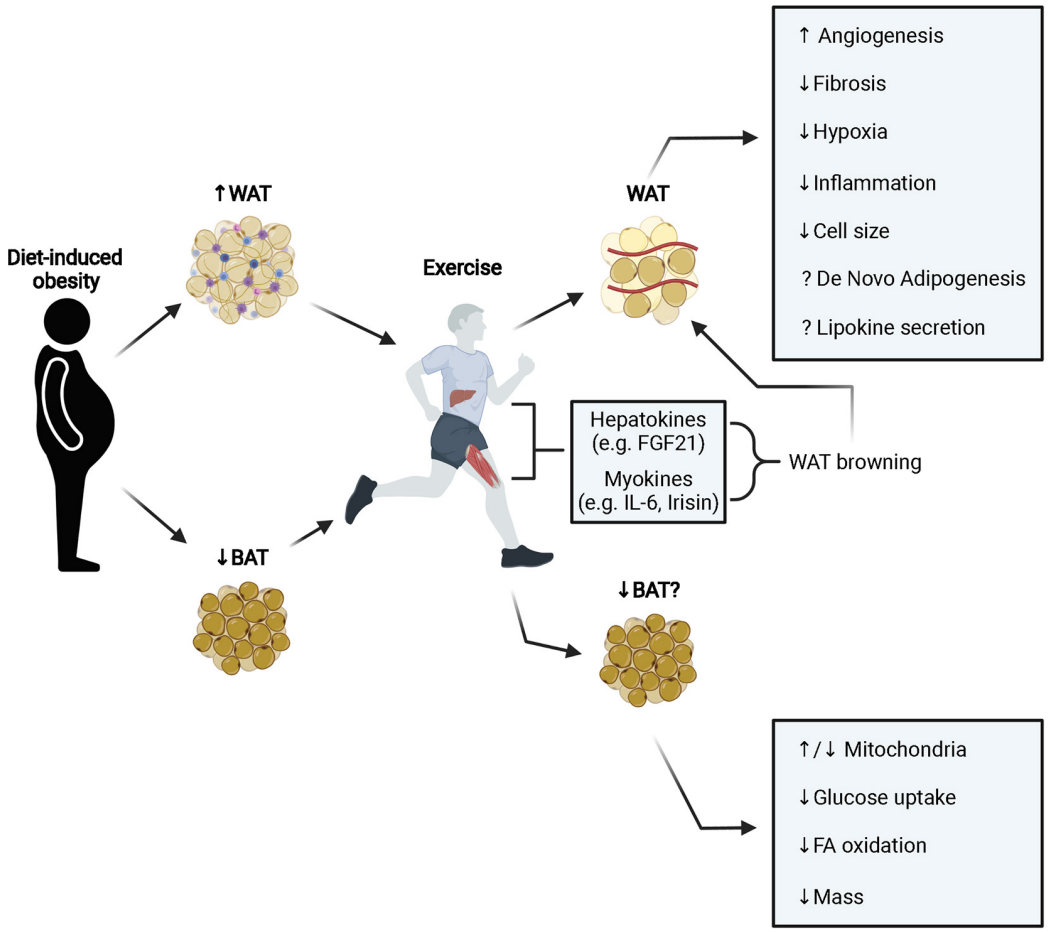

FIGURE 1 | Diet induced obesity leads to pathological expansion and remodeling of WAT resulting in fibrosis, inflammation, and hypoxia. Exercise stimulates browning of WAT (primarily in mice) through the secretion of various hepatokines and myokines that improves metabolic function. Additionally, exercise stimulates angiogenesis, reduces fibrosis, hypoxia, inflammation, and cell size. It is still unclear how exercise may modulate the adipose progenitor niche and influence *de novo* adipogenesis and conflicting results have been observed in terms of lipokine secretion (e.g., adiponectin and leptin). In BAT, obesity decreases tissue mass and metabolic function. Interestingly, exercise appears to also decrease BAT mass and metabolic activity, however, this does not prevent improvements to systemic metabolic health with exercise. White adipose tissue; WAT, brown adipose tissue; BAT, fatty acid; FA, fibroblast growth factor 21; FGF21, interleukin-6; IL-6.

adipose tissue. Macrophages are frequently implicated as a major contributor to adipose tissue inflammation and exercise has been shown to consistently reduce inflammatory macrophages in adipose tissue (Kawanishi et al., 2013a; Geng et al., 2019) and can shift the phenotype to an anti-inflammatory M2 macrophage (Kolahdouzi et al., 2019). In addition to a reduction in macrophage infiltration, exercise can reduce the amount of $CD8^+$ T cell in adipose tissue with obesity (Kawanishi et al., 2013a). While we know that obesity leads to progressive low-grade inflammation associated with impaired insulin action in adipose tissue, even a shorter bout of exercise can reverse these effects. Thus, only 2 h of treadmill running in mice was able to cause noticeable anti-inflammatory effects in SUB fat of mice fed HFD (Macpherson et al., 2015). Mechanistically, acute exercise raised the expression of anti-inflammatory cytokines IL6 and IL10 and shifted the M1 macrophage phenotype toward the M2 phenotype in SUB fat of mice fed the HFD, which led to an insulin sensitizing effect. While this study associates exercise with better immune phenotype in adipose tissue, the causal mechanisms underlying these beneficial effects are still unknown.

Immune infiltrates in response to exercise have not been extensively studied in BAT when compared to WAT, however, one study found that exercise training in mice resulted in increased BAT mass and anti-inflammatory gene expression (Fu et al., 2021), suggesting the anti-inflammatory effects observed in WAT may also occur in BAT.

EFFECTS OF EXERCISE ON WAT METABOLIC FUNCTION

There are well documented effects of exercise on mitochondrial activity (Stanford et al., 2015a,b), gene expression (Stanford et al., 2015a,b), and adipokine secretion (Bradley et al., 2008; Golbidi and Laher, 2014). Most notably, exercise increases peroxisome

proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a) expression in both WAT and BAT (Sutherland et al., 2009), increases UCP1 in WAT (Stanford et al., 2015a), and may increase plasma adiponectin levels (Lehnig and Stanford, 2018). Recent work has also shown that transplanting SUB fat from exercised mice to sedentary mice improves insulin sensitivity (Stanford et al., 2015a,b). The adaptations listed above are usually referred to as WAT beiging, which is specific to SUB depots in different locations. Emerging evidence suggests that the browning effect in WAT seen with exercise may be mediated, in part, by exercise-induced myokines and hormones such as IL-6, fibroblast growth factor 21 (FGF21), and Irisin (Severinsen et al., 2020). For example, FGF21 is known to stimulate WAT browning (Fisher et al., 2012) and acute exercise has been shown to increase circulating FGF21 in both mice and humans (Kim et al., 2013). The pro-browning effect of exercise has been mostly shown in rodents as humans seem refractory to this effect. Indeed, short-term (10 days) endurance training in young lean male subjects did not increase the mRNA expression of brown adipose tissue genes, mitochondrial content or fatty acid oxidation in SUB fat (Camera et al., 2010). Similarly, 6 weeks of endurance training did not increase brown and beige selective gene expression in abdominal or gluteofemoral SUB of obese subjects (Tsiloulis et al., 2018). These discrepant findings in humans and rodents could be due to differences between human and mice in terms of body size, cold tolerance and brown/beige content. The browning effect of exercise has been reviewed extensively and the reader is referred to recent reviews for more info on this topic (Sepa-Kishi and Ceddia, 2016; Townsend and Wright, 2019). Taken together, exercise intervention can improve both local WAT function and systemic metabolic health potentially through improvements in adipokine secretion.

BAT ADAPTATIONS TO EXERCISE

The effects of exercise on BAT are less clear in comparison to WAT adaptations. Considering that both exercise and BAT increase thermogenesis and energy expenditure, some have hypothesized that exercise may downregulate BAT to maintain body temperature (Lehnig and Stanford, 2018). One study found that treadmill exercise in rats resulted in a decrease in BAT mass, UCP1 expression, PGC-1a expression, and fatty acid oxidation (Wu et al., 2014). In human studies, endurance training has been linked to lower metabolic activity in BAT (Vosselman et al., 2015) and decreased insulin-stimulated glucose uptake following training (Motiani et al., 2017). Decreased BAT with exercise may seem counterintuitive to improvements in metabolic health, however, more work is needed to identify mechanisms of exercise-induced adaptions to BAT in both rodent models and humans to fully understand this relationship.

SUMMARY AND CONCLUSION

Exercise improves adipose tissue cellularity, stimulates angiogenesis, and improves metabolic function of WAT (**Figure 1**). Also, exercise may limit fibrosis and pro-inflammatory immune cell infiltration in WAT. Moreover, exercise may improve the endocrine function of WAT by influencing the release of adipokines that attenuate systemic metabolic dysfunction, however, conflicting results have been observed. While many of the adaptations to WAT listed above may occur in BAT, less is known about the exercise-induced adaptations to BAT. While some have observed decreased BAT activity with exercise, it is clear this does not entirely prevent exercise-induced improvements in metabolic health. Taken together, exercise exerts positive changes to adipose tissue that promote healthier adipocytes and improve metabolic homeostasis. However, there are important questions that remain to be addressed: (1) how are stromal vascular cells within adipose tissue including APCs sensing exercise? (2) what are the systemic or local exercise-related signals that these cells are responding to and how is this response mediated at the cellular level? Does exercise impact proliferation/differentiation of APCs? What is the relative contribution of skeletal muscle versus BAT/beige adipose tissue to thermogenesis during exercise? Future studies are needed to address these topics which will help understand the mechanisms underlying the exercise effect on adipose tissue and metabolic health.

AUTHOR CONTRIBUTIONS

JG wrote the manuscript. SB critically revised the manuscript. Both authors approved the submitted version.

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Transcription Factor E2F1 Knockout Promotes Mice White Adipose Tissue Browning Through Autophagy Inhibition

Mingchen Xiong†, Weijie Hu†, Yufang Tan, Honghao Yu, Qi Zhang, Chongru Zhao, Yi Yi, Yichen Wang, Yiping Wu and Min Wu**

Department of Plastic and Cosmetic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Ryan Ceddia, Vanderbilt University Medical Center, United States

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Jin Young Huh, Seoul National University, South Korea

***Correspondence:**
Yiping Wu
tongjiplastic@163.com
Min Wu
wumin@hust.edu.cn

† These authors have contributed equally to this work and share first authorship

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Obesity is associated with energy metabolic disturbance and is caused by long-term excessive energy storage in white adipose tissue (WAT). The WAT browning potentially reduces excessive energy accumulation, contributing an attractive target to combat obesity. As a pivotal regulator of cell growth, the transcription factor E2F1 activity dysregulation leads to metabolic complications. The regulatory effect and underlying mechanism of E2F1 knockout on WAT browning, have not been fully elucidated. To address this issue, in this study, the *in vivo* adipose morphology, mitochondria quantities, uncoupling protein 1 (UCP-1), autophagy-related genes in WAT of wild-type (WT) and E2F1$^{-/-}$ mice were detected. Furthermore, we evaluated the UCP-1, and autophagy-related gene expression in WT and E2F1$^{-/-}$ adipocyte *in vitro*. The results demonstrated that E2F1 knockout could increase mitochondria and UCP-1 expression in WAT through autophagy suppression in mice, thus promoting WAT browning. Besides, adipocytes lacking E2F1 showed upregulated UCP-1 and downregulated autophagy-related genes expression *in vitro*. These results verified that E2F1 knockout exerted effects on inducing mice WAT browning through autophagy inhibition *in vivo* and *in vitro*. These findings regarding the molecular mechanism of E2F1-modulated autophagy in controlling WAT plasticity, provide a novel insight into the functional network with the potential therapeutic application against obesity.

Keywords: E2F1 transcription factor, obesity, white adipose tissue (WAT), browning, autophagy

INTRODUCTION

Obesity is a serious global public health problem and a preventable risk factor for hypertension, diabetes, breast cancer, and other cancers (Marie et al., 2015). Obesity is caused by the long-term excessive energy storage in WAT, including visceral adipose tissue and subcutaneous adipose tissue (Mori et al., 2014). Brown adipose tissue (BAT) possesses the ability to expend energy as heat,

Abbreviations: WAT, White Adipose Tissue; BAT, Brown Adipose Tissues; UCP-1, Uncoupling Protein-1; E2F1, E2f Transcription Factor 1; MAP1LC3, Microtubule-Associated Protein-1 Light Chain-3; SQSTM1/p62, Sequestosome-1; ATG, Autophagy-Related Gene; WT, Wild-Type; ADSCS, Adipose Stem Cells; FBS, Fetal Bovine Serum; PBS, Phosphate-Buffered Saline; H&E, Hematoxylin-Eosin; IHC, Immunohistochemistry; TEM, Transmission Electron Microscopy; CTRP5, C1q/TNF-related protein 5.

and is associated with UCP-1 expression. UCP-1 is located in the inner membrane of brown adipocytes mitochondria and uncouples the respiratory chain from oxidative phosphorylation through a proton conductance pathway (Villarroya et al., 2018). It is well-known that obesity is closely associated with adipose tissues, which play central roles in metabolic regulation (Kaisanlahti and Glumoff, 2019). Thus, further research on adipose physiological effects is needed to improve the understanding of metabolism and restore metabolic health.

When stimulated to a certain extent, brown adipocyte-like cells mainly appear in the subcutaneous storage of WAT, which is called "beige adipose tissue." This metabolic process is called "WAT Browning" (Wang et al., 2015). Similar to BAT, beige adipose tissues present many small multilocular lipid droplets and densely packed mitochondria, with remarkable heat production ability through UCP-1-mediated mechanisms (Wang and Seale, 2016). There are several methods to activate thermogenic beige adipocytes within WAT during browning, including transcriptional and epigenetic regulation, lifestyle and environmental change, and endocrine factors and hormones secretion (Rabiee, 2020). Promoting the formation of beige adipocytes in WAT may potentially reduce the negative effects of excessive energy accumulation and improve overall metabolic health.

E2f Transcription Factor 1, the first member of the E2F transcription factor family, plays an important role in the regulation of the cell cycle, apoptosis, senescence, and DNA-damage response (Xiao et al., 2020). E2F1 has the ability to bind various gene promoter regions to regulate different biological functions (Wu et al., 2014; Ertosun et al., 2016). More importantly, E2F1 has been considered as a key regulator of metabolism both in normal and pathological conditions (Denechaud et al., 2017). Several metabolic tissues, including WAT, express higher E2F1 binding to the promoters of stress signaling genes in human obesity (Haim et al., 2017; Maixner et al., 2020). E2F1 repressed energy homeostasis and mitochondrial functions in muscle and brown adipose tissue, thus the mice lacking E2F1 were resistant to diet-induced obesity (Blanchet et al., 2013). The overall metabolic role of E2F1 in obesity suggests that E2F1 might play a significant role in gene regulation in adipose plasticity, thus needing further research.

Autophagy is a highly conserved cellular self-digestion pathway (Sun et al., 2018). Autophagosomes with bilayer membrane vesicles can trap the degraded substances and then fuse with the lysosome to degrade damaged cellular proteins and dysfunctional organelles (Wang et al., 2017, 2019). Amino acids and other degradation products produced by autophagy are recycled in the cytoplasm and help to maintain homeostasis (Yoshihara et al., 2014). Notably, E2F1 was proved to bind to regions encompassing the promoters of autophagy genes, thereby up-regulating the expression of microtubule-associated protein-1 light chain-3 (MAP1LC3), autophagy-related gene-1 (ATG1), and ATG5, and down-regulating the expression of sequestosome-1 (SQSTM1/p62) (Polager et al., 2008; Korah et al., 2016). In the setting of obesity, E2F1 expression was elevated in adipose tissue, which was relevant to the increased autophagy genes expression and the activated autophagy process (Haim et al., 2015). The correlation between E2F1, autophagy activity, and adipose tissue has great potential for balancing energy metabolism and controlling obesity.

Therefore, in this study, we aimed to investigate the regulatory effect of E2F1 knockout on WAT browning both *in vitro* and *in vivo*, as well as the potential autophagy-related mechanism. In general, we detected the adipose morphology, mitochondria quantities, UCP-1, autophagy-related genes in WT and E2F1$^{-/-}$ mice *in vivo*. Furthermore, we evaluated UCP-1, and autophagy-related gene expression in WT and E2F1$^{-/-}$ adipocytes *in vitro*. Lastly, our results confirm that E2F1-mediated autophagy is a novel pathway to regulate WAT browning, which is conducive to the development of therapeutic strategies for metabolic diseases such as obesity.

MATERIALS AND METHODS

Animals

The E2F1$^{-/-}$ mice were obtained from the Jaxson laboratory[1] and were bred, maintained, and operated in the Animal Experimental Center of Tongji Hospital, Huazhong University of Science and Technology. In this study, the E2F1$^{-/-}$ mice (male, 6–8 w) were compared with WT littermates of the same age and gender. All mice were allowed free access to food and water under controlled conditions (12/12 h light/dark cycle with humidity of 60 ± 5%, and a temperature of 22 ± 3°C). All surgical procedures were performed under anesthesia. The study was performed following guidelines on animal experimentation of the Ethical Committee of the Tongji Hospital.

Isolation and Culture of Adipose Stem Cells (ADSCs)

The subcutaneous adipose tissues were extracted from the inguinal region of mice, and the superficial fascia and blood vessels were removed. Then, the adipose tissues were washed with phosphate-buffered saline (PBS) and minced into small pieces, followed by digestion using 0.15% type I Collagenase (Sigma, United States) under 37°C for 35 min. The Dulbecco's modified Eagle's medium (DMEM) (Gibco, United States) high glucose medium containing 20% fetal bovine serum (FBS) (Gibco, United States) was added to terminate the digestion, followed by filtration using a 75 μm screen mesh and centrifugation at 400 × *g* for 5 min. Lastly, the cells were added to the primary medium with 20% FBS and inoculated into a culture dish, and placed in an incubator with 5% CO_2 at 37°C. After 24 h, the non-adherent cells were removed. These obtained ADSCs were passaged (1:3) using the culture medium containing 10% FBS every 2–3 days when they reached approximately 80–90% confluence.

Adipogenic Differentiation and Oil Red O Staining

In a 6-well plate, 1 × 10^5 ADSCs/cm^2 were plated in complete media for 24 h. Differentiation was initiated by

[1]http://jaxmice.jax.org/strain/002785.html

replacing complete media with adipogenic differentiation media, which contained 1 μM dexamethasone, 10 μg/μL insulin, 0.5 mM isobutylmethylxanthine, and 200 μM indomethacin. Cells were incubated for 21 days, and the media changed every 3 days. Murine 3T3-L1 preadipocyte cells were cultured and differentiated as previously described (Hwang and Lee, 2020). To assess the effect of autophagy on adipocyte differentiation, the autophagic inhibitor (3-MA), an inhibitor of phosphoinositide 3 kinase that specifically inhibits autophagosome formation at 5 mM (MCE, China), were added to the media before adipogenic differentiation.

The Oil Red O staining was used to evaluate adipogenic differentiation. The cells were rinsed with PBS and fixed with 4% formaldehyde, then were rinsed with distilled water, dehydrated with 60% isopropanol, stained with Oil Red O, and finally were observed under a light microscope (SDPTOP, China).

Flow Cytometry Assay

The fourth-passage ADSCs were tested by flow cytometry analysis. Briefly, the adherent cells were harvested by trypsinization, centrifuged and washed with sterile PBS, and finally resuspended in PBS. For biomarker identification, cell suspensions were incubated with anti-CD29-APC, anti-CD90-FITC, anti-CD105-APC, anti-CD34-Alexa Fluor 647, and anti-CD44-PE·Cy^{TM}7 antibodies (Becton Dickinson, United States). Finally, ADSCs were analyzed using a FACS Calibur cytometer (Becton Dickinson, United States) and Flow Jo VX software.

Hematoxylin-Eosin (H&E) Staining

The adipose tissues were fixed in 4% formaldehyde and embedded in paraffin. Tissues were then cut into sections of 3 μm thickness with a microtome, deparaffinized, and stained with H&E. H&E images were taken with the microscope (SDPTOP, China) at 200 × and 400 × magnification. The adipocytes area quantification, represented by adipocyte diameter was performed in 6 fields of each preparation.

Immunohistochemistry (IHC)

For IHC staining of UCP-1 expression level in WAT, formalin-fixed and paraffin-embedded adipose tissues were deparaffinized to prevent non-specific protein binding. Samples were then incubated with diluted primary anti-UCP-1 antibody overnight at 4°C. After the wash steps, the sections were incubated with secondary antibody for 2 h and were visualized by incubating with DAB substrate. The UCP-1 expressions were evaluated by high-power light microscopy examination. Images were taken with the microscope (SDPTOP, China) at 200 × and 400 × magnifications. The expression level of UCP-1 in adipose tissue was indicated with a stained positive index (integrated density/area).

Transmission Electron Microscopy (TEM)

A small portion of WAT sections (1 mm^3) were fixed with fresh TEM fixative at 4°C overnight and then washed the tissues using 0.1 M PB. Tissues were fixed with 1% OsO4 in 0.1 M PB (pH 7.4) for 2 h at room temperature. The tissues were dehydrated by a series of graded concentrations of alcohol and acetone and embedded in araldite. The resin blocks were cut to 60–80 nm thin and then stained with 2% uranium acetate and lead citrate, and observed under the TEM (Hitachi, Japan). The number of mitochondria in a region containing the complete view of an adipocyte was counted.

Cell Transfection

The E2F1 siRNAs were purchased from Ribobio (Guangzhou, China). The sequences for the AGER siRNA used for the experiments were as follows: E2F1 siRNA1: 5′-GCAGAAACG GCGCATCTAT-3′; E2F1 siRNA2: 5′-GGGTGAGGGCATTAGA GAT-3′. ADSCs were plated in six-well plates and transient transfection using Lipofectamine 3000 Reagent (Invitrogen, United States) according to the manufacturer's instructions.

RNA Isolation, cDNA Synthesis, and qRT-PCR

The total RNA was isolated from the inguinal WAT of WT and E2F1$^{-/-}$ mice using TRIzol (Takara, Japan), and 1 μg of RNA in a final reaction volume of 20 μL was then reversed-transcribed into complementary DNA (cDNA) using the 1st Strand cDNA Synthesis SuperMix (Yeason, China) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green Master Mix (Yeason, China) and was detected with an ABI Q1 PCR System (Thermo Fisher Scientific, United States). Threshold cycles of primer probes were normalized to the housekeeping gene β-actin and translated to relative values. The following primer set sequences were used, LC3-II (forward 5′- TTATAGAGCGATACAAGGGGG AG-3′, reverse 5′-CGCCGTCTGATTATCTTGATGAG-3′), p62 (forward 5′-GAGGCACCCCGAAACATGG-3′, reverse 5′-ACTTATAGCGAGTTCCCACCA-3′), ATG5 (forward 5′-TG TGCTTCGAGATGTGTGGTT-3′, reverse 5′-ACCAACGTCA AATAGCTGACTC-3′), ATG7 (forward 5′-TCTGGGAAGCC ATAAAGTCAGG-3′, reverse 5′-GCGAAGGTCAGGAGCAGA A-3′), UCP-1 (forward 5′-GTGAACCCGACAACTTCCGA A-3′, reverse 5′-TGCCAGGCAAGCTGAAACTC-3′), E2F1 (forward 5′-GAGAAGTCACGCTATGAAACCTC-3′, reverse 5′-CCCAGTTCAGGTCAACGACAC-3′), and β-actin (forward 5′-GGCTGTATTCCCCTCCATCG-3′, reverse 5′-CCAGTTGG TAACAATGCCATGT-3′).

Western Blot Analysis

Briefly, the protein concentration was quantified by the BCA method. The proteins were separated using 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for 1.5 h and with 1:1000 dilutions of anti-LC3 antibody (Proteintech, United States), anti-p62 antibody (Proteintech, United States), anti-UCP-1 antibody (Proteintech, United States), anti-E2F1 antibody (ABclonal, China), and anti-β-actin antibody (ABclonal, China) overnight at 4°C. The membrane was then washed with TBST and incubated with a 1: 4000 diluted HRP-conjugated secondary antibody (ABclonal, China) for 1 h at 37°C. At last, the immunoreactive bands were

detected by using an enhanced chemiluminescent (ECL) reagent kit (Yeason, China), and were scanned by ChemiDoc XRS$^+$ imaging system (Bio-Rad, United States) and analyzed by Image Lab software. The analysis represented the ratio of the target protein expression relative to β-actin expression.

Statistical Analysis

All experiments were performed with at least three replicates per group. Data are shown as the means ± standard deviation (means ± SD). Two treatment groups were compared by Student's *t*-test. Statistical analysis was conducted using GraphPad Prism 9.0 software, and statistical significance was declared as $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, respectively.

RESULTS

Effects of E2F1 Knockout on Body Weight and Inguinal Adipose Tissue Morphology in Mice

Firstly, we intended to explore the impact of E2F1 knockout on the general morphology of mice and WAT. There was no obvious difference in the size and weight of subcutaneous inguinal WAT between the WT and E2F1$^{-/-}$ groups (**Figure 1A**). Mice weight growth curve was measured weekly from the birthday, and there was no significant difference in weight between the two groups (**Figure 1B**). Interestingly, the microscopic observation revealed that the E2F1$^{-/-}$ WAT exhibited substantially denser and smaller adipocytes (**Figure 1C**). The adipocyte diameter in the E2F1$^{-/-}$ group was significantly shorter than that in the WT group ($P < 0.001$) (**Figure 1D**). These results certified that E2F1 knockout did not change the body and WAT weight, but reshaped the morphology of adipose. Thus, E2F1$^{-/-}$ WAT might tend to be beige adipose morphology with multiple small lipid droplets.

E2F1 Knockout Increased the Number of Mitochondria and the UCP-1 Expression in WAT *in vivo*

Emerging evidence has confirmed that the increased number of mitochondria and high UCP-1 expression in WAT are strong indicators for browning. Consequently, we hypothesized that E2F1 knockout might induce WAT browning activity, which is related to mitochondria and UCP-1 in WAT. Intriguingly, E2F1$^{-/-}$ WAT showed small intracellular lipid droplets and dramatically increased mitochondria, compared with the WT WAT ($P < 0.01$) (**Figures 2A,F**). In the WT group, the presence of multiple degraded vesicles was consistent with the structure

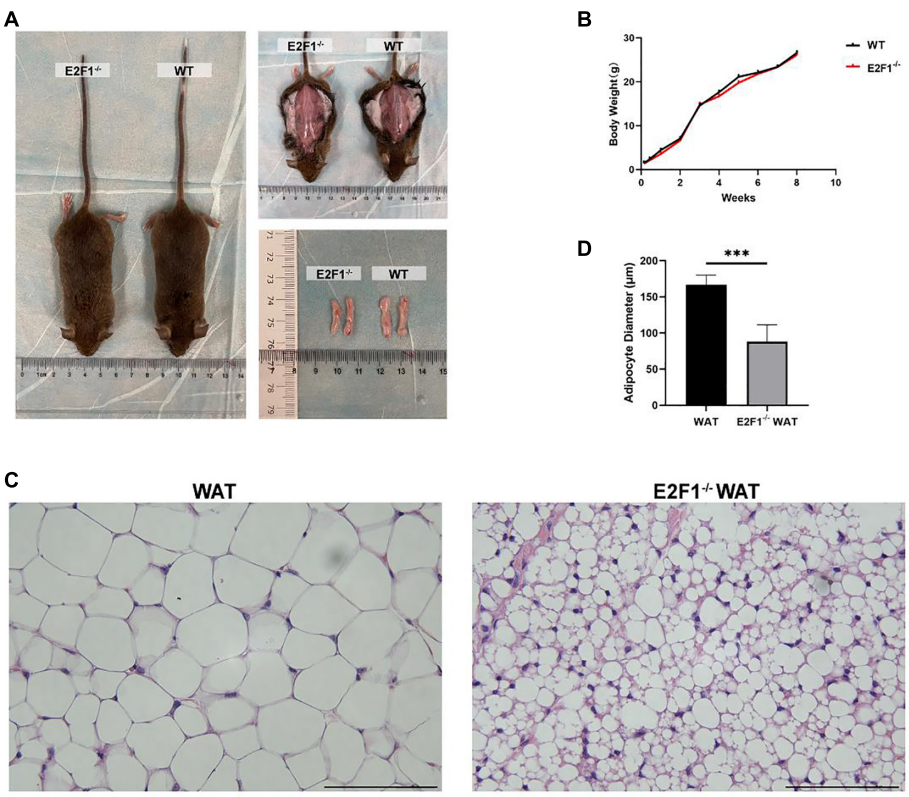

FIGURE 1 | Effects of E2F1 knockout on body weight and inguinal adipose tissue morphology in mice. **(A)** Gross view of mice, *in situ* subcutaneous WAT, and *ex vivo* dissected WAT in WT and E2F1$^{-/-}$ group. **(B)** The bodyweight curve of the WT and E2F1$^{-/-}$ mice ($n = 10$) for 8 weeks. **(C)** Representative H&E staining images of WAT in WT and E2F1$^{-/-}$ mice. Scale bar, 100 μm. Magnification, 400×. **(D)** Quantitative analysis of adipocyte diameter in WT and E2F1$^{-/-}$ WAT. $^{***}P < 0.001$.

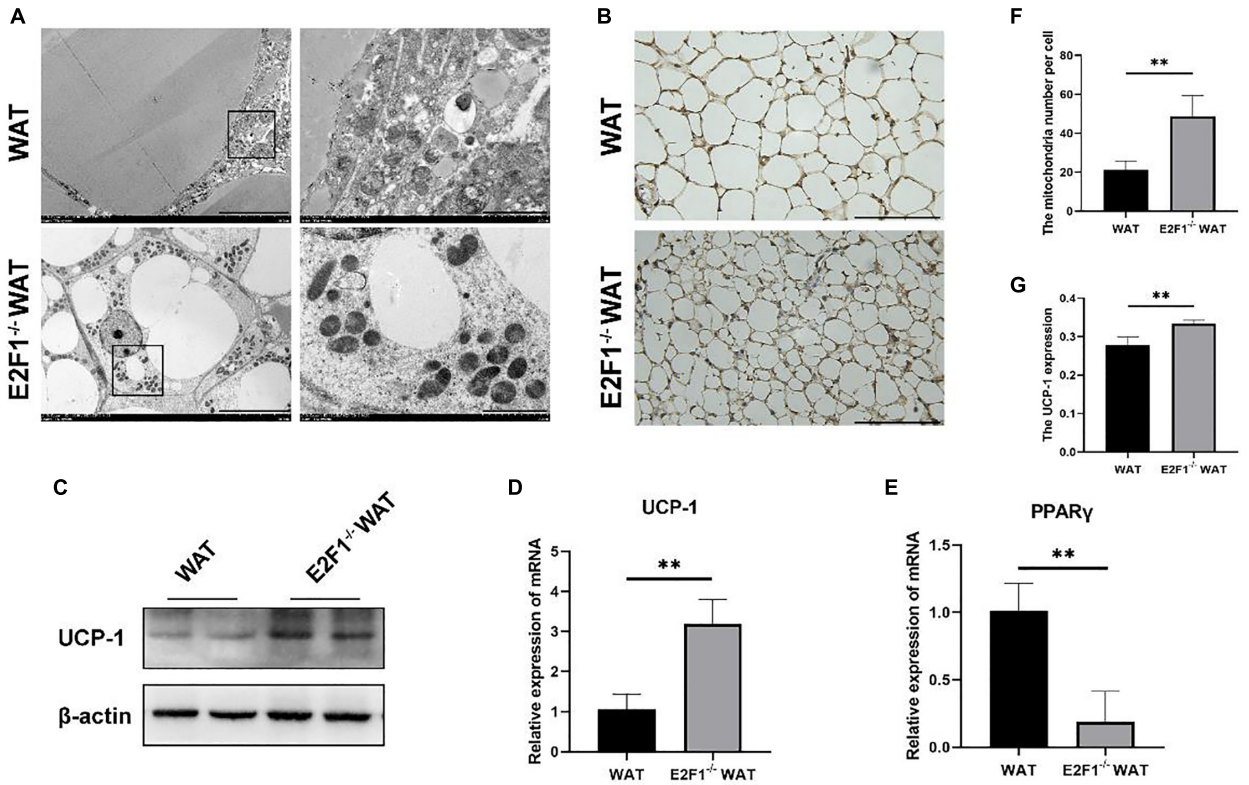

FIGURE 2 | E2F1 knockout increased the mitochondrial number and the UCP-1 expression in WAT *in vivo*. **(A)** Representative TEM images of WAT ultrastructures in WT and E2F1$^{-/-}$ group. Scale bar, 10 μm (left) and 2 μm (right). **(B)** Representative IHC pictures of UCP-1 protein (brown stain) in WAT of WT and E2F1$^{-/-}$ mice. Scale bar, 100 μm. Magnification, 400×. **(C)** Representative Western Blot images of UCP-1 protein level in WT and E2F1$^{-/-}$ WAT, $n = 3$. **(D)** The UCP-1 mRNA levels in WT and E2F1$^{-/-}$ WAT in mice by qRT-PCR. **(E)** The PPARγ mRNA levels in WT and E2F1$^{-/-}$ WAT in mice by qRT-PCR. **(F)** Quantitative analysis of mitochondrial number per cell in WT and E2F1$^{-/-}$ WAT. **(G)** The expression level of UCP1 staining in WAT was indicated with a stained positive index (integrated density/area) in WT and E2F1$^{-/-}$ WAT. *$P < 0.05$, **$P < 0.01$.

of autophagosomes and autophagolysosomes, which suggested that autophagy activity might occur. The UCP-1 of IHC staining demonstrated that there were more UCP-1 expression and beige cells formation in the E2F1$^{-/-}$ group ($P < 0.01$) (**Figures 2B,G**). Moreover, the Western Blot analysis showed that the expression of UCP-1 protein was significantly increased in the E2F1$^{-/-}$ WAT ($P < 0.05$) (**Figures 2C**, **3C**). Similarly, the qRT-PCR results consistently confirmed that E2F1$^{-/-}$ WAT possessed higher mRNA expression of UCP-1 than WT WAT ($P < 0.05$) (**Figure 2D**). In addition, to validate the adipogenic gene expression during WAT browning, the PPARγ mRNA expression in the E2F1$^{-/-}$ WAT group was detected, which exhibited a downward trend compared with the WT group (**Figure 2E**). These data indicated that E2F1 knockout induced WAT into beige adipose tissue by increasing mitochondrial number and expressing highly UCP-1 both on mRNA and protein levels.

E2F1 Knockout Affected the Expression of Autophagy-Related Proteins in WAT *in vivo*

Since the autophagy-related autophagosomes expression was decreased in E2F1$^{-/-}$ WAT, we further explored the underlying autophagy mechanism of E2F1 in regulating WAT browning. The E2F1$^{-/-}$ group demonstrated the protein expressions of E2F1 and LC3-II/I were significantly decreased, and p62 was increased inversely ($P < 0.05$, $P < 0.01$) (**Figures 3A,C**). Compared with the WT group, the reduced mRNA expressions of E2F1, LC3-II, ATG5, and ATG7 in E2F1$^{-/-}$ WAT were further confirmed ($P < 0.05$, $P < 0.01$) (**Figure 3B**). The results suggested that autophagy participated in the intracellular beige adipogenesis, while E2F1$^{-/-}$ WAT exhibited lower autophagy gene expression than WT WAT.

E2F1 Knockout Increased UCP-1 Expression in Adipocytes *in vitro*

The cultured WT and E2F1$^{-/-}$ ADSCs both presented a typical fibroblast-like morphology, which was similar to previous studies (**Figure 4A**). Flow cytometry analysis also verified the surface markers of obtained ADSCs, by staining positively for CD29, CD90, CD105, and CD44, and negatively for CD31 and CD34 (**Figure 4C**). The Oil Red O staining images indicated the presence of oil droplet formation, suggesting the acquisition of mature adipocytes *in vitro* (**Figure 4B**). To demonstrate the role of E2F1 in beige adipocyte modulation *in vitro*, the UCP-1

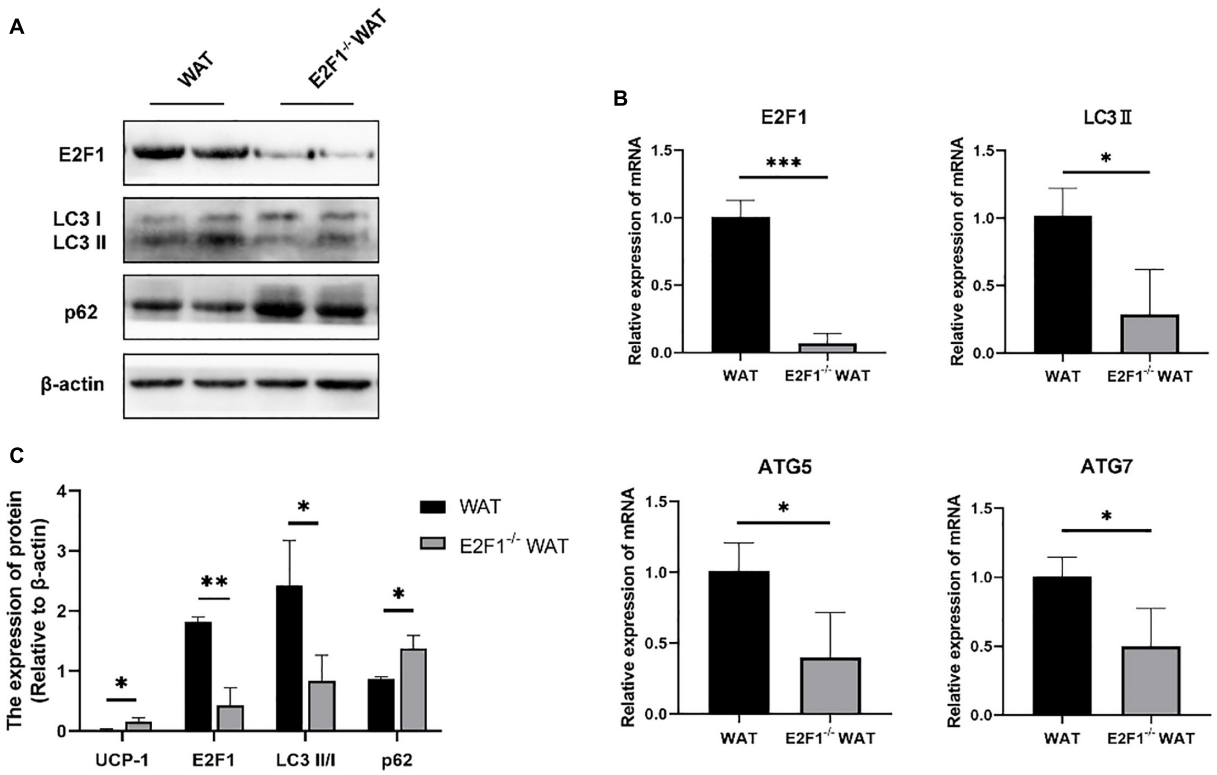

FIGURE 3 | E2F1 knockout affected the expression of autophagy-related proteins in WAT in mice. **(A)** Representative Western Blot image of E2F1, LC3-II/I, and p62 protein levels in WT and E2F1$^{-/-}$ WAT. **(B)** mRNA levels of E2F1, LC3-II, ATG5, and ATG7 genes in WT and E2F1$^{-/-}$ WAT by qRT-PCR. **(C)** Quantitative analysis of the protein expressions relative to β-actin, $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

protein, and mRNA expression were analyzed. The Western Blot analysis testified that E2F1$^{-/-}$ adipocytes possessed significantly high expression of UCP-1 ($P < 0.05$) (**Figures 5A,E**). The qRT-PCR results further proved the higher mRNA expression of UCP-1 in the E2F1$^{-/-}$ group than WT group *in vitro* ($P < 0.01$) (**Figure 5B**). Thus, E2F1 knockout could increase the UCP-1 expression in adipocytes, which was necessary to the white-to-beige process.

E2F1 Knockout Reduced Autophagy Gene Expression in Adipocytes *in vitro*

The autophagy inhibition was proved to occur in the E2F1$^{-/-}$ WAT *in vivo*, thus participating in the WAT browning remodeling. To confirm that the role of E2F1 in beige adipocytes regulation depends on autophagy *in vitro*, the autophagy-related genes were detected. At the protein level, the LC3-II/I expression was decreased, and the p62 expression was increased in the E2F1$^{-/-}$ adipocytes group, compared with the WT adipocyte group ($P < 0.05$, $P < 0.01$) (**Figures 5C,E**). Also, the reduced mRNA expression of LC3-II and the induced mRNA expression of p62 were further verified in E2F1$^{-/-}$ adipocytes ($P < 0.05$, $P < 0.01$) (**Figure 5D**). The results proved that E2F1 could be involved in the upregulation of autophagy-related genes in adipocytes *in vitro*, and E2F1 knockout inhibited autophagy activity to promote WAT browning.

Autophagy Inhibition Increased the UCP-1 Expression, Without Reduced E2F1 Expression in Adipocytes *in vitro*

Firstly, we used the 3T3-L1 cell line as an *in vitro* model to generate adipocyte-like cells. To figure out whether the reduced autophagy could affect the UCP-1 and E2F1 expression, the 3T3-L1 adipocytes were treated with autophagy inhibitor 3-MA at the early differentiation stage. After adipogenic induction, the inhibition of autophagy using 3MA resulted in lower autophagy activity. The LC3-II and ATG5 mRNA expressions were relatively reduced compared to the normal cultured 3T3-L1 cells (**Figures 6A,B,I**). The p62 mRNA expression was increased in the 3T3-L1 + 3MA group (**Figure 6B**). Furthermore, the 3MA-treated 3T3-L1 adipocytes also expressed higher UCP-1 on mRNA and protein levels, and lower PPARγ mRNA expression than the 3T3-L1 adipocytes, which demonstrated that the autophagy inhibition leads to reduced lipogenesis and enhanced WAT browning transition (**Figures 6A,C,I**). Importantly, there was no significant difference in E2F1 expression between the 3T3-L1 + 3MA group and 3T3-L1 group (**Figure 6D**). It was indicated that the autophagy inhibition in the WAT could be induced by E2F1 knockout, leading to the increased mitochondria and UCP1 expression in the browning WAT.

Next, three groups were designed to examine the effect of E2F1 on autophagy inhibition and WAT browning, including

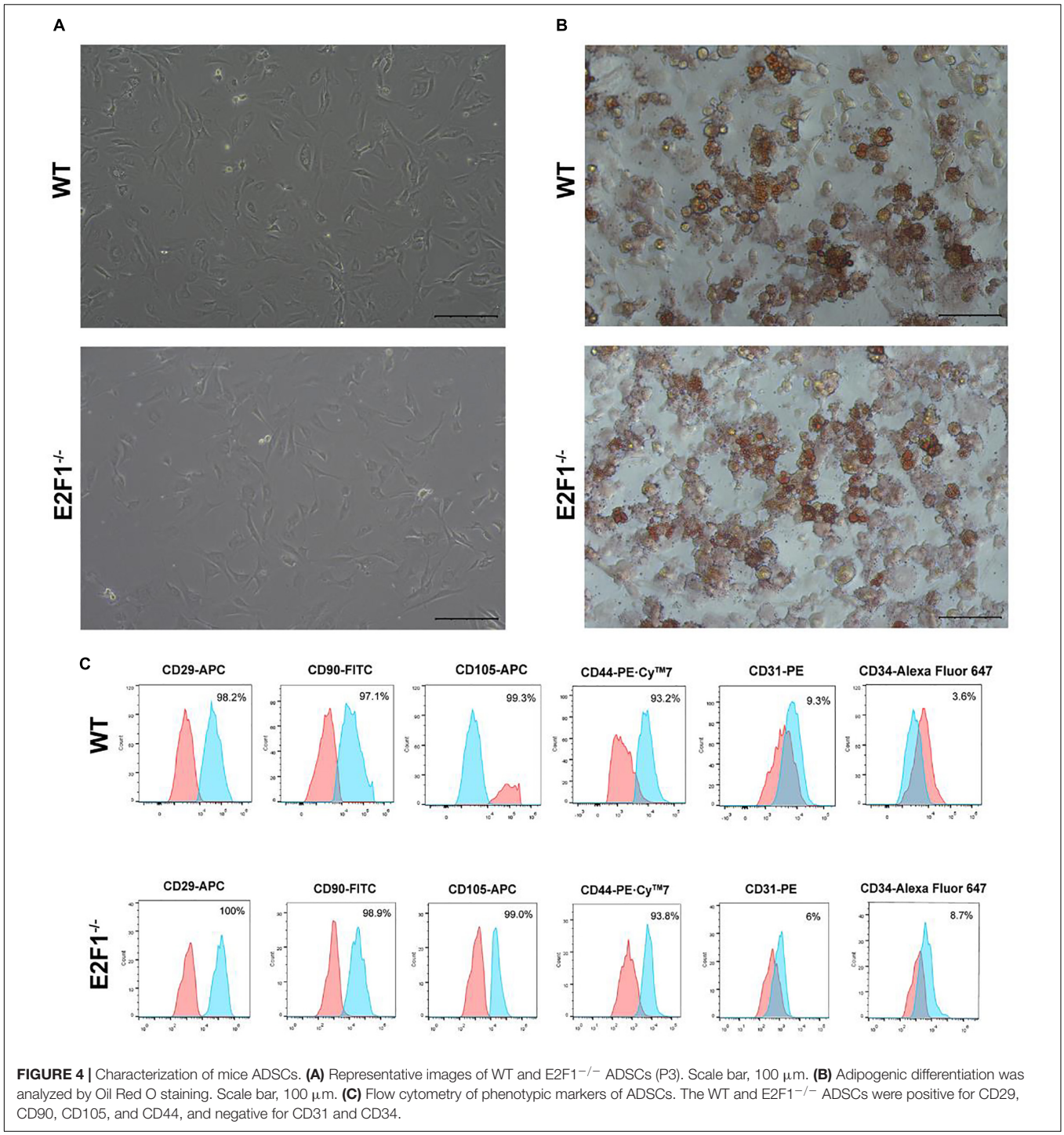

FIGURE 4 | Characterization of mice ADSCs. **(A)** Representative images of WT and E2F1$^{-/-}$ ADSCs (P3). Scale bar, 100 μm. **(B)** Adipogenic differentiation was analyzed by Oil Red O staining. Scale bar, 100 μm. **(C)** Flow cytometry of phenotypic markers of ADSCs. The WT and E2F1$^{-/-}$ ADSCs were positive for CD29, CD90, CD105, and CD44, and negative for CD31 and CD34.

ADSCs + 3MA group, ADSCs + E2F1 siRNA group, and ADSCs + siNC group. After ADSC adipogenic differentiation, the expression levels of E2F1 mRNA were reduced by si-E2F1 in ADSCs (**Figure 6E**). The 3MA addition did not lead to a decrease of E2F1 mRNA expression in the WT ADSCs + 3MA group. The Western Blot analysis proved that the LC3-II/I protein expression indeed was reduced in both ADSCs + 3MA group and ADSCs + E2F1 siRNA group (**Figures 6F,J**). The ADSCs treated with E2F1 siRNA mimicked the effect of autophagy inhibitor to increase the UCP-1 expression at protein and mRNA level (**Figures 6F,G,J**). In addition, the p62 mRNA expression was relatively induced in ADSCs + 3MA group and ADSCs + E2F1 siRNA group, compared to the ADSCs + siNC group (**Figure 6H**). These results further confirmed that E2F1 deficiency could be involved in UCP-1 expression via autophagy inhibition in WAT and adipocytes.

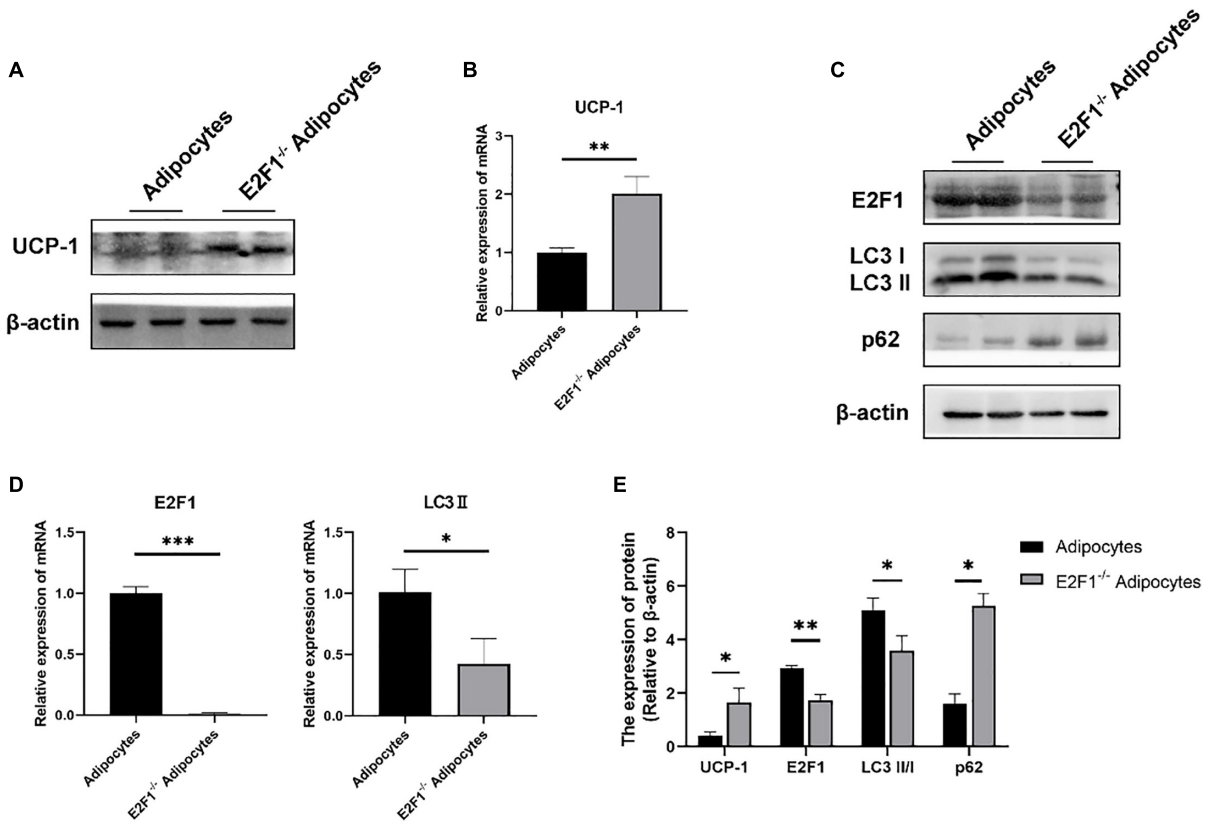

FIGURE 5 | E2F1 knockout increased UCP-1 expression and reduced autophagy gene expressions in adipocytes *in vitro*. **(A)** Representative Western Blot image showing the level of UCP-1 protein in adipocytes and E2F1$^{-/-}$ adipocytes. **(B)** qRT-PCR analyses of UCP-1 expression at the mRNA level in adipocytes and E2F1$^{-/-}$ adipocytes. **(C)** Representative Western Blot image of E2F1, LC3-II/I, and p62 protein levels in adipocytes and E2F1$^{-/-}$ adipocytes. **(D)** The qRT-PCR analyses of E2F1, and LC3-II expressions at the mRNA level in adipocytes and E2F1$^{-/-}$ adipocytes. **(E)** Quantitative analysis of the protein expressions relative to β-actin, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DISCUSSION

Obesity is caused by a metabolic disturbance between energy intake and energy expenditure. Brown and beige fats specialize in adaptive thermogenesis, expend energy as heat by decoupling mitochondrial respiratory chains and ATP synthesis, and play an increasingly important role in glucose homeostasis, insulin sensitivity, and lipid metabolism (Kaisanlahti and Glumoff, 2019). E2F1 is a widely recognized cell cycle regulatory transcription factor, and its role in human obesity is being emphasized (Haim et al., 2015). Imbalanced E2F1 activity leads to metabolic complications associated with obesity. In addition, the regulation of autophagy activity adapted to individual metabolism is another important factor in adipose tissue metabolism and function balance (Ro et al., 2019). Autophagy inhibition in the early stage could alter WAT characteristics into a "browning" state and improves glucose tolerance at later stages (Ghosh et al., 2018). In this study, we utilized the WT and E2F1$^{-/-}$ mice to investigate the possible role and E2F1-modulated autophagy mechanism.

In the present study, primarily, there were no differences in body sizes, weights, the *in situ* subcutaneous WAT, and the *ex vivo* dissected WAT. Interestingly, Choi et al. reported that HFD-fed and ob/ob mice exhibited more expression levels of mRNA, including fibroblast growth factor receptor 1, cyclin D, and E2F1, compared to those of the normal diet-fed and lean control mice, respectively (Choi et al., 2016). Thus, the E2F1 knockout did not change the bodyweight, which might attribute to that we did not establish the obesity settings in mice. Besides, H&E staining revealed significantly smaller adipocytes size in the E2F1$^{-/-}$ WAT compared with WT WAT, which presented "beige" adipose-like morphology. As Xue et al. reported that after exposure to cold temperatures, subcutaneous WAT of mice possessed relatively large, round, and condensed mitochondria with numerous transverse cristae surrounding smaller lipid droplets, indicating browning of WAT (Xue et al., 2018). Here, E2F1$^{-/-}$ WAT showed smaller intracellular lipid droplets and more mitochondria quantities than WT WAT, revealing the beige adipose shape. High expression of UCP-1 is one of the important characteristics of WAT browning (Devlin, 2015). Blanchet et al. illustrated that E2F1$^{-/-}$ BAT showed increased expression in the mitochondrial respiratory chain and uncoupling respiration (UCP-1, 2), compared to the WT BAT (Blanchet et al., 2013). In this study, the highly

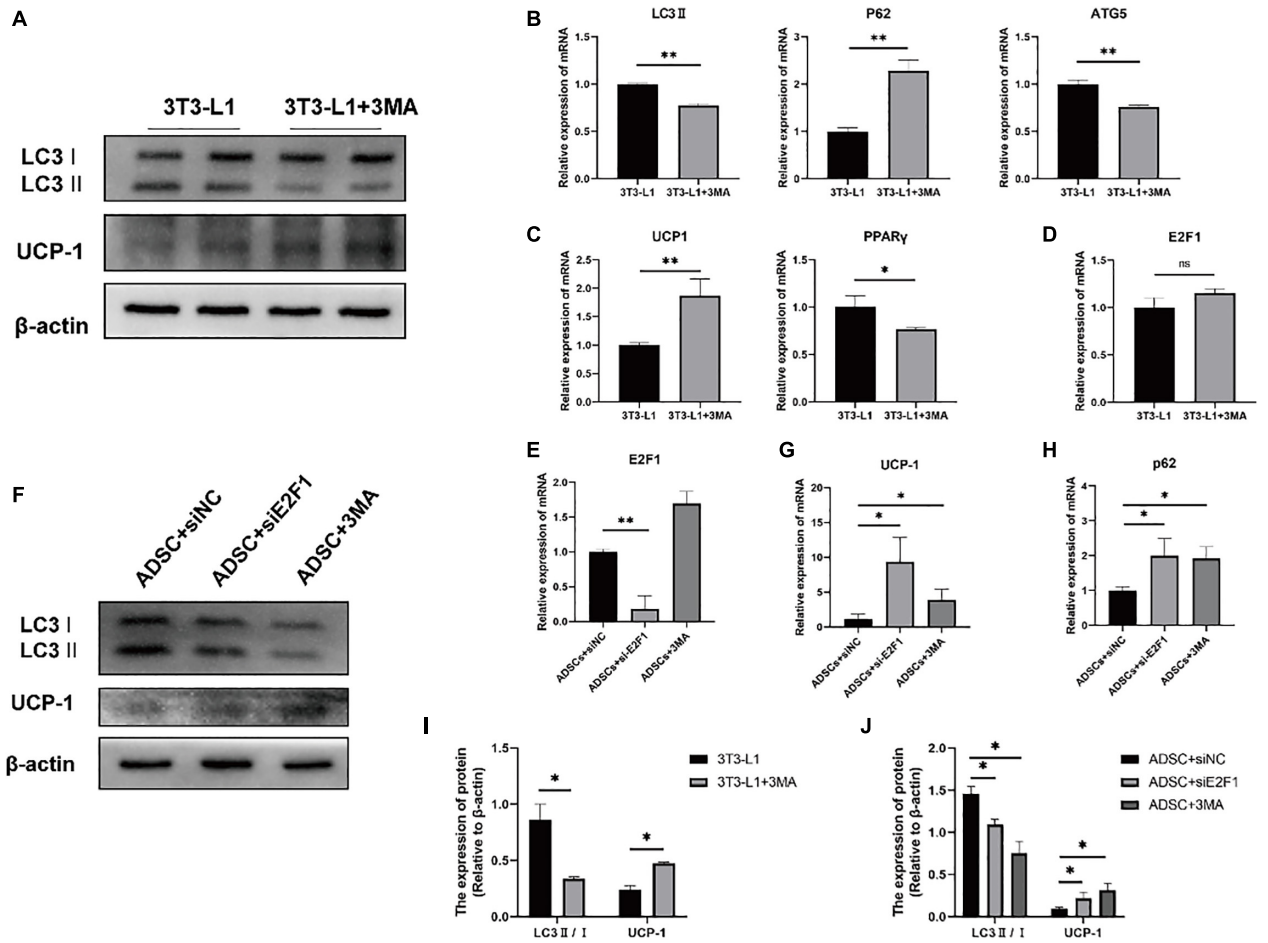

FIGURE 6 | Autophagy inhibition increased the UCP-1 expression, without reduced E2F1 expression in adipocytes *in vitro*. **(A)** Representative Western Blot image showing the level of LC3-II/I and UCP-1 protein in 3T3-L1 adipocytes and 3T3-L1 + 3MA adipocytes. **(B–D)** qRT-PCR analyses of LC3-II, p62, ATG5, UCP-1, PPARγ, and E2F1 expression at the mRNA level in 3T3-L1 adipocytes and 3T3-L1 + 3MA adipocytes. **(E)** The qRT-PCR analysis of E2F1 expression at the mRNA level in ADSCs + siNC group, ADSCs + E2F1 siRNA group, and ADSCs + 3MA group. **(F)** Representative Western Blot image of LC3-II/I, and UCP-1 protein levels in ADSCs + siNC group, ADSCs + E2F1 siRNA group, and ADSCs + 3MA group. **(G,H)** The qRT-PCR analyses of p62, and UCP-1 expressions at the mRNA level in ADSCs + siNC group, ADSCs + E2F1 siRNA group, and ADSCs + 3MA group. **(I)** Quantitative analysis of the LC3-II/I and UCP-1 protein expressions relative to β-actin in 3T3-L1 adipocytes and 3T3-L1 + 3MA adipocytes, $n = 3$. **(J)** Quantitative analysis of the LC3-II/I and UCP-1 protein expressions relative to β-actin in ADSCs + siNC group, ADSCs + E2F1 siRNA group, and ADSCs + 3MA group, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

UCP-1 expression and beige cells formation were observed in E2F1$^{-/-}$ WAT *in vivo* and E2F1$^{-/-}$ adipocytes *in vitro*. These results demonstrated that E2F1 knockout promoted WAT browning through increasing UCP-1 expression and mitochondria, which might lead to energy expenditure in a thermogenic manner.

The C1q/TNF-related protein 5 (CTRP5) was reported as a novel adipokine that was significantly down-regulated in subcutaneous WAT of mice after being exposed to cold temperatures (Rao et al., 2019). The overexpressing CTRP5 elevated the autophagy level and suppressed UCP-1 expression, whereas the autophagy inhibitor could rescue the suppression. E2F1 acted as a key transcription factor and its knockout induced WAT browning along with the decrease in autophagy activity. We observed that the autophagosomes occurred with cytoplasm-like substances enclosed by a multilayered membrane in WT WAT under TEM. These results were similar to their counterparts observed by Kim et al. (2018). Besides, autophagolysosomes with monolayered structures also could be seen in the WT group. Instead, there were nearly no autophagosomes and autophagolysosomes appearing in E2F1$^{-/-}$ WAT. The LC3 in the cytosolic form (LC3-I) was conjugated to phosphatidylethanolamine to form LC3-II, and then recruited to autophagosomal membranes. Hence, LC3-II/I is used as a key marker of autophagy (Tao et al., 2016). As an autophagic adapter, p62 interacting with LC3 was determined as another indicator of autophagy. Maixner et al. summarized that promotor binding, promoter activity, and autophagic flux measurement results identified the capacity of E2F1 to transcriptionally regulate autophagy genes and to activate autophagy in adipose tissue and adipocytes (Maixner et al., 2016). More interestingly, in the E2F1$^{-/-}$ group, the LC3-II/I at the

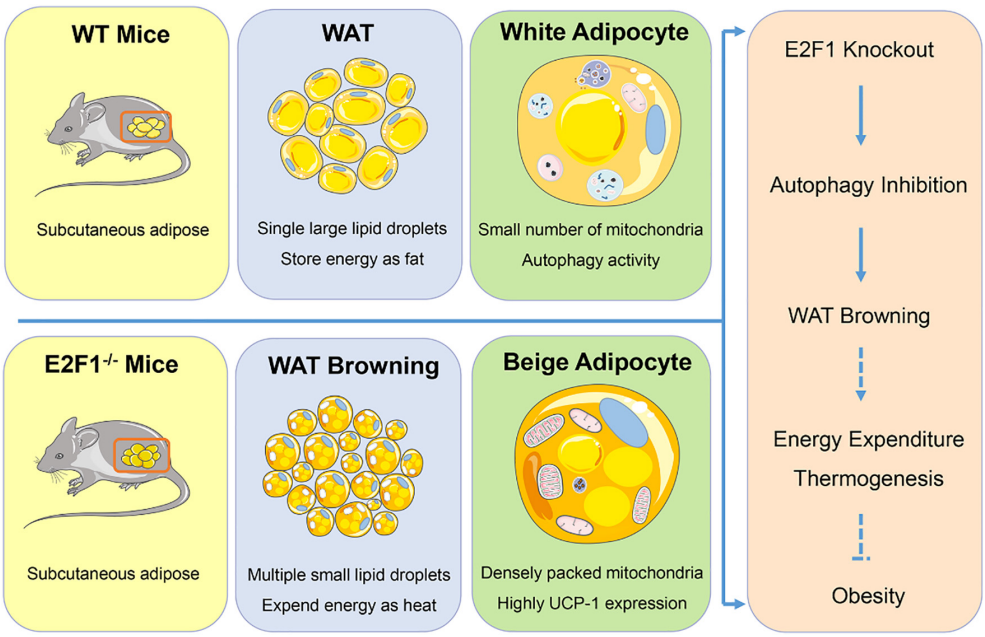

FIGURE 7 | Graphical summary. E2F1$^{-/-}$ WAT exhibited multiple small lipid droplets, densely packed mitochondria, and highly UCP-1 expression, indicating the browning form. Mechanically, E2F1 knockout suppressed the autophagy activity to promote WAT browning in mice, which was beneficial to expend energy in thermogenic manner for combating obesity.

protein level was significantly decreased, indicating the reduction of autophagosomes. Consequently, the expression of mitophagy receptor p62 was increased due to the blockage of autophagy.

Autophagy has been confirmed as a key regulator involved in adipose tissue physiology, especially in WAT and BAT adipogenesis (Zhang et al., 2012). Mitophagy is an important type of autophagy that selectively removes excess or damaged mitochondria, positively regulating the white adipogenesis and negatively regulating the beige and brown adipogenesis (Altshuler-keylin et al., 2017). It is well-recognized that WAT maintains more autophagy activity to keep a lower number of mitochondria, whereas BAT and beige adipose tissue possessed higher mitochondrial biogenesis and lower mitophagy (Ferhat et al., 2019). The autophagy and mitophagy occurred dynamically depending on browning status. For instance, Cairó et al. (2016) demonstrated that autophagy inhibition in brown adipocytes resulted in the increase of UCP-1 protein and uncoupled respiration, suggesting that autophagy inhibition activated thermogenesis and was part of the adaptive mechanism of brown adipocytes. Moreover, in the course of transition from white to beige adipose tissue, the inhibition of mitophagy could help to maintain higher mitochondrial content for the beige adipose tissue remodeling. Singh et al. found that Atg7 knockout mice showed a decreased white adipose mass and enhanced insulin sensitivity (Singh et al., 2009). Besides, the defective autophagy in WAT promoted a phenotype with reduced WAT mass and increased browning adipocyte features. In the study of Armani et al., they also verified that mineralocorticoid receptor antagonism markedly reduced the autophagic rate both in murine preadipocytes *in vitro* and WAT depots *in vivo*, with a concomitant increase in UCP-1 protein expression (Armani et al., 2014). In keeping with the fact that our study also E2F1$^{-/-}$ group *in vivo* and *in vitro* possessed decreased autophagy-related LC3-II, ATG5, ATG7 mRNA expression, and certainly increased p62 mRNA expression. It further proved that E2F1 knockout could alleviate the autophagy activity in WAT of mice. In our study, the reasons for E2F1-involved autophagy or mitophagy inhibition in WAT browning were not fully examined, but the observed enhanced mitochondrial content suggested a failure to clear mitochondria or impaired mitophagy, indicating that the thermogenic program inversely correlates with the autophagy program. It speculated that mitochondrial autophagy activity might be inhibited after E2F1 knockout in the WAT, contributing to an increase in the mitochondria number and higher mitochondria function. Thus, E2F1 knockout in WAT repressed the autophagy to up-regulate UCP-1 expression and promote WAT browning, which was expected to be used for fat burning to dissipate extra energy and obesity prevention.

CONCLUSION

In summary, our findings provided evidence that E2F1 knockout induced WAT and adipocyte browning through reducing autophagy activity both *in vivo* and *in vitro* (**Figure 7**). E2F1 could serve as a potential target to trigger mitochondrial activation in WAT and to promote WAT browning, which is conducive to increased energy expenditure and subsequent weight loss. The involvement of E2F1 in autophagy may be

the entry point affecting WAT browning, providing a novel explanation for the autophagy regulation by transcription factors to determine energy metabolism balance and obesity control. On the other hand, some issues still should be addressed in further study Firstly, the supervised basic body temperature and basal metabolic rate are evidence for beige and brown adipose thermogenesis, which was not acquired in the results. Secondly, cold exposure or β-adrenergic stimulation is necessary to activate WT and E2F1$^{-/-}$ WAT, contributing to more pronounced phenotypic and functional changes. Then, it will be of value to explore the role of E2F1 overexpression in E2F1$^{-/-}$ adipocyte, which can be further confirmed E2F1 regulation by the rescue experiments. Lastly, the actual function of E2F1 needs to be explored in mice with a high-fat diet under obesity settings.

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 Animal Experimental Center of Tongji Hospital, Huazhong University of Science and Technology.

AUTHOR CONTRIBUTIONS

MX, WH, YT, and HY performed the experiment. MX and WH wrote the manuscript and finished the result analysis. QZ, YWu, and MW conceived the project and revised the manuscript. CZ, YWa, and YY edited the manuscript. All authors reviewed the manuscript and all approved of the final version.

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The Insulin-Sensitizer Pioglitazone Remodels Adipose Tissue Phospholipids in Humans

*Juan P. Palavicini[1,2], Alberto Chavez-Velazquez[1], Marcel Fourcaudot[1], Devjit Tripathy[1], Meixia Pan[1,2], Luke Norton[1], Ralph A. DeFronzo[1] and Christopher E. Shannon[1]**

[1]Division of Diabetes, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States, [2]Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States

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Metabolic Solutions Development
Company, United States

***Correspondence:**
Christopher E. Shannon
shannonce@uthscsa.edu

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The insulin-sensitizer pioglitazone exerts its cardiometabolic benefits in type 2 diabetes (T2D) through a redistribution of body fat, from ectopic and visceral areas to subcutaneous adipose depots. Whereas excessive weight gain and lipid storage in obesity promotes insulin resistance and chronic inflammation, the expansion of subcutaneous adipose by pioglitazone is associated with a reversal of these immunometabolic deficits. The precise events driving this beneficial remodeling of adipose tissue with pioglitazone remain unclear, and whether insulin-sensitizers alter the lipidomic composition of human adipose has not previously been investigated. Using shotgun lipidomics, we explored the molecular lipid responses in subcutaneous adipose tissue following 6 months of pioglitazone treatment (45 mg/day) in obese humans with T2D. Despite an expected increase in body weight following pioglitazone treatment, no robust effects were observed on the composition of storage lipids (i.e., triglycerides) or the content of lipotoxic lipid species (e.g., ceramides and diacylglycerides) in adipose tissue. Instead, pioglitazone caused a selective remodeling of the glycerophospholipid pool, characterized by a decrease in lipids enriched for arachidonic acid, such as plasmanylethanolamines and phosphatidylinositols. This contributed to a greater overall saturation and shortened chain length of fatty acyl groups within cell membrane lipids, changes that are consistent with the purported induction of adipogenesis by pioglitazone. The mechanism through which pioglitazone lowered adipose tissue arachidonic acid, a major modulator of inflammatory pathways, did not involve alterations in phospholipase gene expression but was associated with a reduction in its precursor linoleic acid, an effect that was also observed in skeletal muscle samples from the same subjects. These findings offer important insights into the biological mechanisms through which pioglitazone protects the immunometabolic health of adipocytes in the face of increased lipid storage.

Keywords: adipose, obesity, lipidomics, pioglitazone, type 2 diabetes

INTRODUCTION

Adipose tissue is the primary site for fat storage and quantitatively the most important energy reservoir in the body. The coordinated expansion and breakdown of adipose lipid stores is crucial to the dynamic regulation of circulating nutrient availability and plays a central role in the control of whole-body metabolism. Excessive nutrient storage in adipose tissue (i.e., obesity) promotes adipocyte dysfunction, inflammation, and insulin resistance and is therefore strongly implicated in the etiology of type 2 diabetes (T2D; DeFronzo, 2004).

Pioglitazone is an insulin-sensitizing drug approved for the treatment of T2D. The molecular effects of pioglitazone are primarily mediated through the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), a transcriptional regulator of adipocyte differentiation and lipid storage which is highly abundant in adipose tissue (Spiegelman, 1998). Although its precise therapeutic mechanism remains controversial, the disease-modifying actions of pioglitazone are traditionally ascribed to the PPARγ-mediated expansion of subcutaneous adipose tissue, resulting in a reduction in systemic lipid concentrations and the subsequent reversal of "lipotoxicity" in non-adipose tissues (Bays et al., 2004). In the absence of a negative energy balance (i.e., weight loss), the mobilization of ectopic lipids, especially from skeletal muscle (Bajaj et al., 2010) and liver (Bajaj et al., 2003), as well as the redistribution of visceral fat, is accompanied by a reciprocal increase in the subcutaneous adipose tissue depots (Miyazaki et al., 2002) and in adiponectin levels (Gastaldelli et al., 2021). Mechanistically, recent estimates of adipogenesis in obese individuals treated with pioglitazone (White et al., 2021) confirm earlier morphological observations (McLaughlin et al., 2010) that thiazolidinediones drive adipose expansion by stimulating the formation of new adipocytes (i.e., hyperplasia). As a result of this mode of action, pioglitazone paradoxically causes weight gain (specifically fat mass) despite improving dyslipidemia, insulin sensitivity, and glycemic control (Miyazaki et al., 2001; Shadid and Jensen, 2003).

Recent reports have suggested that the expansion of adipose tissue following pioglitazone treatment could be driven by the formation of new adipocytes (i.e., adipogenesis) in subcutaneous adipose depots (White et al., 2021). This is consistent with observations that pioglitazone increases the proportion of smaller adipocytes in subcutaneous adipose tissue (de Souza et al., 2001; McLaughlin et al., 2010) which likely contributes to the enhancement of adipocyte glucose uptake and greater overall capacity for lipid storage (Olefsky, 1976). Importantly, whereas adipose tissue expansion in obesity is coupled with metabolic dysfunction and chronic, low-grade inflammation (Zatterale et al., 2019), the pioglitazone-mediated increase in lipid storage is associated with the promotion of anti-inflammatory pathways in human adipose (Koppaka et al., 2013; Spencer et al., 2014).

The lipid composition of human adipose tissue is dominated by triglyceride species (Al-Sari et al., 2020), but also includes numerous less abundant lipid molecules that may nevertheless be important effectors of inflammatory and insulin signaling pathways. For example, it was recently found that changes in adipose glycerophospholipids, rather than triglycerides, more closely reflect the transcriptional and metabolic adaptations occurring during adipose expansion with diet-induced obesity (Liu et al., 2020). Alterations in certain glycerophospholipid species, including those enriched in arachidonic acid, have been directly implicated in the inflammatory milieu of adipose tissue in human obesity (Pietiläinen et al., 2011). How the molecular lipid profile of adipose tissue responds to pioglitazone therapy has not previously been investigated and, as such, the events involved in adipose tissue remodeling following pioglitazone treatment remain poorly characterized.

The objective of the present study was to determine the impact of pioglitazone treatment on molecular lipids in adipose tissue from obese type 2 diabetics, using a multi-dimensional mass spectrometry-based shotgun lipidomics approach (Han and Gross, 2005), which facilitated the class-targeted analysis of all glycerophospholipid, sphingolipid, acylcarnitine, free fatty acid, triacylglycerol, and diacylglycerol species. Understanding how adipose tissue biology influences the resolution of human insulin resistance can identify novel pathophysiological lipid species and represents an important step toward developing more effective therapeutic strategies to combat the clinical and socioeconomic burden of soaring rates of obesity and T2D.

MATERIALS AND METHODS

Human Studies

Seven obese individuals with T2D (male/female 6/1; Mexican American/Caucasian 5/2; age 57 ± 7 years; BMI 32 ± 6 kg/m^2; HbA_{1c} 8.0 ± 0.6%) treated with diet alone or diet plus metformin and/or sulfonylurea participated in the study, which was approved by the Institutional Review Board of the South Texas Veterans Healthcare System, University of Texas Health Science Center San Antonio. After providing fully informed consent and completing a routine health screening visit, eligible subjects reported to The Bartter Clinical Research Unit of the South Texas Veterans Healthcare System following an overnight ~10-h fast. A baseline blood sample was drawn for the measurement of fasting blood glucose, HbA1c, and triglycerides, and subcutaneous abdominal adipose tissue and vastus lateralis muscle biopsies were obtained under local anesthesia (1% Lidocaine) for lipid profiling and gene expression analysis. Baseline measurements were repeated after 6 months of pioglitazone treatment (45 mg/day). Due to biopsy sample availability, lipidomic analyses were carried out on six of the seven subjects for adipose tissue and five of the seven subjects for skeletal muscle tissue, such that lipidomic analyses in at least one tissue are presented for all subjects. Clinical and adipose gene expression data are presented for all seven subjects.

Multi-Dimensional Mass Spectrometry-Based Shotgun Lipidomics

Adipose tissue or skeletal muscle samples (10–20 mg) were homogenized in ice-cold diluted (10%) phosphate-buffered saline, and lipids were extracted by a modified Bligh and Dyer procedure in the presence of internal standards added based

on total protein content, as previously described (Han and Gross, 2005; Wang et al., 2017; Palavicini et al., 2020). A triple-quadrupole mass spectrometer (Thermo Scientific TSQ Altis, CA, United States) and a Quadrupole-Orbitrap™ mass spectrometer (Thermo Q Exactive™) equipped with a Nanomate device (Advion Biosciences Ltd., NY, United States) and Xcalibur system software were used as previously described (Wang et al., 2017). Briefly, diluted lipid extracts were directly infused into the electrospray ionization source through a Nanomate device. Signals were averaged over a 1-min period in the profile mode for each full-scan mass spectrometry (MS) spectrum. For tandem MS, a collision gas pressure was set at 1.0 mTorr, but the collision energy varied with the classes of lipids. Similarly, a 2- to 5-min period of signal averaging in the profile mode was employed for each tandem MS mass spectrum. All full and tandem MS mass spectra were automatically acquired using a customized sequence subroutine operated under Xcalibur software. Data processing, including ion peak selection, baseline correction, data transfer, peak intensity comparison, 13C deisotoping, and quantitation, was conducted using a custom programmed Microsoft Excel macro as previously described after considering the principles of lipidomics (Yang et al., 2009).

Adipose Tissue Gene Expression

Target mRNA expression was determined in adipose tissue lysates by qRT-PCR as previously described (Shannon et al., 2017) using the following pre-designed SYBR green human primer assays from Sigma (MO, United States): *FADS1*, *FADS2*, *ELOVL5*, *CDS1*, *CDIPT*, *PLA2G4A*, *PLA2G4C*, *PLA2G7*, and *PLA2G16*. Data were normalized to the geometric mean of the reference genes *ACTB* and *GAPDH* and expressed as a fold change relative to the baseline (pre-treatment) mean.

Statistical Analyses

Clinical data and lipid class totals are expressed as mean ± standard error and were compared using paired *t* tests. Overall lipidomics data patterns were initially visualized by principal component analyses (PCA) performed on raw data for quantified species (in nmol/mg protein). Lipid data were then scaled to the total molar content of all detected species in that lipid class. Compositional changes in lipid classes were subsequently evaluated by comparing individual species or groups of individual species (for acyl chain length and saturation profiles) with multiple paired *t* tests, controlling for a < 10% false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger, and Yekuieli (GraphPad Prism 9). Gene expression changes were assessed by multiple paired *t* tests controlling for FDR.

RESULTS

Clinical Responses to Pioglitazone Treatment

In agreement with the established clinical effects of thiazolidinediones (Aronoff et al., 2000; Miyazaki et al., 2001), 6 months of pioglitazone treatment lowered fasting blood glucose (**Figure 1A**) and HbA1c (**Figure 1B**), although the decline in fasting triglycerides (**Figure 1C**) did not reach statistical significance ($p = 0.085$). Subjects gained an average of 3.2 kg body weight following pioglitazone treatment (**Figure 1D**).

Glycerophospholipid Profile Responds to Pioglitazone Treatment in Adipose Tissue

Our class-targeted shotgun lipidomics approach determined the adipose tissue concentration of over 300 molecular lipid species across 15 functional lipid classes. In general, the total amount of each lipid class remained constant following pioglitazone treatment, apart from the free fatty acid and cardiolipin pools, which were both significantly decreased post treatment (**Figure 2A**). These observations were preserved regardless of whether lipid class totals were normalized to protein content or tissue weight (**Supplementary Figure 1**).

A wide inter-individual variability was observed for many lipid classes and, indeed, PCA of each major structural family of lipids (glycerolipids, glycerophospholipids, sphingolipids, and fatty acyl lipids) revealed that much of the variance in adipose lipid species was driven by between-subject differences (**Figure 2B**). However, baseline and post-pioglitazone samples clustered separately along PC1 for glycerophospholipids, with this vector explaining 36% of variance in all glycerophospholipid species (**Figure 2B**). This parallels previous observations that, in comparison with the dominant pool of adipose triglycerides, the composition of glycerophospholipids appears to be more responsive to changes in adipose tissue mass and/or metabolic function (Pietiläinen et al., 2011; May et al., 2017; Liu et al., 2020). As such, our subsequent analyses focused predominantly on the 141 measured lipid species comprising glycerophospholipid classes.

Pioglitazone Increases the Saturation of Membrane Lipids in Adipose Tissue

Glycerophospholipids are critical components of cell membranes and the preferential trafficking of polyunsaturated fatty acids (PUFA) toward these complex lipids plays an important role in the regulation of membrane fluidity (Pietiläinen et al., 2011). Changes in the composition of glycerophospholipids, particularly their fatty acyl chain saturation, could therefore have important implications for adipocyte function. We considered the relative amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA in the total glycerophospholipid pool as an index of adipose cell membrane saturation. Pioglitazone treatment was associated with a significant decrease in the PUFA fraction, but an increase in the SFA fraction, in glycerophospholipids (**Figure 3A**). This shift toward a greater saturation of membrane lipids was similarly reflected in the free fatty acid pool (**Figure 3B**).

The fatty acid desaturases (FADS1 and FADS2) catalyze the rate-limiting enzymatic steps in the generation of PUFA, and an index of the combined flux through these pathways can be estimated from the product-to-substrate ratio of arachidonic acid to linoleic acid in tissue lipids (Martinelli

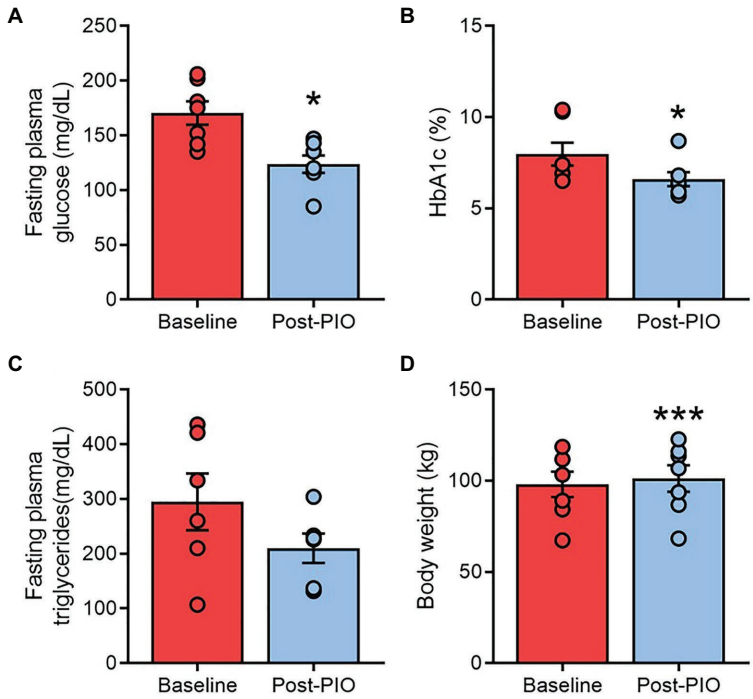

FIGURE 1 | Clinical responses to pioglitazone treatment. Fasting plasma glucose **(A)**, glycated hemoglobin **(B)**, plasma triglycerides **(C)**, and body weight **(D)** at Baseline (red) and following 6 months of 45 mg/day pioglitazone treatment (blue). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for $n = 7$ subjects.

et al., 2008; Del Pozo et al., 2020). Consistent with the increased saturation of membrane lipids, the FADS ratio calculated from the glycerophospholipid fatty acyl pool was significantly lower following pioglitazone treatment (**Figure 3C**), which may be indicative of a lower desaturase activity. However, no changes were observed in the saturation profile of the triglyceride pool (**Figure 3D**), which represents the overwhelming site of fatty acyl esterification in adipose tissue. These findings suggest that, consistent with our PCA plots (**Figure 2B**), the glycerophospholipid pool is highly responsive to pioglitazone-induced changes in unsaturated free fatty acid availability, whereas the triglyceride pool is more resistant to these changes.

Another process closely linked to fatty acid desaturation is *de novo* lipogenesis (DNL), which can be similarly estimated from the ratio of non-essential fatty acids (which can be synthesized) to essential fatty acids (obtained exclusively from the diet) in the glycerophospholipid pool (Yew Tan et al., 2015). In agreement with previous reports that thiazolidinediones enhance DNL in human adipose tissue (de Souza et al., 2001; McTernan et al., 2002), the DNL index was significantly increased by pioglitazone treatment (**Figure 3E**), a finding that was again replicated in the free fatty acyl pool (**Figure 3F**). Moreover, these observations were substantiated by another common metric of DNL, the palmitate (C16:0) to linoleate (C18:2) ratio, in both the glycerophospholipid (**Supplementary Figure 2A**) and free fatty acid pools (**Supplementary Figure 2B**).

Pioglitazone Lowers Arachidonic Acid Enrichment in Adipose Glycerophospholipids

To ascertain whether certain functional lipid groups or species were responsible for the observed changes in membrane lipid saturation following pioglitazone treatment, each molecular species was next normalized to the respective total glycerophospholipid content determined in each sample. After controlling for multiple comparisons (see *Statistical Analyses* in Methods), 26 species (15 downregulated, 11 upregulated) were found to be altered by pioglitazone treatment (**Figure 4A**). Strikingly, two-thirds of downregulated glycerophospholipids were species containing the long-chain polyunsaturated fatty acid arachidonic (C20:4) acid (AA). Indeed, the cumulative amount of AA esterified in glycerophospholipids was 40% lower following pioglitazone treatment (**Figure 4B**), while AA-containing species also accounted for many of the strongest positive loadings on PC1 for glycerophospholipids (**Supplementary Figure 3A**). In contrast, no change was observed in the glycerophospholipid levels of docosahexaenoic acid (C22:6; **Supplementary Figure 3B**), another major long-chain polyunsaturated fatty acid. Moreover, the AA content of adipose triglycerides also remained unchanged following pioglitazone treatment (**Supplementary Figure 3C**). Together, these findings highlight a selective decrease in glycerophospholipid AA enrichment as a key feature of the molecular lipid response to pioglitazone treatment in adipose tissue.

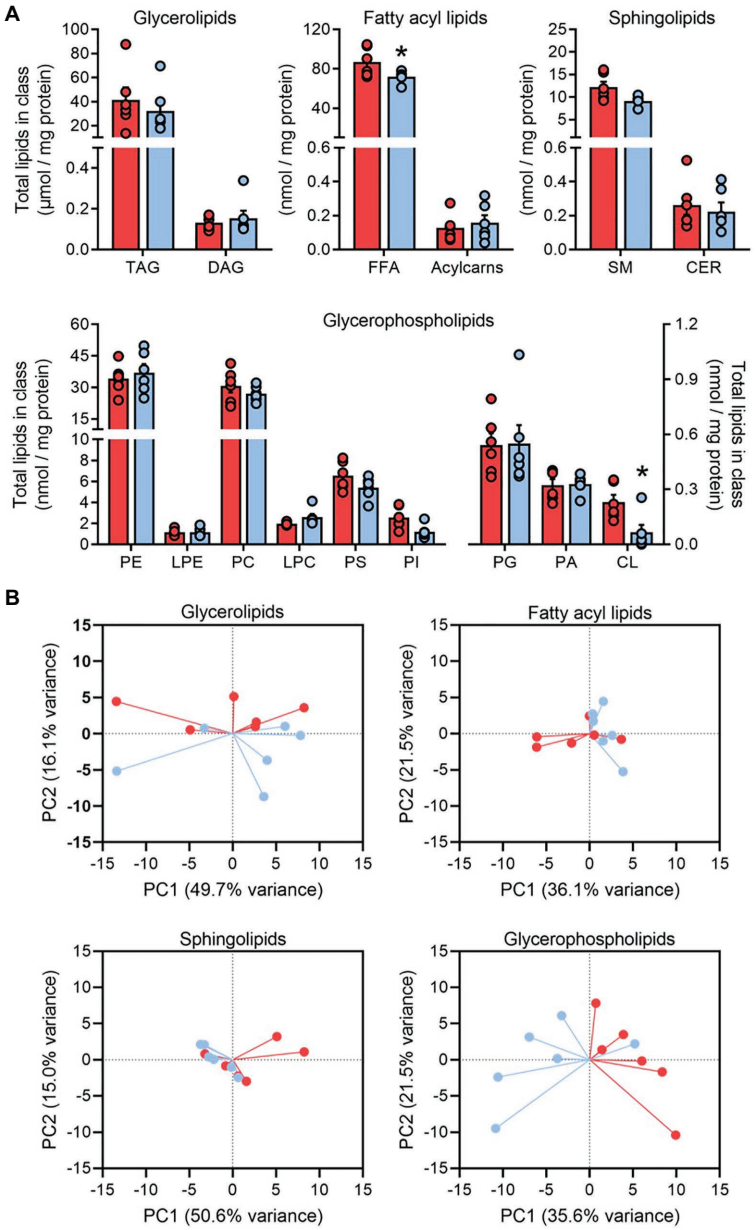

FIGURE 2 | The glycerophospholipid profile responds to pioglitazone treatment in adipose tissue. Lipid class totals in adipose tissue **(A)** for triglyceride, diacylglyceride, free fatty acids, acylcarnitine (acylcarns), sphingomyelin (SM), ceramide, phosphatidylethanolamine (PE), lyso-phosphatidylethanolamine, phosphatidylcholine (PC), lyso-phosphatidylcholine, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA), and cardiolipin (CL) normalized to adipose protein content. *$p < 0.05$ vs. Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for $n = 6$ subjects. Principal component analysis score plots of the first two principal components **(B)** summarizing the overall pattern of variance in glycerolipid, fatty acyl, sphingolipid, and glycerophospholipid pools from human adipose tissue at Baseline (red) and following 6 months of 45 mg/day pioglitazone treatment (blue). Note that pioglitazone-treated samples cluster separately along the x-axis (PC1) for glycerophospholipid scores.

Changes in the arachidonic acid content of glycerophospholipids could be related to alterations in free arachidonic acid availability and its subsequent esterification into phosphatidic acid, the obligate precursor for all glycerophospholipids. Accordingly, pioglitazone treatment resulted in a ~45% reduction in free AA availability (**Figure 4C**), which was paralleled by a decrease in 18:0–20:4 phosphatidic acid (**Figure 4D**), the major AA-containing phosphatidic acid species. Moreover, free AA concentrations were positively and significantly correlated with the AA content of glycerophospholipids across all samples and timepoints (**Figure 4E**). The cellular pool of free AA is partially dependent upon its synthesis from the essential fatty acid linoleic acid (Hanna and Hafez, 2018). Adipose

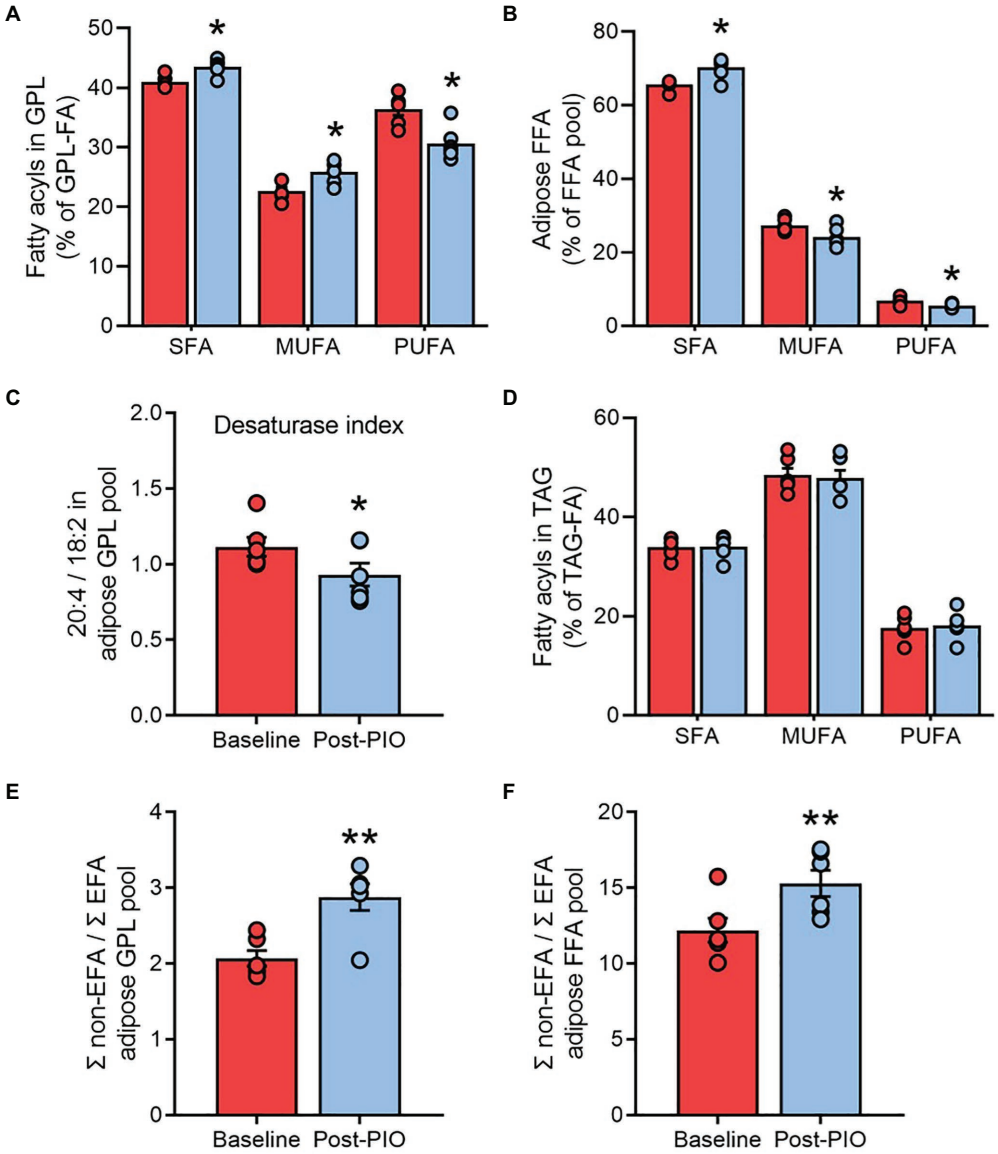

FIGURE 3 | Pioglitazone increases the saturation of membrane lipids in adipose tissue. Contribution of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids to total fatty acyl groups in the glycerophospholipid **(A)**, free fatty acid **(B)**, and triglyceride **(D)** pools; ratio of 20:4 to 18:2 fatty acyl groups in glycerophospholipids as an index of fatty acid desaturase activity **(C)**; ratio of non-essential fatty acids (non-EFA) to essential fatty acids as an index of *de novo* lipogenesis in the glycerophospholipid **(E)** and free fatty acid **(F)** pools from adipose tissue at Baseline (red) and following 6 months of 45 mg/day pioglitazone treatment (blue). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for $n = 6$ subjects.

tissue levels of free linoleic acid were also significantly reduced after pioglitazone treatment (**Figure 4F**), supporting a decrease in AA synthesis as a possible mechanism through which pioglitazone influences glycerophospholipid remodeling.

Adipose Tissue Plasmenylethanolamines Are Lowered by Pioglitazone Treatment

Phosphatidylethanolamines (PE) are the most abundant glycerophospholipids in adipose tissue (**Figure 2A**) and represent the major site of AA accumulation in most mammalian cell membranes, specifically at the *sn-2* fatty acyl position. Consistent with the profile changes of total glycerophospholipids (**Figure 3A**), pioglitazone treatment was associated with an increased saturation of the *sn-2* fatty acyl chain in PE (**Figure 5A**), as well as replacement of fatty acyl species containing 20 carbons by predominantly shorter (C16 and C18) species (**Figure 5B**). In contrast, the composition of the fatty acyl chain in the *sn-1* position of PE, which does not accrue AA, was unaltered by pioglitazone treatment (**Supplementary Figures 4A,B**).

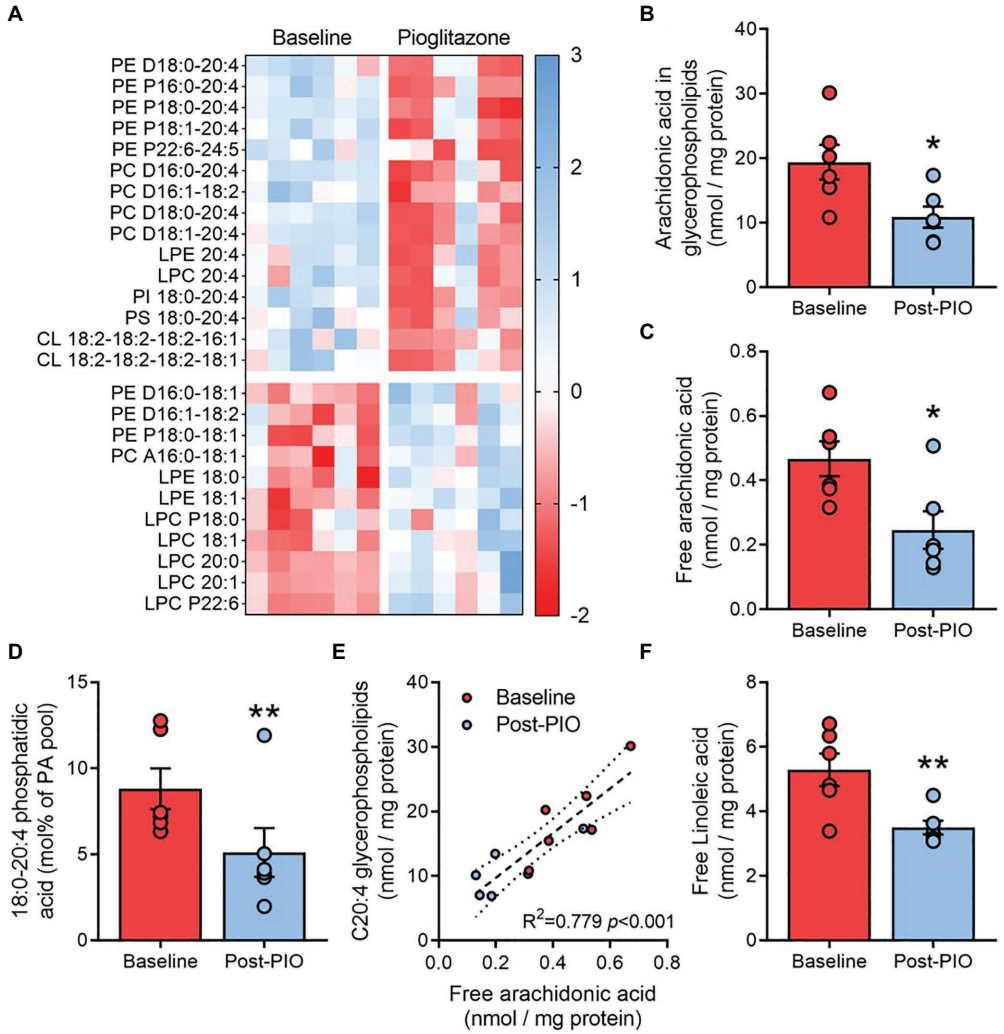

FIGURE 4 | Pioglitazone lowers arachidonic acid enrichment in adipose glycerophospholipids. Heatmap depicting fold change (*z* scores) for significantly altered molecular glycerophospholipid species in paired adipose tissue samples **(A)**, with blue indicating an upregulation and red indicating a downregulation following 6 months of 45 mg/day pioglitazone treatment. Lipids were first normalized to total glycerophospholipid content detected in each respective sample and compared with multiple paired *t* tests corrected for a false discovery rate of 10%. Absolute content of arachidonic acid esterified in glycerophospholipids **(B)** and in unesterified form **(C)**, and its fractional contribution in phosphatidic acid **(D)**, as well as the correlation between free and glycerophospholipid esterified arachidonic acid **(E)** and absolute content of linoleic acid **(F)** in paired adipose tissue samples at Baseline (red) and Post-PIO (blue). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for $n = 6$ subjects.

Compared with other glycerophospholipid classes, PE contain a greater portion of plasmalogen species (plasmenylet hanolamines), which are especially highly enriched in AA (Brites et al., 2004) and have previously been associated with adipose tissue expansion in human obesity (Pietiläinen et al., 2011). Numerous plasmenylethanolamines were altered by pioglitazone (**Figure 4A**), culminating in a decrease in the contribution of plasmalogens to the total PE pool relative to diacyl and alkyl forms (**Figure 5C**). A more modest pattern of increased acyl chain saturation was reflected in the *sn-2* position of phosphatidylcholine (PC), another major constituent of cellular membranes, consistent with the lower enrichment of AA in PC vs. PE (**Figure 5D**). The increased remodeling of cell membrane lipids was further reflected by alterations in the lyso-phospholipid pool. Whereas the relative levels of 20:4 lyso-PE and lyso-PC were reduced following pioglitazone treatment, many other (non-20:4) species were upregulated (**Figure 4A**).

Pioglitazone Influences Lipid Mediators of Insulin Signaling and Mitochondria in Adipose Tissue

Another group of lipids tightly linked to AA metabolism is the phosphatidylinositols (PI; Anderson et al., 2016), serving as a precursor pool for the downstream generation of phosphatidyl

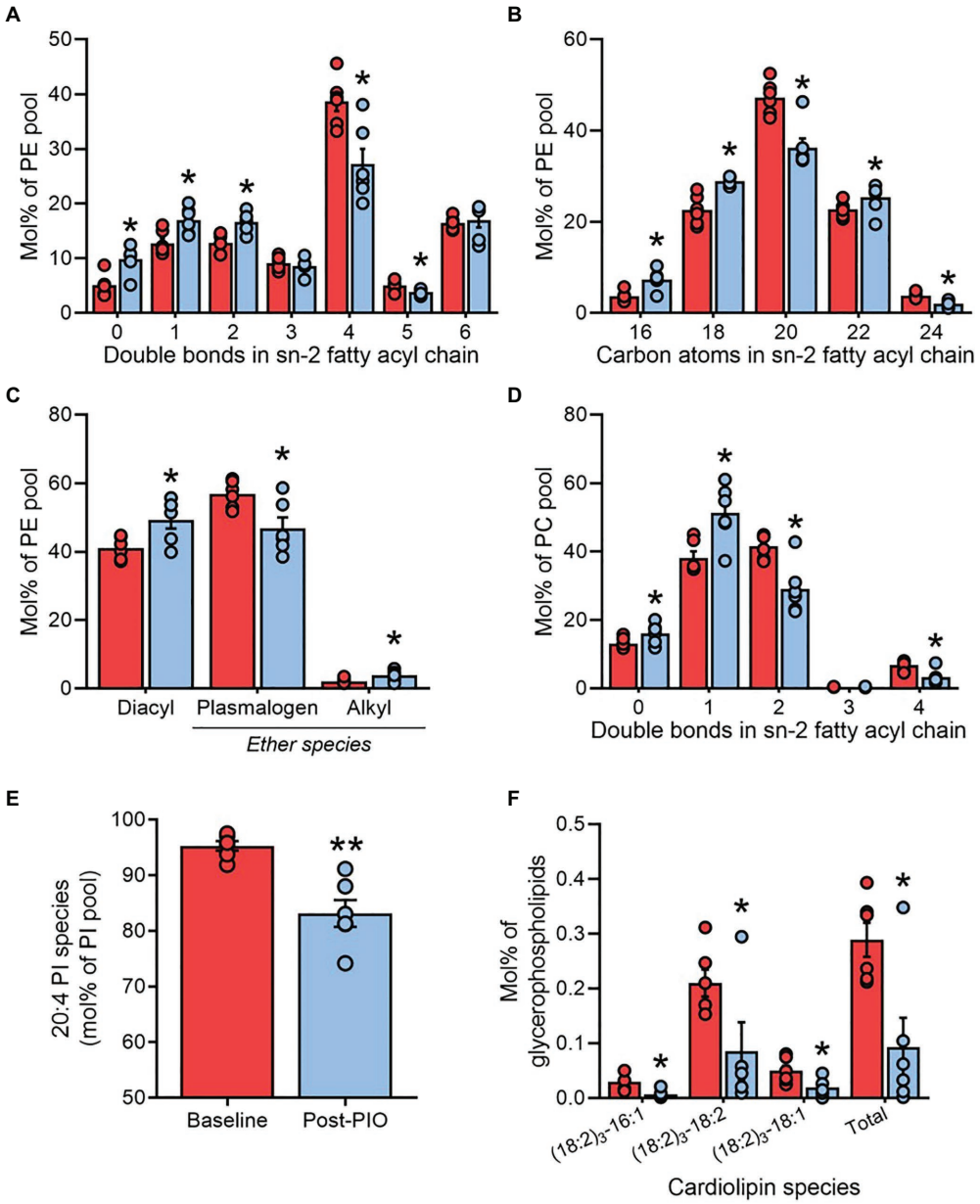

FIGURE 5 | Adipose tissue plasmenylethanolamines are lowered by pioglitazone treatment. Saturation number of double bonds; **(A)** and length number of carbon atoms; **(B)** of fatty acyl chains in the *sn-2* position, and ether chain content **(C)**, of phosphatidylethanolamine (PE); Saturation of fatty acyl chains in the *sn-2* position of phosphatidylcholine **(D)**; arachidonic acid enrichment in phosphatidylethanolamine **(E)**; content of cardiolipins **(F)** in paired adipose tissue samples at Baseline (red) and Post-PIO (blue). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles), normalized to the total molar content for each respective lipid class **(A–E)**, or to the total glycerophospholipid pool **(F)**, for $n = 6$ subjects.

3,4,5-triphosphate (PIP_3) and therefore playing a vital role in the regulation intracellular insulin signaling (Sandra and Marshall, 1986). In agreement with conserved observations across mammalian cell types (Patton et al., 1982; Kurvinen et al., 2000; Wang et al., 2016), PI 18:0–20:4 predominated as the major molecular PI species in adipose tissue (**Supplementary Figure 4C**). However, consistent with the reduction in AA availability (**Figure 4C**), PI species containing 20:4 acyl moieties were markedly lower following pioglitazone treatment (**Figure 5E** and **Supplementary Figure 4C**), whereas the contribution of other species to the total PI pool increased (**Supplementary Figure 4C**).

As well as contributing to AA synthesis, linoleic acid is a crucial component of the mitochondrial membrane lipid cardiolipin. Consistent with the lower mitochondrial content in human white adipose tissue, total cardiolipin concentrations detected in adipose tissue were nearly 10-fold lower than that measured in skeletal muscle from the same subjects (**Figure 2A** and **Supplementary Figure 4D**). As previously acknowledged,

and in agreement with our previous findings in livers from pioglitazone-treated mice (Shannon et al., 2021), adipose tissue cardiolipin was significantly reduced by pioglitazone treatment (**Figure 2A**). This decrease was not driven by changes in particular molecular species of cardiolipin but was instead related to a universal reduction in all detected species (**Figure 5F**).

Pioglitazone-Induced Changes in Adipose Tissue Are Partially Recapitulated in Skeletal Muscle

Skeletal muscle is another major tissue targeted by the insulin-sensitizing effects of pioglitazone (Bajaj et al., 2010). We evaluated whether the pioglitazone-induced changes observed in adipose tissue were also evident in skeletal muscle biopsies from a subset ($n=5$) of the same subjects. Free linoleic acid concentrations in skeletal muscle were ~15% lower following pioglitazone treatment (**Figure 6A**), although this was not associated with alterations in either the level of free AA (**Figure 6B**) or its enrichment in the total glycerophospholipid pool (**Figure 6C**). Nevertheless, AA enrichment in phosphatidylinositol declined significantly with pioglitazone (**Figure 6D**). Thus, part of the lipid remodeling effects of pioglitazone observed in human adipose tissue are paralleled by changes in skeletal muscle.

Impact of Pioglitazone on Phospholipase Gene Expression

Since many of the disease-modifying actions of pioglitazone have been attributed to transcriptional modulation of lipid metabolism pathways (Spiegelman, 1998), we explored prospective changes in genes involved in the regulation of AA metabolism. The adipose tissue mRNA expression of genes involved in AA synthesis from linoleic acid (*ELOVL5*, *FADS1*, and *FADS2*; **Figure 7A**), or AA removal from glycerophospholipids (*PLA2G4*, *PLA2G4B*, *PLA2G4C*, *PLA2G7*, and *PLA2G16*; **Figure 7B**), remained unchanged after 6 months of pioglitazone treatment. Similarly, no differences were observed in the genes responsible for AA incorporation into phosphatidylinositol, including *CDS1* or *CDIPT* (**Figure 7C**).

DISCUSSION

Despite being prescribed as an anti-diabetic agent for over 20 years, the precise molecular mechanisms of pioglitazone remain unresolved. The results of the current study demonstrate that the clinical benefits seen in T2D patients treated with pioglitazone are accompanied by robust compositional changes in adipose tissue glycerophospholipids, with minimal alterations observed in other lipid classes. Specifically, pioglitazone-induced adipose remodeling was characterized by an increased saturation of membrane lipids, driven primarily by reductions in glycerophospholipid species enriched for arachidonic acid (AA), including phosphatidylinositols and plasmenylethanolamines. These changes were not dependent upon transcriptional activation of phospholipase genes but were associated with a decrease in adipose tissue levels of free AA and its parent precursor linoleic acid. Notably, despite weight gain, which

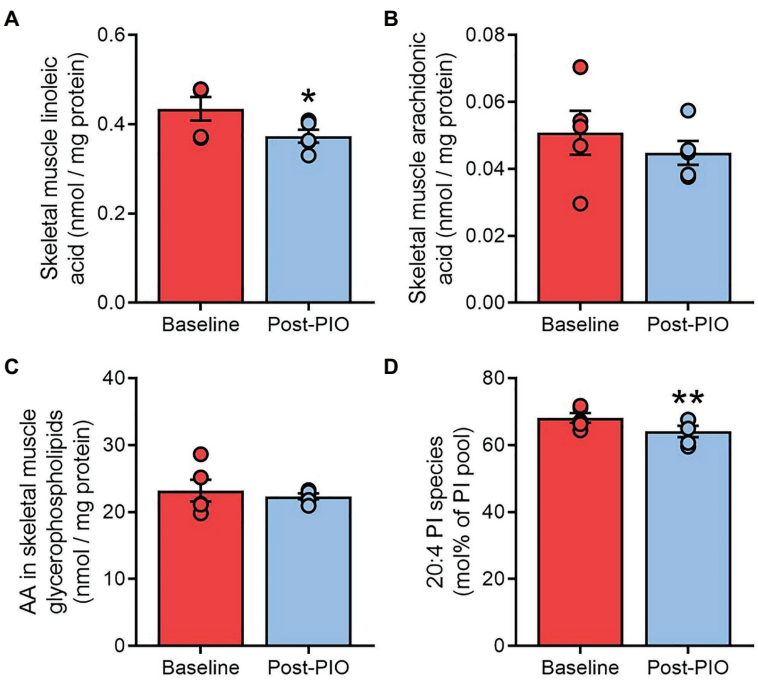

FIGURE 6 | Pioglitazone-induced changes in adipose tissue are partially recapitulated in skeletal muscle. Content of free linoleic acid **(A)** and arachidonic acid in unesterified form **(B)** or esterified in glycerophospholipids **(C)**, or as a fraction of the phosphatidylinositol pool **(D)**, in paired skeletal muscle samples at Baseline (red) and Post-PIO (blue). *$p<0.05$, **$p<0.01$ vs. Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for $n=5$ subjects.

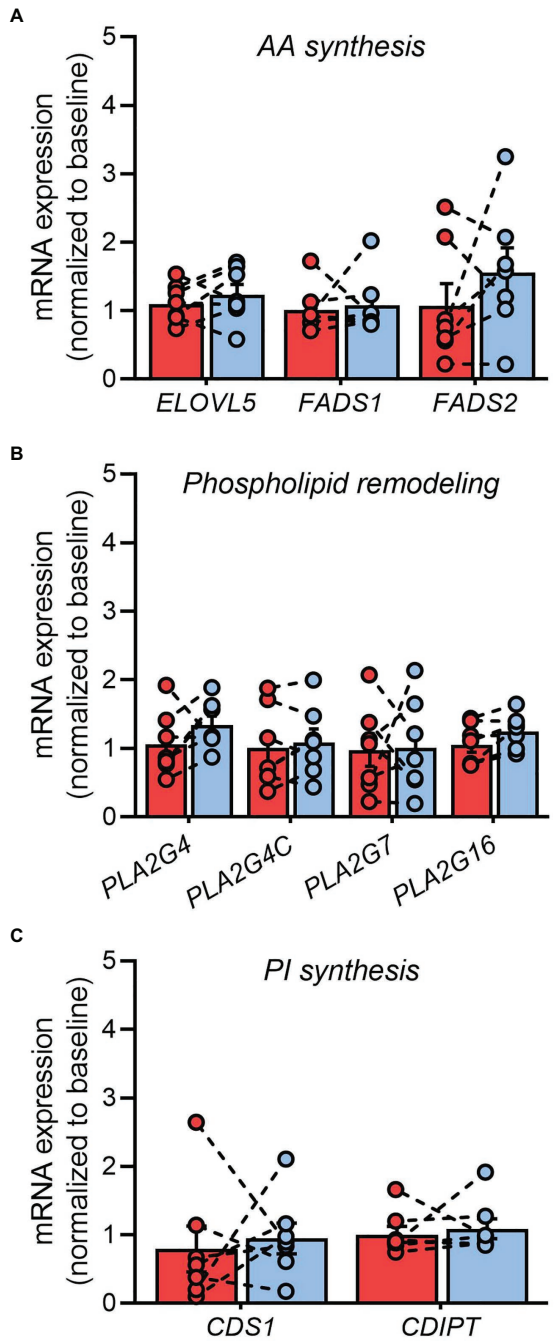

FIGURE 7 | Pioglitazone does not influence phospholipase gene expression in adipose tissue. Fold change in the adipose tissue mRNA expression of genes involved in arachidonic acid synthesis **(A)**, glycerophospholipid hydrolysis **(B)**, and phosphatidylinositol synthesis **(C)** following 6 months of 45 mg/day pioglitazone treatment. Target genes were first normalized to the geometric mean of *ACTB* and *GAPDH* and subsequently normalized to the average of Baseline samples. *ELOVL5* (fatty acid elongase 5), *FADS1* (fatty acid desaturase 1), *FADS2* (fatty acid desaturase 2), *PLA2G4* (cytosolic phospholipase A2), *PLA2G4C* (cytosolic lysophospholipase), *PLA2G7* (lipoprotein-associated phospholipase A2), *PLA2G16* (adipose-specific phospholipase A2), *CDS1* (CDP-diacylglycerol synthase 1), *CDIPT* (CDP-diacylglycerol-inositol-3-phosphatidyltransferase/phosphatidylinositol synthase). Data are mean ± standard error (filled bars) and individual values (filled circles) for $n = 7$ subjects.

is likely indicative of an expansion of the subcutaneous adipose tissue depots (Bray et al., 2013), the molecular profile of lipid storage in adipose triglycerides was unchanged by pioglitazone, suggesting a targeted remodeling of lipids within cellular membranes. These data provide evidence that the (mal)adaptive remodeling of adipose tissue glycerophospholipids observed in human obesity (Pietiläinen et al., 2011; Liu et al., 2020) is reversible by insulin-sensitizing therapy and, moreover, identify AA metabolism as a central node in this process.

Pioglitazone is classically understood to improve insulin sensitivity and cardiometabolic health by reversing lipotoxicity in muscle and liver, second to increasing lipid storage in adipose tissue. The results from our current study offer support for this paradigm. Indeed, pioglitazone caused a shortening and increased saturation of fatty acyl chains in glycerophospholipids, a trait that was recently shown to characterize the differentiation of preadipocytes into mature adipocytes, at least for *in vitro* models of adipogenesis (Miehle et al., 2020). Moreover, pioglitazone treatment increased indices of DNL, which is another demonstrable feature of smaller, more insulin sensitive adipocytes (Roberts et al., 2009). By contrast, the enlargement of existing adipocytes under obesogenic conditions (i.e., hypertrophy) is associated with a more elongated, unsaturated fatty acyl profile in adipose tissue, particularly for membrane lipids (Pietiläinen et al., 2011; Yew Tan et al., 2015), as well as a downregulation of DNL (Roberts et al., 2009). The changes in membrane lipids observed here may thus reflect a reversal of obesity-associated processes and are consistent with a pioglitazone-mediated *de novo* formation of smaller adipocytes (McLaughlin et al., 2010).

Studies in both rodents (Grzybek et al., 2019; Liu et al., 2020) and humans (Pietiläinen et al., 2011) have reported an obesity-associated decrease in the fatty acyl saturation of adipose tissue glycerophospholipids. This remodeling of cell membrane lipids is believed to be necessary to accommodate the sustained expansion of adipose tissue depots with progressive weight gain. One prior lipidomic investigation of twin pairs discordant for obesity identified an increase in polyunsaturated PEs, specifically plasmenylethanolamine species, as an important feature of this adipose remodeling (Pietiläinen et al., 2011). Supported by computer simulations, the authors speculated that the increase in vinyl-ether bonds present in plasmalogens (which decrease membrane fluidity) served to maintain healthy cell membrane function despite the opposing effects of glycerophospholipid desaturation (which increases membrane fluidity). Reciprocally, therefore, the selective reduction in plasmenylethanolamines following pioglitazone treatment could be expected to offset potential alterations in membrane fluidity that might otherwise accompany the increased saturation of glycerophospholipids. Although we did not measure adipose tissue mass in the current study, the observed weight gain was comparable to previous studies in which increased adiposity with pioglitazone was directly demonstrated by dual X-ray absorptiometry (Bray et al., 2013). Our findings thus provide evidence that the parallel expansions of adipose tissue with obesity vs. pioglitazone are likely associated with opposing effects on membrane lipid remodeling.

A high content of plasmalogen lipids in the adipose tissue of obese individuals has been implicated in the activation of inflammatory pathways (Pietiläinen et al., 2011) since these species are particularly vulnerable to oxidative stress (Scherrer and Gross, 1989). As such, the observed reduction in plasmenylethanolamines may also represent an important, novel mechanism through which pioglitazone treatment corrects adipose dysfunction to lower systemic inflammation in obesity and T2D (DeFronzo, 2004). The decrease in AA, a crucial component of plasmenylethanolamines, may be especially pertinent to the therapeutic actions of pioglitazone in adipose tissue. Indeed, adipose tissue levels of AA are reportedly increased in obesity (Williams et al., 2007; Pietiläinen et al., 2011) and this omega-6 PUFA, as well as its lipid derivatives, has been shown to activate pro-inflammatory pathways (Schreiber and Zechner, 2014) and antagonize insulin-mediated glucose uptake (Tebbey et al., 1994) in adipocytes. By contrast, the omega-3 PUFA DHA, which was unchanged by pioglitazone, is believed to promote anti-inflammatory signaling (Kuda et al., 2016). As such, the shift toward a lower adipose AA/DHA ratio following pioglitazone treatment likely represents a more metabolically protective PUFA profile (Pietiläinen et al., 2011).

The selective decrease in AA-containing glycerophospholipids with pioglitazone, including plasmenylethanolamines, could result from either an increase in glycerophospholipid turnover (i.e., hydrolysis) or from the suppression of AA synthesis and/or esterification. In alignment with the former possibility, one previous study demonstrated that acute incubation of fibroblasts with pioglitazone accelerated the release of AA from cell membranes (Tsukamoto et al., 2004). Moreover, and in agreement with the current findings, this was not mediated by transcriptional or post-translational regulation of phospholipase activity but was instead attributed to a pioglitazone-mediated inhibition of AA reuptake. However, chronic blockade of membrane AA reuptake over 6 months of pioglitazone treatment would be expected to promote the accumulation of free AA or at least its diversion into other pathways. On the contrary, adipose tissue AA availability was lower following pioglitazone treatment and was tightly correlated with the decrease in glycerophospholipid AA esterification. Similarly, 20:4 lyso-PE, a product of the partial de-acylation of AA-containing PE species, was also lower following pioglitazone. Taken together with the decline in 18:0–20:4 phosphatidic acid, these findings suggest that the reduction in glycerophospholipid AA content, and subsequent suppression of plasmenylethanolamine concentrations, was likely related to a reduction in the partitioning of AA into glycerophospholipids rather than an increase in their turnover.

Another interesting finding from the current study was that levels of linoleic acid were reduced following pioglitazone treatment, both in adipose and skeletal muscle tissues. Since linoleic acid is an obligate precursor for the synthesis of other omega-6 PUFAs, it seems likely that its reduction contributed to the decrease in AA available for esterification into glycerophospholipids. Notably, the lower linoleic acid level was also mirrored by a significant reduction in adipose tissue cardiolipins, a mitochondrial membrane lipid highly enriched in linoleoyl side chains. Cardiolipin is often used as a marker of mitochondrial mass and, as such, this finding appears to contradict previous reports that thiazolidinediones increase mitochondrial content and respiratory complex expression in human adipose tissue (Bogacka et al., 2005; Xie et al., 2017). However, considering that our lipidomics analysis was performed on bulk adipose tissue and that white adipocytes are relatively poor in mitochondria (Cedikova et al., 2016), while infiltrating immune cells are relatively rich in mitochondria (e.g., lymphocytes; Picard, 2021) and activated macrophages possess higher mitochondrial mass than resting cells (Yu et al., 2020), the observed differences in cardiolipin content could potentially reflect differences in the cellular makeup of the adipose tissue. Indeed, previous studies have demonstrated a reduction in human adipose tissue inflammation following pioglitazone treatment through reduction of macrophages and mast cells (Spencer et al., 2014). Thus, the observed reduction in total cardiolipin levels after pioglitazone treatment may reflect fewer infiltrating macrophages. Moreover, we recently reported that pioglitazone treatment reduced hepatic cardiolipin content in obese mice and was associated with a suppression of mitochondrial fluxes (Shannon et al., 2021). Accumulating evidence suggests that pathological cardiolipin remodeling characterizes conditions of high oxidative stress, including diabetic cardiomyopathy (Han et al., 2007) and non-alcoholic fatty liver disease (Wang et al., 2015). This remodeling is typified by an increased abundance of long acyl chain cardiolipin species and was reversed by pioglitazone treatment in our previous study (Shannon et al., 2021). The low abundance of mitochondria in adipose tissue precluded the detection of these long acyl chain cardiolipin species in the current study and may thus have masked patterns of cardiolipin remodeling following pioglitazone treatment. As such, future studies are needed to reconcile discrepancies between cardiolipin profiles and the mitochondrial effects of pioglitazone treatment in adipose tissue.

The mechanism through which pioglitazone treatment lowered tissue linoleic acid is unclear. Since we did not monitor dietary patterns in the current study, we cannot exclude the possibility that the intake of linoleic acid, an essential fatty acid, declined over the six-month intervention period. However, alterations in dietary linoleic acid do not appear to influence the systemic availability of either linoleic acid or arachidonic acid (Rett and Whelan, 2011), suggesting that a decrease in linoleic acid intake is unlikely to be responsible for the lower tissue concentrations of linoleic acid following pioglitazone treatment. A more likely explanation for the decline in both linoleic acid and AA concentrations involves the activation of PPARγ, which is the primary molecular target of thiazolidinediones in adipose tissue (Spiegelman, 1998). Indeed, PPARγ agonism by thiazolidinediones is known to upregulate both lipid storage and lipid oxidation pathways in adipose tissue of T2D patients (Boden et al., 2005). Additionally, since AA and its derivatives can act as natural ligands for PPARγ (Nikolopoulou et al., 2014), it is feasible that a negative feedback loop exists coupling a reduced production

of endogenous ligand metabolites with the sustained activation of PPARγ by exogenous ligands such as pioglitazone.

It should be acknowledged that the reduction in PUFA-containing lipids could be indicative of their selective metabolism by peroxisomal β-oxidation, since the master driver of peroxisomes PPARα (Lee et al., 1995) has been implicated in the therapeutic efficacy of pioglitazone (Orasanu et al., 2008). However, peroxisomes serve as the exclusive site of plasmalogen synthesis (Thai et al., 2001) and, therefore, their putative upregulation by pioglitazone cannot explain the observed reduction in plasmalogens following pioglitazone treatment. Indeed, while the effects of PPARα agonists in human adipose tissue are poorly defined, it is notable that fenofibrates do not influence peroxisomal density (size or number) in human liver (Gariot et al., 1983). Moreover, human studies dissecting the divergent effects of pioglitazone and fenofibrate (monotherapy and combination) revealed that improvements in insulin sensitivity, glycemic control, and lipid metabolism following pioglitazone are near-exclusively driven by PPARγ and not PPARα (Bajaj et al., 2007; Belfort et al., 2010).

A final noteworthy effect of pioglitazone treatment related to AA metabolism was the reduction in AA-containing phosphatidylinositol. Although this effect was more robust in adipose tissue, it was also observed in skeletal muscle and is consistent with *in vitro* evidence that free AA availability can influence its enrichment in phosphatidylinositol (Anderson et al., 2016). At present, very little is known about how the acyl chain composition of phosphatidylinositol impacts upon its function. However, given the integral role of phosphatidylinositol derivatives (e.g., polyphosphoinositides PIP_2 and PIP_3) in the insulin signaling pathway (Barneda et al., 2019), our data raise the intriguing possibility that the molecular link between AA availability and phosphatidylinositol remodeling may contribute to the insulin-sensitizing effects of pioglitazone in peripheral tissues.

In summary, our study provides the first lipidomic characterization of the chronic effects of pioglitazone on adipose tissue in humans. The findings identify the glycerophospholipid pool as a central transducer of the responses to pioglitazone treatment, highlighting a potential role for adipose cell membrane remodeling in the immunometabolic benefits of thiazolidinediones. Our results derive from a relatively small and mostly (86%) male sample and thus warrant further validation in expanded populations, especially given the increasingly appreciated role of sexual dimorphism in adipose tissue metabolism and health (MacCannell et al., 2021; Pan and Chen, 2021). The lack of a control group is also a shortcoming, although it is partially compensated by assessment of the lipidome at baseline (pre-treatment). Nevertheless, our findings are consistent with known physiological effects of pioglitazone that have been robustly demonstrated in larger human studies (Miyazaki et al., 2002; Bray et al., 2013; White et al., 2021). Overall, our data support the targeting of pathways involved in the regulation of adipocyte cell membrane function as a novel approach to combat the metabolic and inflammatory sequelae of obesity and T2D.

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 Institutional Review Board of the South Texas Veterans Healthcare System, University of Texas health Science Center San Antonio. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the data analysis and interpretation of the data, drafting, and revising the manuscript and approved the final version of the manuscript. The study design was conceptualized by JP, DT, RD, and CS. Methodology was performed by JP, AC-V, DT, MP, and MF. Funding acquisition, RD and CS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.784391/full#supplementary-material

Supplementary Figure 1 | Lipid class totals in adipose tissue for triglyceride (TAG), diacylglyceride (DAG), free fatty acids (FFA), acylcarnitine (acylcarns), sphingomyelin (SM), ceramide (CER), phosphatidylethanolamine (PE), lyso-phosphatidylethanolamine (LPE), phosphatidylcholine (PC), lyso-phosphatidylcholine (LPC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA), and cardiolipin (CL) normalized to adipose tissue wet weight. $^*p < 0.05$ versus Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for n=6 subjects.

Supplementary Figure 2 | Adipose tissue ratio of C16:0 (palmitate)/C18:2 (linoleate) in glycerophospholipids **(A)** and free fatty acids **(B)** as an index of *de novo* lipogenesis. $^*p < 0.05$ versus Baseline. Data are mean ± standard error (filled bars) and individual values (filled circles) for n=6 subjects.

Supplementary Figure 3 | Strongest (top 15%) loadings from the first principal component of glycerophospholipids **(A)** which differentiated Baseline from Post-PIO adipose samples. Positive loadings (downregulated by pioglitazone) are in red and negative loadings (upregulated by pioglitazone) are in blue. Loadings which identify an arachidonic acid (20:4)-containing glycerophospholipid species are in bold text. Adipose tissue content of docosahexaenoic acid (C22:6) in glycerophospholipids **(B)** and arachidonic acid (C20:4) in triglycerides

(C). Data are mean ± standard error (filled bars) and individual values (filled circles) for *n*=6 subjects.

Supplementary Figure 4 | Saturation [number of double bonds; **(A)**] and length (number of carbon atoms; **(B)** of fatty acyl chains in the *sn-1* position of phosphatidylethanolamine; Fatty acyl composition of phosphatidylinositol **(C)** in adipose tissue, expressed as a molar fraction of total lipids in respective class. Skeletal muscle total cardiolipin content **(D)**. Data are mean ± standard error (filled bars) and individual values (filled circles) for *n*=6 **(A–C)** or *n*=5 **(D)** subjects.

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Lipid Transport in Brown Adipocyte Thermogenesis

*Gina Wade, Ayren McGahee, James M. Ntambi and Judith Simcox**

Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, United States

Non-shivering thermogenesis is an energy demanding process that primarily occurs in brown and beige adipose tissue. Beyond regulating body temperature, these thermogenic adipocytes regulate systemic glucose and lipid homeostasis. Historically, research on thermogenic adipocytes has focused on glycolytic metabolism due to the discovery of active brown adipose tissue in adult humans through glucose uptake imaging. The importance of lipids in non-shivering thermogenesis has more recently been appreciated. Uptake of circulating lipids into thermogenic adipocytes is necessary for body temperature regulation and whole-body lipid homeostasis. A wide array of circulating lipids contribute to thermogenic potential including free fatty acids, triglycerides, and acylcarnitines. This review will summarize the mechanisms and regulation of lipid uptake into brown adipose tissue including protein-mediated uptake, lipoprotein lipase activity, endocytosis, vesicle packaging, and lipid chaperones. We will also address existing gaps in knowledge for cold induced lipid uptake into thermogenic adipose tissue.

Keywords: brown adipose tissue (BAT), fatty acid, fatty acid binding protein (FABP), triglycerides (TGs), CD36, thermogenesis, lipoprotein, fatty acid transport protein (FATP)

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****Correspondence:***
Judith Simcox
jsimcox@wisc.edu

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INTRODUCTION

Endotherms maintain their body temperature by producing heat through both shivering and non-shivering thermogenesis. The cells primarily involved in non-shivering thermogenesis include brown and beige adipocytes, both of which stimulate heat production through disruption of the mitochondrial proton gradient by uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 2004; Cannon et al., 2020). These cells leverage other mechanisms to produce heat including futile cycling of phosphocreatine, calcium, and free fatty acids (Prentki and Madiraju, 2008; Kazak et al., 2015; Ikeda et al., 2017, 2018). The high energy demand of these futile cycles makes cells reliant on peripheral sources of stored fuels including glucose and lipids.

Research on peripheral fuel sources for non-shivering thermogenesis has focused on glucose uptake by brown and beige adipose tissue. Until the late 1980s it was thought that only human infants contained significant quantities of brown adipose tissue (BAT) (Lean et al., 1986). However, innovation in imaging with positron emission tomography with contrast tomography (PET-CT) using ^{18}F-fluorodeoxyglucose demonstrated that adult humans also have BAT capable of cold-induced heat production (active BAT) (Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009; Von Bank et al., 2021a). The use of glucose uptake to image and quantify BAT mass and function has led to a glucose centric view of thermogenesis. This has been fortified by the discovery that BAT is able to regulate whole body glucose homeostasis in humans and takes up 8-fold more glucose than skeletal muscle when activated (Chondronikola et al., 2014; Sidossis and Kajimura, 2015; Carpentier et al., 2018).

Moreover, adult humans that are exposed to repeated cold exposure have significantly lower blood glucose and hemoglobin A1C levels, the effects of which are dependent on the presence and quantity of BAT (Matsushita et al., 2014; Hanssen et al., 2016). Activation of thermogenesis with β3-adrenergic receptor agonists showed a similar response, leading to improved glucose levels in obese and diabetic individuals (Cypess et al., 2015; Finlin et al., 2020). Recent studies in humans and mice demonstrate that cold activation of BAT leads to import of fuel sources other than glucose including non-esterified fatty acids, branch chain amino acids, and triglycerides (TGs) (Bartelt et al., 2011; Ouellet et al., 2012; Yoneshiro et al., 2019).

Circulating lipids are also required as a fuel source for BAT (**Figure 1**). The most common circulating lipids are triglycerides, cholesterol and cholesteryl esters, and phospholipids. These lipids make up lipoproteins that are assembled by the intestine (chylomicrons and HDL) or liver (LDL, IDL, and HDL). Lipoprotein transport and uptake will be discussed further in section Extracellular Lipolysis and LDL Receptor Endocytosis Facilitates Triglyceride and Cholesterol Uptake into BAT. Free fatty acids (FFAs) also circulate at high levels bound to albumin. FFAs are released from white adipose tissue (WAT) after induction of an intracellular lipolysis cascade and taken up by peripheral tissues either passively (diffusion) or actively (protein-mediated). TG-rich lipoproteins (TRLs) such as VLDL, are the main source of circulating FFAs for BAT during thermogenesis (Festuccia et al., 2011; Hoeke et al., 2016). However, FFAs from WAT are necessary for body temperature regulation in mice (Schreiber et al., 2017). These FFAs can be directly imported into BAT or taken up by the liver where they are processed into TGs or acylcarnitines that are then shuttled to BAT (Górski et al., 1988; Simcox et al., 2017; Grefhorst et al., 2018). Moreover, hepatic uptake of FFA from WAT is necessary for activation of the transcription factor HNF4α, which positively regulates expression of genes involved in acylcarnitine synthesis. This suggests that circulating lipids are also essential signaling molecules for thermogenic activation, and lipid import into liver and BAT is required for the thermogenesis. The liver exports lipoproteins, cholesterol, and acylcarnitines into the circulation following import of FFA in response to cold exposure which are preferentially taken up by brown adipocytes (Bartelt et al., 2011; Berbée et al., 2015; Hoeke et al., 2016; Simcox et al., 2017). Collectively these studies show that a variety of lipids are taken up by BAT, which is reflected in the changing composition of circulating lipids upon cold exposure (Simcox et al., 2017; Lynes et al., 2018). This review will focus on the emerging knowledge that circulating lipids are an important mediator of thermogenic potential with a focus on how their transport into brown adipocytes is facilitated.

CIRCULATING LIPIDS ARE NECESSARY FOR BAT THERMOGENESIS

The utilization of circulating lipids by cold-activated BAT was not appreciated until 2011, when Bartelt et al. demonstrated a shuttling of TRLs into activated murine BAT (Bartelt et al., 2011). This uptake of TRLs was found to be necessary for thermogenesis. Mice unable to transport FFAs and TRLs generated by whole body knockout (KO) of the putative FA transport protein cluster of differentiation 36 (*Cd36*$^{-/-}$) were cold intolerant. The reliance of BAT thermogenesis on circulating lipids was further demonstrated by genetic ablation of the first enzyme in intracellular TG lipolysis, adipose triglyceride lipase (ATGL) encoded by *Pnpla2*. Whole body or adipose tissue specific ATGL KO led to accumulation of TGs in BAT and an inability to maintain body temperature (Haemmerle et al., 2006). Conversely, loss of ATGL in brown and beige adipocytes alone had no effect on body temperature or thermogenic transcripts (Schreiber et al., 2017). The results from the ATGL KO mouse models tell a compelling story that lipolysis in brown and beige adipocytes is dispensable for body temperature maintenance, but lipolysis in WAT is required. These findings were supported in mice unable to synthesize TGs and subsequently lipid droplets in BAT due to a UCP1-cre driven knockout of acyl-CoA:diacylglycerol transferase (*Dgat*) 1 and 2 (Chitraju et al., 2020). In this model, body temperature was maintained in the cold and utilization of circulating FFAs, and glucose was increased in BAT. Together, these studies supported the need for lipid uptake from plasma into BAT for thermogenic regulation.

Just as BAT uptake of glucose regulates systemic glucose homeostasis, uptake of lipids into BAT also regulates the circulating lipid pool. In hyperlipidemic mice modeled by KO of the extracellular lipolysis stimulator apolipoprotein A-V (*Apoa5*), cold exposure led to reduced levels of TGs and cholesterol in the plasma accompanied by an influx into the BAT (Bartelt et al., 2011; Berbée et al., 2015). These data demonstrate that BAT is an important regulator of the circulating lipid pool in mice (Hoeke et al., 2016). The presence of BAT in humans also regulates circulating TG and high-density lipoprotein (HDL) cholesterol levels. Moreover, repeated cold exposure has been shown to normalize circulating lipid levels and decrease hepatic lipid accumulation in humans (Wang et al., 2015; Bartelt et al., 2017; Wibmer et al., 2021). Although there is overwhelming evidence that cold activated BAT relies on lipid uptake for thermogenesis and can modulate circulating lipid levels, little is known about the regulation of transporters and the mechanisms that control lipid uptake. The next sections will focus on the protein-mediated uptake of FFAs, TGs, and other circulating lipids into activated BAT.

FREE FATTY ACID UPTAKE INTO BAT IS MEDIATED BY FABP, FATP, AND CD36

FA transport is divided into 3 steps: (1) adsorption, (2) translocation, and (3) desorption (Hamilton, 1998; Chmurzyńska, 2006). FAs are first delivered to the cell surface where they intercalate between outer leaflet phospholipids (adsorption). Next, a transition between outer and inner leaflet occurs (translocation) followed by exit from the plasma

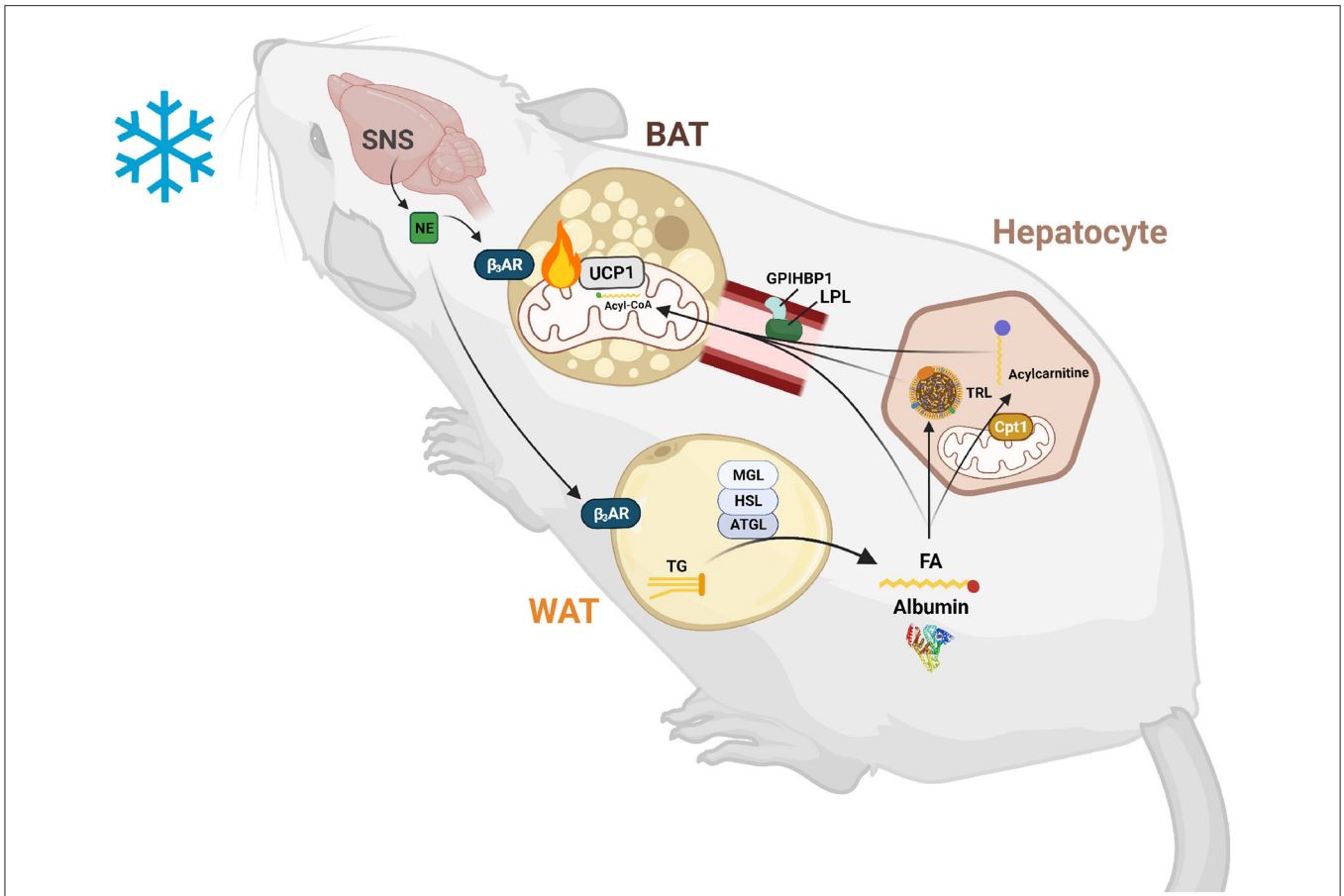

FIGURE 1 | Lipid-mediated crosstalk between the liver, WAT, and BAT is required for non-shivering thermogenesis. After exposure to cold temperatures, the sympathetic nervous system (SNS) releases norepinephrine (NE) to signal a need for thermogenesis. NE binds to β3-adrenergic receptors (β_3ARs) in white and brown adipose tissue (WAT and BAT). In WAT, this signal induces lipolysis of triglycerides (TGs) into fatty acids (FAs) and glycerol by an intracellular neutral lipase cascade involving adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoglyceride lipase (MGL). These FAs are exported into the circulation and are either taken up by BAT to activate uncoupling protein 1 (UCP1) and be broken down by β-oxidation or are taken up by hepatocytes to serve as substrates and transcriptionally activate acylcarnitine production. These FAs are also used to synthesize TGs for packaging into triglyceride-rich lipoproteins (TRLs) for export into the circulation and use by BAT to fuel thermogenesis. Hepatic acylcarnitines synthesized after signaling by WAT through FAs are exported into the circulation to be used as both a signal and fuel for heat production.

membrane into the cytoplasm (desorption). Acylation concomitant to desorption, termed vectorial acylation, may serve as a final step in FA transport and a means of directing the FA toward its target organelle (Zou et al., 2003). The integral membrane protein and putative lipid transporter CD36 along with fatty acid transport proteins (FATPs) have been suggested to carry out the first two steps and act as a docking site for plasma membrane associated fatty acid binding protein ($FABP_{pm}$), which has been proposed to facilitate the desorption step. However, $FABP_{pm}$ has also been proposed to function as a buffering system, maintaining the concentration of unbound fatty acids across the plasma membrane (**Figure 2**) (Abumrad et al., 1999; Glatz and Luiken, 2020). Cytoplasmic FABPs shuttle FAs to various organelles including the mitochondria for β-oxidation, the ER for lipid synthesis, or the nucleus where FAs regulate transcription.

Fatty Acid Transport Proteins (FATPs)

The FATP family is considered the primary transporter in the putative protein-mediated FA transport mechanism. There are six isoforms in mammals, and each displays a unique pattern of tissue expression. They are members of the solute carrier protein superfamily, classified specifically as Slc27a1-6. FATP1 was the first family member discovered in 1994 and is most highly expressed in skeletal muscle and adipose tissue. FATP1 was shown to localize to the plasma membrane and significantly increased radiolabeled oleic acid uptake when overexpressed in the adipocyte 3T3-L1 cell line (Schaffer and Lodish, 1994). The structure of FATPs is unknown but based on sequence and protease protection assays of epitope tagged FATP1, FATPs are oriented with an extracellular N-terminus and a cytoplasmic C-terminus (Lewis et al., 2001; Stahl, 2004). FATPs contain one or more membrane-spanning regions, multiple membrane-associated regions, and an AMP-binding motif in the intracellular

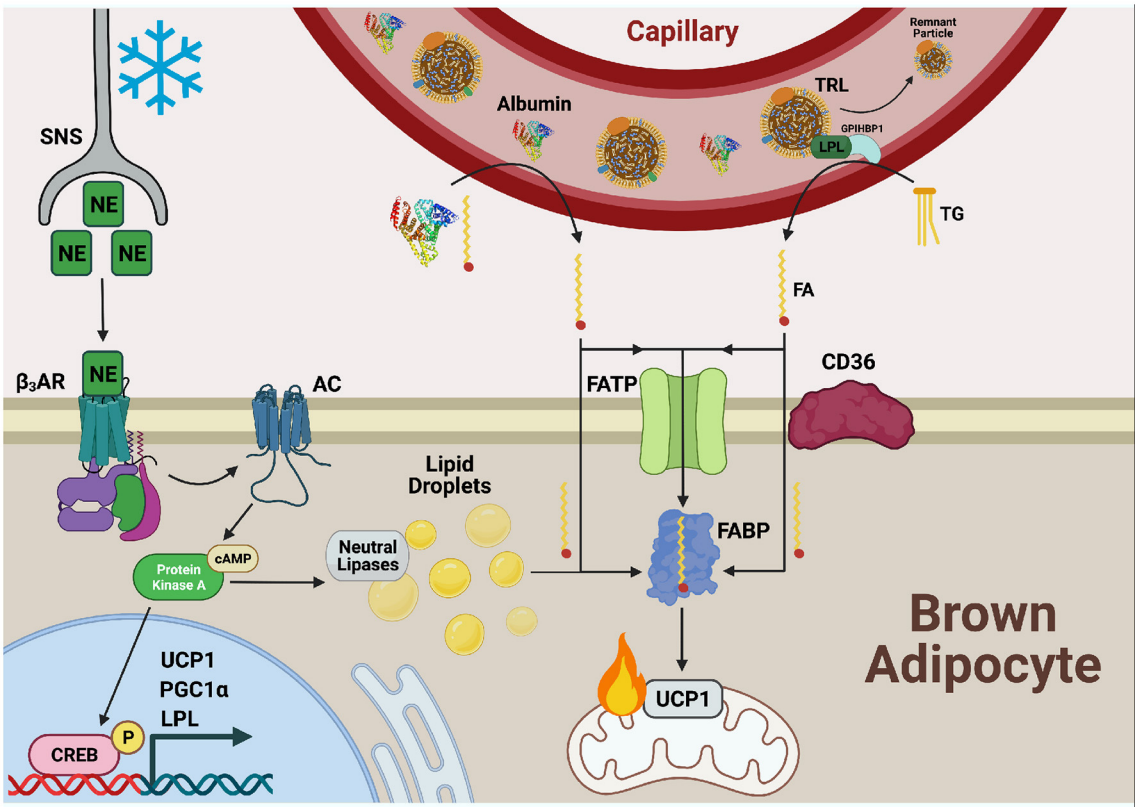

FIGURE 2 | Fatty acid uptake is facilitated by FATP, CD36, and $FABP_{PM}$ in cold-activated BAT. Cold exposure stimulates the release of norepinephrine (NE) from the sympathetic nervous system (SNS) which is sensed by β3-adrenergic receptors (β_3ARs) on the cell surface of adipocytes. Adenylate cyclase (AC) is activated and produces cyclic AMP (cAMP) for binding to protein kinase A (PKA). This upregulates transcriptional programs to support thermogenesis, including uncoupling protein 1 (UCP1), proliferator-activated receptor-gamma co-activator (PGC1α), and lipoprotein lipase (LPL) through cAMP-responsive element binding protein (CREB). PKA also activates lipolysis of lipid droplets to liberate fatty acid (FAs), fuel FA β-oxidation, and activate UCP1 through direct binding. Necessary to this process is the transport of exogenous FAs into BAT by the combined action of fatty acid transport proteins (FATPs), CD36, and fatty acid binding proteins (FABPs). FAs bound to albumin are released from the circulation into the brown adipocyte and can either diffuse across the plasma membrane or be actively transported by the FA transport machinery. Triglycerides (TGs) from circulating TG rich lipoproteins (TRLs) are hydrolyzed to FAs via LPL tethered to the vascular lumen by glycophosphatidylinositol-anchored HDL-binding protein 1 (GPIHBP1) for transport into and use by the cell as well.

region. FA transport through FATP is ATP-dependent, and the sequence of the AMP-binding region is required for transport. Loss-of-function mutations in the AMP-binding domain prevent FA uptake, suggesting AMP-binding is directly involved in or coupled to the transport mechanism (Stuhlsatz-Krouper et al., 1998). Further, overexpression of FATP1 increased intracellular FA acylation, thus suggesting a role for FATPs in cellular acyl-coA synthetase activity (Steinberg et al., 1999). These results, along with sequence similarity to acyl-CoA synthetases, suggested that FATPs function to transport FAs through the plasma membrane and esterify them as they enter the cell. Evidence has emerged suggesting some FATPs do not actually transport FAs, but rather enhance FA uptake through esterification at the ER, thus lowering intracellular levels of non-esterified FAs and encouraging FA uptake (Milger et al., 2006). Whether the transport and esterification functions of FATP are coupled or independent is widely debated and may depend on the cellular context.

FATP1 is the primary member expressed in adipocytes. Translocation to the plasma membrane is stimulated by insulin and causes an increase in FA uptake (Kim et al., 2004). This insulin stimulation is consistent with the current model that inhibition of lipolysis through the intracellular hormone sensitive lipase (HSL) signals a shift toward glucose utilization and storage of TGs. FATP1 KO mice showed delayed clearance of serum FAs following insulin injection. FA uptake in primary brown adipocytes from these mice was diminished following insulin treatment, suggesting FATP1 is needed to transport FA from the circulation into tissue. Radiolabeled oleic acid uptake into WAT in FATP1 KO mice was diminished, while uptake by the liver and heart was increased, demonstrating a systemic compensation for disruption of FA transport by specific FATPs (Wu et al., 2006). Single nucleotide polymorphisms (SNPs) in FATP1 are linked to increased plasma TG levels in humans, and high expression of other FATPs in peripheral tissue correlates with obesity and insulin resistance

owing to an accumulation of intracellular FAs (Lobo et al., 2007).

FATP1 in BAT is necessary for maintaining core body temperature after prolonged cold exposure (Wu et al., 2006). It is primarily localized to the plasma membrane upon cold activation, but a small pool is detected on intracellular membranes as well. Besides uptake of exogenous FAs, FATP1 may aid in lipid transport between organelles in brown adipocytes or esterify incoming FAs for quick turnover into β-oxidation. FATP1 expression is significantly induced in cold-activated BAT, and FATP1 KO mice exhibit a significant drop in body temperature after ∼12 h of cold exposure. This was accompanied by a rise in serum FA levels during cold exposure, likely owing to the inability of activated BAT to efficiently take up exogenous FAs. Peroxisome proliferator-activated receptor alpha (PPAR-α) and gamma (PPAR-γ), two transcriptional regulators of cell differentiation and lipid metabolism, were shown to control expression of FATP1 in 3T3-L1 adipocytes (Frohnert et al., 1999). Treatment with activators of PPAR-γ, such as linoleic acid, induced expression of FATP1 and FA uptake. This is consistent with the induction of both PPAR-γ and FATP1 in BAT during cold exposure, although a direct path of regulation has not been demonstrated in cold-activated BAT. Similarly, BAT-specific KO of FATP1 has yet to be explored to elucidate the function of its FA transport role in thermogenesis. Future studies are required to contextualize the role of FATPs in exogenous FA uptake by BAT.

Fatty Acid Binding Proteins (FABPs)

FABPs are a family of intracellular protein chaperones that bind long chain fatty acids (LCFAs) to expedite movement through membranes. There are currently 9 FABPs named for the tissue of discovery, but they are not exclusively expressed in the tissue for which they are named. The cytoplasmic FABP family differs in sequence, structure, and function from the plasma membrane associated FABP ($FABP_{PM}$). First described in 1985, the putative fatty acid transporter $FAPB_{PM}$ was shown to have a high affinity for fatty acids (Stremmel et al., 1985). Later, $FABP_{PM}$ was found to be identical to mitochondrial aspartate aminotransferase, a member of the malate-aspartate shuttle, although expression at the plasma membrane was associated with fatty acid uptake (Berk et al., 1990; Bradbury and Berk, 2000). Inhibition of $FABP_{PM}$ by antibody treatment in multiple tissues yielded a decrease in LCFA uptake while overexpression in skeletal muscle yielded an increase, thus supporting $FABP_{PM}$ as a lipid transporter (Berk et al., 1990; Clarke et al., 2004). More work is needed to understand the importance of $FABP_{PM}$ expression in brown adipocytes and how it changes with cold exposure. The remainder of this section will focus on other FABP family members in the cytoplasm.

Cytoplasmic FABPs solubilize hydrophobic molecules in the aqueous cellular environment, acting as chaperones and delivery systems for lipids between organelles. Structural characterization has revealed an indirect role in LCFA uptake; that is, FABPs do not facilitate transport across the plasma membrane but are essential for delivery into the cell (Storch and McDermott, 2009). All FABPs share a common tertiary structure comprised of antiparallel beta sheets forming a small beta barrel within which a hydrophobic molecule may bind (Sacchettini et al., 1988). There are subtle structural differences between isoforms that dictate ligand specificity. For example, adipocyte FABP (FABP4), also known as adipocyte protein 2 (AP2), exclusively binds LCFA while liver FABP (FABP1) can additionally bind eicosanoids, lysophospholipids, and acyl-CoA (Rolf et al., 1995; Thompson et al., 1997; Furuhashi and Hotamisligil, 2008). These cytoplasmic FABPs have also been shown to be secreted into the bloodstream with several physiological stresses including high fat diet, insulin resistance, and cold exposure (Hotamisligil and Bernlohr, 2015; Shu et al., 2017).

The adipocyte associated FABP (FABP4) is the best characterized in its family. FABP4 was first isolated from mouse embryonic fibroblast (3T3)-derived adipocytes, although it is highly expressed in macrophages as well (Hunt et al., 1986; Hotamisligil et al., 1996). FABP4 KO mice were viable, developed normally, and were indistinguishable from control mice in appearance and metabolic health (Hotamisligil et al., 1996). Like control mice, they were sensitive to dietary and genetic obesity, but were protected from insulin resistance and diabetes. This implicates FABP4 in suppression of insulin signaling, perhaps through increasing the intracellular lipid pool. Additionally, these mice exhibited a compensatory increase in epidermal FABP (E-FABP or FABP5) expression in adipose tissue, despite basal FABP5 expression being almost 100-fold lower than FABP4. Besides binding FAs, FABP4 has been shown to suppress PPAR-γ, a master adipocyte transcription factor that activates genes involved in lipid synthesis and uptake to enhance adipogenesis (Garin-Shkolnik et al., 2014). FABP4 also stimulates intracellular lipolysis through direct binding of HSL as suggested by a reduction in lipolysis in FABP4 KO mice and demonstrated by fluorescence resonance energy transfer of FABP4 mutagenized within the putative HSL binding site (Smith et al., 2008). At a chemical level, FABP4 binds FAs entering the cell. However, substantial evidence illustrates its function as an intracellular signal for proper adipocyte function and systemic fuel utilization.

FABP4 is increased with cold exposure in BAT and in the blood plasma and was shown to be necessary for FFA uptake into BAT (Shu et al., 2017). Knockout of FABP4 and HFD led to cold sensitivity and lower UCP1 expression that could be rescued with recombinant FABP4. FABP4 was shown to be sufficient to drive increased oxygen consumption in mice and elevated UCP1 expression. This increase required FA binding to FABP4, as a mutant R126Q did not have the same rescue capacity. Mechanistically, FABP4 in the BAT was shown to drive the intracellular conversion of T4 to T3 which is necessary for the thermogenic program (Ahmadian et al., 2011). FABP4 has also been shown to be increased in the BAT of hibernating mammals (Hittel and Storey, 2001; Eddy and Storey, 2004). Other studies have shown that FABP4 and 5 are necessary for proper metabolic adaptation in cold-activated BAT and that fasting greatly impacts this contribution to BAT function as well. When fasted for 20 h prior to a 4-h cold exposure, FABP4/5 KO mice were cold intolerant but indistinguishable from control mice in a fed state (Syamsunarno et al., 2014). This suggests a requirement of FABP4/5 for proper adjustment of systemic fuel

utilization during physiological stress, such as long-term fasting and cold.

Curiously, heart-type FABP (FABP3) has been shown to be the dominant isoform in BAT upon cold activation. Expression of FABP3 was significantly increased in BAT after 4 h of cold despite a basal level of expression 6-fold lower than FABP4. Further, FABP3 KO mice were cold intolerant but displayed similar changes in thermogenic transcriptional programs to WT mice, such as induction of the coactivator PGC1α, which regulates mitochondrial biogenesis. Expected physiological changes were observed in FABP3 KO mice as well, such as depletion of TGs in BAT and increased FFAs in plasma (Vergnes et al., 2011). FABP3 was also shown to increase in hibernating ground squirrels (Hittel and Storey, 2001). Together, these data highlight an essential role for FABP3 in the uptake of exogenous lipids into BAT, the dysfunction of which results in an inability to maintain body temperature. Although FABP3 is not the dominant isoform in BAT, its function in cold exposure suggests a dynamic response network of FABPs in certain cell types depending on the environmental stimulus (Daikoku et al., 1997; Yamashita et al., 2008). This also underscores the gaps in current knowledge of FABPs in BAT function.

Cluster of Differentiation 36 (CD36)

CD36, also referred to as fatty acid translocase (FAT), is an integral membrane protein belonging to the scavenger receptor superfamily. SRs bind a host of ligands including LDL, oxidized LDL, phospholipids, cholesterol esters, collagen, thrombospondin, carbohydrates, and microbial pathogens. There are 11 classes of scavenger receptors categorized based on primary sequence (A-L). CD36 belongs to the scavenger receptor B class (SR-B), the members of which contain two transmembrane domains and a single extracellular loop (Pepino et al., 2014). Diversity in the extracellular loop allows SR-Bs to regulate a variety of signal transduction pathways. Loss of function mutations or genetic deficiencies of SR-B members causes dysregulation of apoptosis, inflammatory response, and intracellular metabolism resulting in metabolic disease (Bonen et al., 2007; Glatz et al., 2010; McFarlan et al., 2012).

Although originally identified in human platelets, CD36 was characterized as a high affinity long-chain fatty acid (LCFA) receptor and transporter in rat adipocytes (Abumrad et al., 1993; Harmon and Abumrad, 1993). Treatment with highly charged FA analogs (N-sulfosuccinimidyl LCFA esters) inhibited oleate transport into adipocytes, and covalent protein labeling identified the 88 kDa CD36 as the receptor candidate (Harmon and Abumrad, 1993). Northern blotting revealed transcript abundance across tissues including adipose, heart, intestine, and skeletal muscle. Oleate uptake was limited to cell lines expressing CD36, further implicating the membrane protein as a lipid transporter (Abumrad et al., 1993). Modern proteomics and transport kinetics studies in transgenic Chinese hamster ovary cells identified a definitive FA binding pocket in CD36 that supports a direct function in lipid binding (Kuda et al., 2013). This pocket contains lysine 164 which binds the CD36 inhibitor N-hydroxysuccinimidyl ester of oleate (SSO) and, when mutagenized to alanine, diminished FA uptake. Despite a host of evidence supporting its lipid binding function, its role as a transporter has long been disputed.

Transmembrane LCFA transport has been at the center of a contentious debate for decades. The two opposing sides are comprised of "diffusionists," who argue for rapid diffusion-mediated LCFA transport, and "translocationists," who suggest transport is facilitated by proteins (Pownall and Moore, 2014; Glatz and Luiken, 2020). There is a myriad of evidence supporting both sides. The diffusionists argue that in biological membranes, lipids constantly diffuse laterally and often exchange between membrane leaflets without proteins. The FFAs would transfer from albumin to the outer leaflet of the plasma membrane and then flip-flop to the inner leaflet (Hamilton, 1998). This hypothesis is supported by demonstration of diffusion through fluorescent LCFA uptake in lipid vesicles pre-treated with inhibitors of FA transporters, two of which were direct competitive inhibitors for CD36 (Jay et al., 2020). The rate of uptake in rat adipocytes was unaffected by inhibitor treatment, suggesting that proteins are not required for transport (Abumrad et al., 1993; Harmon and Abumrad, 1993; Coburn et al., 2000; Kuda et al., 2013). However, these are imperfect systems to study lipid transport. Artificial membranes do not reflect true biological lipid or protein composition, and cultured cells do not reflect membrane dynamics. For example, fatty acid chain composition is different in culture, having lower levels of poly-unsaturated fatty acids compared to cells in organisms (Else, 2020).

The translocationists have been supported by several key findings including the observation that FFA uptake can be saturated in multiple cell types at levels within physiological range and that the flip-flop between the outer and inner leaflet does not occur at high enough levels to support metabolic demand (Kleinfeld and Storch, 1993; Kleinfeld et al., 1997). Moreover, an antibody to CD36 blocked FFA uptake into mouse adipocytes, and mutation of Lys164 in the FA binding pocket demonstrates site specific functional regulation (Abumrad et al., 1993; Pepino et al., 2014). Recent work has found that palmitoylation of CD36 leads to caveolae-dependent internalization, and inhibition of palmitoylation prevents this uptake (Hao et al., 2020). While the mechanism of lipid transport remains controversial, the *in vivo* role of CD36 in regulating FA uptake and metabolism has been demonstrated across many models. Moreover, in humans, common SNPs in the promotor of *CD36* cause protein deficiency and have been associated with high levels of FAs and LDL in serum, a phenotype recapitulated in $Cd36^{-/-}$ mice (Miyaoka et al., 2001; Ma et al., 2004; Goudriaan et al., 2005; Yamashita et al., 2007; Love-Gregory et al., 2011). Newer models favor both diffusion and translocation that are important in different ranges and energy demands of the cell (Abumrad et al., 1999).

CD36 expression is known to be highest in adipose tissue, and it is required for body temperature regulation in mice (Putri et al., 2015). Additionally, its expression is significantly increased in BAT following cold exposure (Bartelt et al., 2011). CD36 is required for lipoprotein uptake in BAT, and global CD36 KO in mice results in TG buildup in BAT and cold intolerance (Yamashita et al., 2007; Bartelt et al., 2011). CD36 is necessary for transport of lipids via lipoproteins and FFA-bound albumin

in BAT as well. Clearance of both lipoproteins and FFAs from plasma and uptake into BAT were diminished in cold-exposed Cd36 KO mice (Bartelt et al., 2011). CD36 is also required for uptake of lipid-related molecules into BAT, such as coenzyme Q (CoQ) (Anderson et al., 2015). CoQ contains an isoprenoid tail and is a necessary electron transporter between complexes in the electron transport chain. Therefore, it is required for both ATP synthesis and heat production in BAT. High levels of CoQ are present in BAT despite low levels of endogenous synthesis, suggesting a requirement for uptake of CoQ from the circulation. Whole-body *Cd36*$^{-/-}$mice displayed a 2-fold increase in serum CoQ accompanied by CoQ deficiency, TG accumulation, and diminished mitochondrial size and metabolic capacity in BAT. Although CD36 is present in other tissues, these phenotypes were not reflected throughout the mice. Cold intolerance in both whole-body and BAT-specific *Cd36*$^{-/-}$was driven by CoQ insufficiency causing reduced FA β-oxidation in BAT. These studies highlight the requirement of CD36 for proper uptake of lipids and hydrophobic molecules into cold-activated BAT.

EXTRACELLULAR LIPOLYSIS AND LDL RECEPTOR ENDOCYTOSIS FACILITATES TRIGLYCERIDE AND CHOLESTEROL UPTAKE INTO BAT

Lipoprotein Metabolism and Regulation by BAT

Lipoproteins are means of transporting lipids of varying polarity from centers of lipid processing, such as the intestine and liver, to peripheral tissues through the circulation. They are comprised of a phospholipid monolayer interlaced with apolipoproteins that serve as structural reinforcements and as LDL receptor (LDLR) recognition sites to aid in endocytosis. Lipoproteins fall into four main classes depending on their relative composition of proteins and lipids: high density (HDL), low-density (LDL), very low-density (VLDL), and chylomicrons. In addition to the characteristic structure of each class, different lipoproteins also serve different functions. For example, LDL carries circulatory cholesterol and easily enters arterial walls, while chylomicrons are essential for dietary lipid transport to the liver and peripheral tissues. Nonetheless, each class has a vital role in lipid transport, endocytosis, and hydrolysis (Zanoni et al., 2018).

LDL endocytosis begins with recognition at the cell membrane by a low-density lipoprotein receptor (LDLR). Following recognition, a clathrin-coated pit forms a vesicle around the endocytosed lipoprotein. The vesicle is directed to endosomes where a drop in pH causes release of LDL from LDLR. Acidic lipases in the newly formed lysosome hydrolyze the contents of LDL to release FAs from TGs and unesterified cholesterol from cholesteryl esters (**Figure 3**). Cholesterol is incorporated into cell membranes and negatively regulates its own synthesis by preventing translocation of sterol regulatory binding protein (SREBP) from the ER to the Golgi, thereby blocking transcriptional activation of HMG-CoA reductase (Brown and Goldstein, 1997). FAs released from LDL are shuttled to the ER for synthesis into membrane lipids, the Golgi for protein acylation, and the mitochondria for FA β-oxidation.

In brown adipocytes, LDLR endocytosis fuels the electron pool needed for proton gradient uncoupling and heat production (**Figure 4**). LDLR is recycled back to the plasma membrane for addition rounds of endocytosis (Ikonen, 2008). LDLRs are also necessary in systemic cholesterol and triglyceride balance during cold exposure. *Ldlr*$^{-/-}$ mice displayed a reduction in plasma TG upon activation of BAT by cold or with a β3 adrenergic receptor agonist but did not show a reduction in plasma cholesterol normally observed after BAT activation (Dong et al., 2013; Berbée et al., 2015). In this model, the liver is unable to clear lipoprotein remnants produced from FA uptake in BAT following LPL-mediated TG hydrolysis and thus cholesterol remains in the circulation. Coordinated plasma lipid clearance between the liver and BAT during cold exposure requires proper protein-mediated lipid uptake, such as LDLR endocytosis.

Triglyceride Uptake Into BAT

TGs are the neutral storage form of FAs and travel between tissues through the bloodstream via chylomicrons and VLDL. Chylomicrons are the largest form of lipoprotein and are assembled by the small intestine following emulsification of dietary fats. TGs packaged into chylomicrons mainly enter adipose tissue and skeletal muscle due to high lipoprotein lipase (LPL) activity. The remaining TG is taken up by the liver and can be repackaged into VLDL for use throughout the body. Alternatively, FAs are synthesized by the liver through *de novo* lipogenesis, assembled into TG, and mobilized in VLDL. As TGs are liberated from VLDL for use by peripheral tissues, smaller remnants of decreasing TG content are formed such as intermediate-density lipoproteins (IDL) and LDL. Like chylomicrons, VLDL is utilized by tissues after lipolysis by LPL.

LPL is the predominant lipase in tissues with high levels of exogenous lipid uptake, such as adipose tissue, heart, and skeletal muscle. LPL is a dimeric enzyme localized to the vascular lumen where it hydrolyzes plasma TGs into glycerol and FAs for tissue uptake. TG-derived FA uptake into BAT is reliant on localized LPL activity, as injection of an LPL inhibitor (tetrahydrolipstatin) or treatment with heparin to release LPL from the vascular wall in mice prior to cold exposure almost completely abolished labeled TRL uptake into BAT.[20] LPL is ferried and anchored to the endothelium via glycophosphatidylinositol-anchored HDL-binding protein (GPIHBP1). Mice lacking GPIHBP1 have increased plasma TGs due to reduced tissue uptake (Cushing et al., 2018). As circulating lipoprotein levels are highly modulated in response to metabolic state, such as fasting and cold exposure, tight regulation of LPL activity is required. Besides localization, LPL is primarily regulated post-translationally by several extracellular proteins. Angiopoietin-like proteins (ANGPTL) are the main class of LPL regulators. ANGPTLs are secreted into the lumen and directly interact with LPL, preventing its dimerization and inhibiting lipolysis (Hegele, 2016).

ANGPTL4 is the predominant isoform in brown adipose tissue. In BAT, ANGPTL4 expression is induced during periods of nutrient deprivation (fasting) and suppressed when substantial

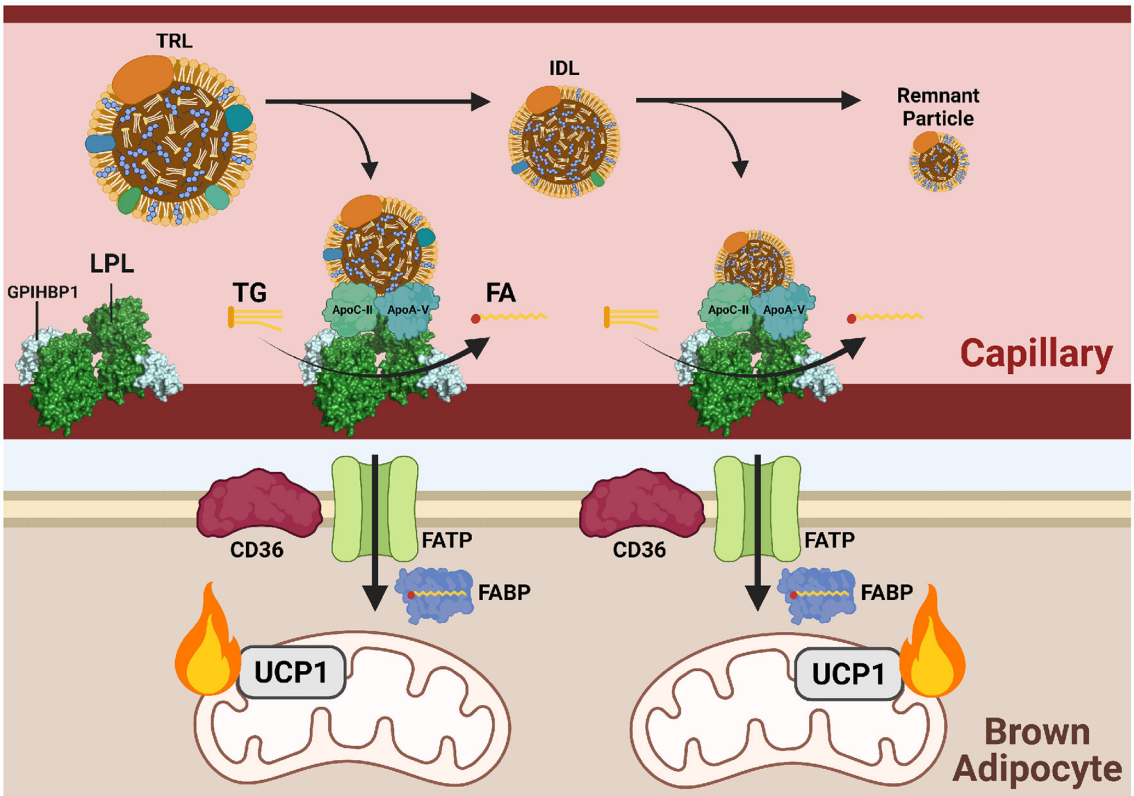

FIGURE 3 | Triglycerides are delivered to cold-activated BAT through LPL hydrolysis at the endothelial wall. Following β3-adrenergic receptor (B_3AR) activation by norepinephrine (NE) signaling a drop in temperature, hepatocytes and enterocytes mobilize triglyceride (TG)-rich lipoproteins (TRLs) into the blood for transport to brown adipocytes. Lipoprotein lipase (LPL) at the vascular endothelium hydrolyzes TGs from TRLs into fatty acids (FAs) for subsequent FATP/CD36-mediated transport into brown adipose tissue (BAT). Hydrolysis is dependent on the co-factor apolipoprotein C-II (apoC-II) and activator apolipoprotein A-V (apoA-V). As hydrolysis occurs, TRLs reduce into intermediate lipoproteins (IDLs), low-density lipoproteins (LDL), and finally remnant particles to be cleared by the liver. Each reduction in size is accompanied by a loss of triglycerides (TGs). Imported FAs are funneled into mitochondrial β-oxidation and eventual heat production.

nutrients are available or needed, such as postprandially or during cold exposure (Singh et al., 2018). In cold-activated WAT, ANGPTL4 is upregulated to shift away from fat storage and toward output of FAs to support thermogenesis. BAT-specific ANGPTL4 KO mice exhibit reduced plasma TGs in the fed and fasted state, accumulation of ^{3}H from labeled triolein in BAT, and increased expression of CD36 in BAT (Cushing et al., 2018). LPL expression was unchanged, but activity was significantly increased in BAT from ANGPTL4 KO mice. ANGPTL4 KO mice had higher rectal temperature over time during a 4-h cold exposure, likely due to enhanced LPL activity in BAT fueling FA uptake and β-oxidation. In contrast, *Gpihbp1*$^{-/-}$ mice show no change in labeled triolein uptake into BAT. Double GPIHBP1/ANGPTL4 KO mice show a partial correction in plasma TG levels compared to GPIHBP1 KO mice (Dijk et al., 2015). However, this correction is lost after a 2-week HFD, with both GPIHBP1 KO and GPIHBP1/ANGPTL4 KO showing a similar increase in plasma TGs compared to mice of the same genotype on a normal chow diet. In sum, these studies highlight the necessity of tight LPL regulation in BAT to modulate TG and FA uptake during metabolic stress, including cold exposure.

It has been shown that BAT relies more heavily on LPL-based uptake of TG-derived FAs rather than particle endocytosis, but cold exposure significantly enhances the uptake of TGs through both methods (Khedoe et al., 2015). This was observed through tracing of ^{3}H-triolein and ^{14}C cholesteryl esters which allow measurement of TG uptake by lipolysis and lipoprotein uptake by endocytosis, respectively.

While VLDL is overwhelmingly shuttled to BAT when activated by exposure to cold, transport of HDL to the liver is drastically increased (Schaltenberg et al., 2021). HDL balances cholesterol flux between peripheral tissues and is both synthesized and excreted by the liver. This hepatic processing is dependent on endothelial lipase (EL). Following extended cold exposure (1 week), expression of the gene encoding EL, *Lipg*, was increased in murine BAT. Cold exposure was also shown to enhance HDL clearance from plasma. Moreover, *Lipg*$^{-/-}$ mice displayed higher plasma levels of HDL, indicating poor cholesterol clearance. The HDL particles from *Lipg*$^{-/-}$ mice were enriched in phospholipids but lacking in cholesterol and TG compared to WT mice both at room temperature and after prolonged cold exposure. EL is known to promote TRL uptake,

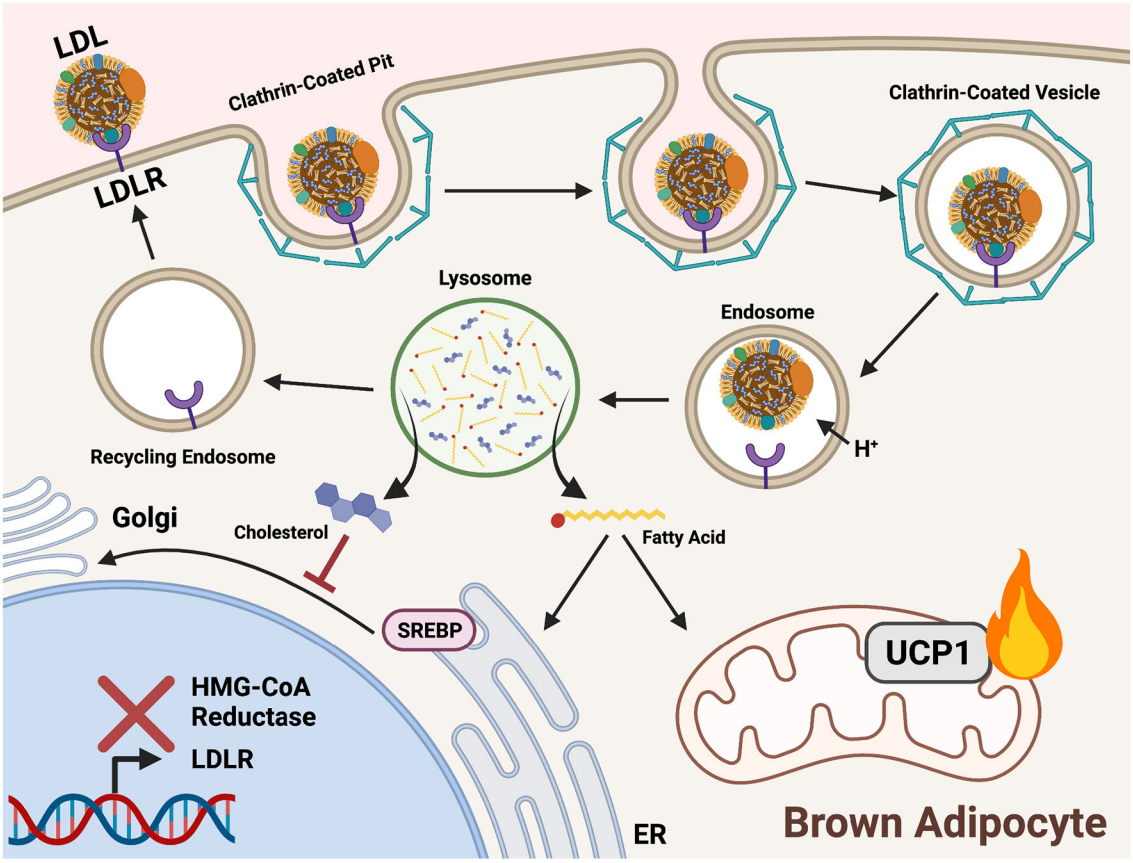

FIGURE 4 | Triglycerides and cholesterol are delivered to cold-activated BAT through LDLR mediated endocytosis. Low-density lipoprotein receptors (LDLRs) recognize apolipoprotein B on the surface of low-density lipoproteins (LDL). A clathrin-coated pit forms around the bound receptor and an endocytotic vesicle forms. An internal drop in pH prompts release of LDL from LDLR for hydrolysis in the lysosome. Acid hydrolases liberate fatty acids (FAs) from triglycerides (TGs) and cholesterol within LDL. In brown adipocytes, FAs are shuttled into mitochondrial β-oxidation to fuel downstream heat production. Cholesterol is incorporated into cell membranes and signals a negative feedback loop of biosynthesis by preventing sterol-regulatory-element binding proteins (SREBP) translocation to the Golgi from the ER. This prevents proteolytic processing and localization to the nucleus, thereby inhibiting expression of genes encoding cholesterol synthesis enzymes (HMG-CoA reductase) and lipoprotein receptors (LDLR).

much like LPL. While EL expression is induced in BAT after cold exposure, it was not required for proper thermogenic activation, nor did its loss affect thermogenic transcriptional programs such as those controlled by PPAR-γ.

VARIOUS LIPIDS ALTERED IN THE PLASMA WITH COLD EXPOSURE

The advent and expansion of mass spectrometry based lipidomics has broadened the field of circulating lipids, and several lipid classes have been shown to be increased in blood plasma with cold exposure including acylcarnitines, ceramides, 12,13-dihydroxy-9z-octadecenoic acid (12,13-diHOME), and fatty acid esters of hydroxy fatty acids (FAHFAs). Little is known about how theses lipids are transported across plasma membranes, what functional roles they serve in non-shivering thermogenesis, and in what complex structure they are mobilized in the circulation. While this remains an emerging field, it is rapidly progressing with new mass spectrometry-based technology such as ion mobility, matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry, heavy isotope labeling, and several new chemical probes (Kyle et al., 2018; Kirkwood et al., 2021).

Acylcarnitines

Acylcarnitines are fatty acids conjugated to a carnitine through esterification. At the cellular level, acylcarnitines function as intermediaries facilitating transport of FFAs into the mitochondria for β-oxidation. Carnitine palmitoyltransferase 1 (CPT1) is embedded on the outer-surface of the mitochondrial membrane and esterifies the fatty acid from an acyl-CoA to carnitine. This acylcarnitine can then diffuse into the porous outer mitochondrial member. Acylcarnitine is then brought into the inner mitochondria by carnitine acylcarnitine transferase (CACT) and de-carnitylated by CPT2. Besides their cellular role for fatty acid transport, acylcarnitines are also found in the blood plasma. Plasma acylcarnitines increase with chronic diseases such as type 2 diabetes, cardiovascular disease, and inborn errors of

metabolism as well as in acute metabolic stresses such as fasting, exercise, and cold exposure (Muoio et al., 2012; Schooneman et al., 2013; McCoin et al., 2015; Simcox et al., 2017). The functional role of acylcarnitines in the plasma has been proposed to range from protection from toxicity to a distinct storage pool that can be pulled from during energy demanding conditions (Muoio et al., 2012).

In cold exposure, short chain, medium chain, and long chain acylcarnitines are increased in the plasma while carnitine levels decrease (Simcox et al., 2017; Pernes et al., 2021). This cold induction of increased plasma acylcarnitines is mediated by β3-adrenergic receptor induced WAT lipolysis, since adipose tissue-specific ATGL knockout mice had no changes in acylcarnitine levels with β3-adrenergic receptor agonist treatment. Once FFAs are released from the WAT, they are taken up into the liver, where they transcriptionally activate CPT1, CACT, and CPT2 through an HNF4α-mediated mechanism as well as serve as substrate for acylcarnitine production (Simcox et al., 2017; Jain et al., 2021). These liver-produced acylcarnitines are then taken up into the BAT, skeletal muscle, and heart. In the BAT, the acylcarnitines are catabolized as a fuel source for thermogenesis. Beyond the liver, there are other potential sources for cold induced plasma acylcarnitines; while ablation of acylcarnitine production in the liver causes cold intolerance, it is not sufficient to completely block the rise in plasma acylcarnitines with cold exposure. Recently it has been shown that the kidney may also contribute to the plasma acylcarnitine pool (Jain et al., 2021). These studies collectively demonstrate that plasma acylcarnitines are produced through a multi-tissue processing, and that they function as a fuel source for cold-activated BAT.

Several questions remain in understanding the regulation and transport of plasma acylcarnitines including how they are transported through the plasma membrane in the liver and in the brown adipose tissue. Studies in Xenopus oocytes have demonstrated that acylcarnitines require a transporter to cross the plasma membrane, and cDNA libraries from mouse liver have demonstrated that these unknown transporters are present in the liver (Berardi et al., 1998; Nakanishi et al., 2001). SLC22a1 was recently identified as an acylcarnitine exporter in the liver, and knockout of SLC22a1 led to decreased short and medium chain acylcarnitines in plasma but had no impact on long chain acylcarnitine levels (Kim et al., 2017). Moreover, there has been no identified BAT acylcarnitine transporter and SLC22a1 has low expression in brown adipocytes. Plasma long chain acylcarnitines have been shown to travel bound to albumin, while short and medium chain acylcarnitines are unbound. Whether albumin bound acylcarnitines are the dominant form of acylcarnitine in the circulation during cold exposure is unknown. Future work will be needed to understand their entry into brown adipocytes and the kinetics of their uptake compared to FFAs of the same acyl chain.

Ceramides

Ceramides are a long chain sphingoid base conjugated to a fatty acid through an amide bond and are the precursor to all sphingolipids. Ceramides are known to circulate during tissue dysfunction and metabolic disease in both mice and humans. Plasma ceramide levels have been shown to correlate with risk of diabetes and coronary artery disease in a species-specific manner across human cohorts (Tippetts et al., 2021). Reduction of plasma and WAT ceramides in mice via increased degradation (ceramidase overexpression) or inhibition of ceramide synthesis (SPTLC2 KO) ameliorated HFD-induced obesity, insulin resistance, and hepatic steatosis (Xia et al., 2015; Chaurasia et al., 2016). SPTLC2 KO in WAT also enhanced adipocyte browning and resulted in an increase in beige adipocyte differentiation (Chaurasia et al., 2016). This suggests that ceramides act as signals to increase lipid storage in WAT and inhibit the beige program. Moreover, liver SPTLC2 expression is upregulated in response to SPTLC2 KO in WAT, suggesting a means of communication to balance tissue ceramide levels.

Despite the wealth of literature on ceramide function in metabolic disease and its regulation of the adipocyte differentiation program, little is known about how ceramides control brown and beige adipocyte maintenance or their direct role in thermogenic metabolism. We have observed significant increases in plasma ceramides following acute cold exposure, with computational assessment revealing that these plasma levels are regulated by the BAT and the kidney (Jain et al., 2021). More work is needed to characterize how these plasma ceramides are regulated in acute cold exposure, what their functional role may be, and what complexes facilitate their transport in the plasma during cold exposure. At ambient temperature, ceramides are known to be associated with lipoproteins (primarily LDL) and have been shown to transfer between cells via extracellular vesicles (EVs) (Hammad et al., 2010; Crewe et al., 2018). These vesicles act as carriers for intercellular signaling molecules during metabolic stress, and many ceramide species act as second messengers for key metabolic pathways including insulin sensing and cell growth. For example, during fasting, white adipose tissue traffics EVs containing signaling molecules such as caveolin 1 and very long chain ceramides to neighboring endothelial cells (and vice versa) (Crewe et al., 2018). EVs are also produced by adipose-derived stem cells during beige adipocyte differentiation and were shown to be sufficient to differentiate these stem cells into beige adipocytes (Jung et al., 2020). Additionally, BAT has been shown to be a significant contributor of exosomes into the circulation (Thomou et al., 2017). It is unknown whether export of these vesicles is upregulated or if ceramides are enriched in these vesicles during cold exposure. Moreover, there are no known plasma membrane transporters of ceramides. Ceramides in cold exposure remain an exciting area of research with many outstanding questions, including the role of ceramides in thermogenic metabolism as well as the function of plasma ceramides compared to ceramides produced in brown and beige adipocytes.

12, 13-Dihydroxy-9Z-Octadecenoic Acid (12,13-DiHome)

12,13-diHOME is produced in brown adipose tissue and can function as an autocrine or paracrine signal to increase mitochondrial oxidation rates (Lynes et al., 2017). Upon β3-adrenergic receptor activation, linoleic acid is oxidized by

cytochrome P450 and soluble epoxide hydrolase to produce 12,13-diHOME. Beyond BAT, other tissues are known to produce 12,13-diHOME, including the skeletal muscle, and contribute to the circulating pool to regulate body weight, energy expenditure, insulin sensitivity, and plasma lipid levels (Vasan et al., 2019). The uptake of 12,13-diHOME into brown adipocytes is regulated by CD36 and FATP1, and treatment of brown adipocytes with 12,13-diHOME is sufficient to increase translocation of CD36 and FATP1 (Lynes et al., 2017, 2019). Further studies are needed to understand how 12,13-diHOME regulates mitochondrial oxidation, determine the mechanism of secretion from cells, and understand how this lipid circulates in the plasma.

Fatty Acid Esters of Hydroxy Fatty Acids (FAHFAs)

FAHFAs are a recently discovered class of signaling lipids that regulate brown and beige adipocyte differentiation and maintenance. Structurally, FAHFAs are fatty acids complexed to a hydroxy fatty acid through an ester bond (Yore et al., 2014). There are numerous types of FAHFAs named for the acyl chains and the location of the hydroxylation including stearic-acid-9-hydroxy stearic acid (9-SAHSA), oleic-acid-9-hydroxy stearic acid (9-OAHSA), and palmitic-acid-9-hydroxy stearic acids (9-PAHSA). Both 5- and 9- PAHSA have been shown to increase brown adipocyte differentiation, insulin sensitivity, decrease inflammation in adipose tissue, and improve whole body glucose tolerance. Treatment of 3T3-L1 adipocytes or leptin deficient mouse models with 9-PAHSA led to increased expression of thermogenic genes including UCP1 (Wang et al., 2018). Part of this signaling is mediated through binding and activating G-protein coupled receptor 120 (GRP120), and knockdown of GPR120 in 3T3-L1 cells abrogated the effect of 9-PASHA treatment (Oh et al., 2014; Wang et al., 2018). Cold exposure induced the production of 5- and 9-PAHSA from WAT, with this production being mediated by lipolysis from triglycerides since knockout of ATGL led to ablated the cold-induced production (Paluchova et al., 2020). Many outstanding questions remain on how various species of FAHFAs impact brown and beige adipocytes and how they are transported into cells.

The numerous plasma lipids that act upon BAT is still an open area of study. For the purpose of this review, we have chosen to focus on plasma lipids that are transported into brown adipocytes, however there are a number of other lipids that are altered in brown adipocytes themselves that regulate thermogenesis. These include ether lipids (such as plasmalogens) and cardiolipins (Lynes et al., 2018; Park et al., 2019; Von Bank et al., 2021b). Although FFAs produced in the BAT are not necessary for thermogenesis (Schreiber et al., 2017), they have been shown to be sufficient to drive the thermogenic program through mediation of GPCR signaling (Sveidahl Johansen et al., 2021). Interestingly, ether lipids have recently been observed to increase with cold exposure in studies where mice were acclimated to thermoneutrality then placed for 24 h in thermoneutrality, room temperature (22°C), and cold exposure (5°C) as well as fasted for the final 5 h of temperature stress (Pernes et al., 2021). More work is needed to understand these various lipids in the plasma and how their transport is regulated.

PERSPECTIVES

BAT is an important regulator of whole-body glucose and lipid homeostasis. Cold exposure increases the uptake of lipids into the BAT by 12-fold and, in models of hyperlipidemia, can normalize plasma triacyclglycerol and cholesterol levels (Bartelt et al., 2011). Not only are thermogenic adipocytes able to regulate systemic lipid metabolism, but they are also reliant on the plasma lipid pool for fuel availability. The importance of peripheral lipid storage for non-shivering thermogenesis has now been established through use of the ATGL KO studies and DGAT1 and 2 double KO studies (Schreiber et al., 2017; Chitraju et al., 2020). The uptake of these lipids into BAT from the circulation is dependent upon facilitated transport through dedicated protein transporters, chaperones, and endocytosis. This review focused on known mechanisms of lipid uptake into BAT, beginning with FFA uptake which is regulated in three distinct steps: CD36 and FATPs regulating (1) adsorption and (2) translocation, while FABP facilitates (3) desorption (Hamilton, 1998; Chmurzyńska, 2006). Loss of CD36, FATP or FABP led to cold intolerance and an inability for cold exposure to regulate circulating FFA levels. TGs and cholesterol can also be imported into BAT through LDL endocytosis, or for TGs, through LPL mediated lipolysis from TRL. While the majority of work has focused on uptake of FFAs, TGs, and cholesterol into BAT, questions remain on the import mechanisms that regulate other plasma lipids during cold exposure. Recent work on plasma acylcarnitines has shown that they are taken up by BAT and are necessary for thermogenic capacity (Simcox et al., 2017). Other work has shown that lipid containing exosomes are increased in the plasma with cold exposure and reflect brown adipocyte activity (Chen et al., 2016). More work is needed to understand how these lipids and lipid-containing vesicles are trafficked into cells, and to determine the tissues where these various plasma lipids are produced.

One existing challenge in the modeling of lipid uptake into brown adipocytes is standardization of protocols for cold exposure and mouse models. Many studies have a range in cold exposure from 3 h to 1 week. Longer cold exposure, such as 72 h to 1 week, is associated with beige adipocyte differentiation, increased BAT mass, and increased food intake (Ikeda et al., 2018). The variations in cold exposure timing and added variable of fasting during a traditional cold tolerance test make comparison difficult due to differences in thermogenic capacity and contribution of the beige depot. Moreover, variations in housing temperature also impact the brown and beige adipocyte population and alter body weight in response to shifts in energy expenditure (Fischer et al., 2018; Corrigan et al., 2020). Many of the studies associated with lipid uptake in brown adipocytes focus on 1 week including characterization of FABP and FATP. Standardization would enable an understanding of the impact of BAT on the circulating lipid pool.

Another barrier for lipid transport in thermogenic adipocytes is depot-specific gene modulation. Many mouse models for

lipid transport assessed the function in BAT using ablation of the gene in all adipose tissue with cre expression driven by the adiponectin promoter or in whole body KO models. All of the work to assess FATP1 function in BAT was performed in FATP1 null mice, as were several of the seminal studies on CD36 (Lobo et al., 2007; Bartelt et al., 2011). Models that use cre drivers target both the brown and beige adipocytes using UCP1-cre for genetic modulation of various genes. An important step in furthering our understanding of lipid transport in thermogenesis will be the development of mouse models that target only the brown or beige adipocytes. Single cell sequencing has uncovered numerous unique markers of beige vs. brown adipocytes, while also identifying numerous sub-populations of adipocytes in brown and beige depots (Merrick et al., 2019; Henriques et al., 2020; Sun et al., 2020). These challenges are particularly important since the uptake of lipids into each of these cell types may be mediated by distinct membrane composition and expression of transporters.

Finally, although the majority of this review focused on lipids being transported into BAT for catabolism, lipids are capable of playing a number of signaling roles that regulate thermogenic potential. Recent work by the Seale group has demonstrated that FA oxidation is an important mediator of beige adipocyte differentiation driven by transcriptional regulator PRDM16. The breakdown of these FAs into ketone bodies was necessary and sufficient to induce differentiation of pre-adipocytes into beige adipocyte (Wang et al., 2019). Beige adipocyte differentiation was also shown to be regulated by ceramide signaling which inhibits the beige program while promoting lipid accumulation needed for white adipocytes (Chaurasia et al., 2016). More work is needed to understand how lipids influence metabolism in brown and beige adipocytes and how they contribute to the thermogenic potential, as well as how these signals are mediated by transport into the BAT.

Lipid import from the circulation into brown adipocytes is necessary for thermogenesis. Once in the brown and beige adipocytes, these lipids can be catabolized as an energy source or serve as signaling molecules. While there are fairly established mechanisms and function for FFA and TG uptake into BAT, more work is needed to characterize the uptake, circulating form, and functional role in thermogenesis for other lipids such as acylcarnitines, ceramides, and FAHFAs. The continued exploration and development of new technology to probe lipid uptake in brown and beige adipocytes will enable distinction of catabolism, storage, and signaling capabilities. Lipid transport proteins are essential to proper systemic lipid metabolism, and tight regulation of this transport is necessary to prevent disease.

AUTHOR CONTRIBUTIONS

GW, AM, and JS contributed to the conception, writing, literature searches, and first draft of the manuscript. GW created the figures for the manuscript. JN and JS contributed editing, revisions, literature searches, and final draft overview. All authors contributed to manuscript revisions, read, and approved the submitted version.

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The Effects of Mild Intermittent Hypoxia Exposure on the Abdominal Subcutaneous Adipose Tissue Proteome in Overweight and Obese Men: A First-in-Human Randomized, Single-Blind, and Cross-Over Study

*Rens L. J. Van Meijel[1], Ping Wang[2], Freek Bouwman[1], Ellen E. Blaak[1], Edwin C. M. Mariman[1] and Gijs H. Goossens[1]**

[1]Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center+, Maastricht, Netherlands, [2]Department of Clinical Genetics, Maastricht University Medical Center+, Maastricht, Netherlands

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Ryan Ceddia, Vanderbilt University Medical Center, United States

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***Correspondence:**
Gijs H. Goossens
g.goossens@maastrichtuniversity.nl

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Adipose tissue (AT) oxygen tension (pO_2) has been implicated in AT dysfunction and metabolic perturbations in both rodents and humans. Compelling evidence suggests that hypoxia exposure alters metabolism, at least partly through effects on AT. However, it remains to be elucidated whether mild intermittent hypoxia (MIH) exposure impacts the AT proteome. We performed a randomized, single-blind, and cross-over study to investigate the effects of seven consecutive days of MIH (FiO_2 15%, 3x2h/d) compared to normoxia (FiO_2 21%) exposure on the AT proteome in overweight/obese men. *In vivo* AT insulin sensitivity was determined by the gold standard hyperinsulinemic-euglycemic clamp, and abdominal subcutaneous AT biopsies were collected under normoxic fasting conditions following both exposure regimens (day 8). AT proteins were isolated and quantified using liquid chromatography-mass spectrometry. After correction for blood contamination, 1,022 AT protein IDs were identified, of which 123 were differentially expressed following MIH ($p < 0.05$). We demonstrate for the first time that MIH exposure, which markedly reduces *in vivo* AT oxygen tension, impacts the human AT proteome. Although we cannot exclude that a single differentially expressed protein might be a false positive finding, several functional pathways were altered by MIH exposure, also after adjustment for multiple testing. Specifically, differentially expressed proteins were involved in redox systems, cell-adhesion, actin cytoskeleton organization, extracellular matrix composition, and energy metabolism. The MIH-induced change in AT TMOD3 expression was strongly related to altered *in vivo* AT insulin sensitivity, thus linking MIH-induced effects on the AT proteome to metabolic changes in overweight/obese humans.

Keywords: adipose tissue, proteomics, mild intermittent hypoxia, obesity, RCT

INTRODUCTION

The prevalence of obesity has increased drastically over the last decades, with nearly a third of the world's population living with overweight or obesity (Chooi et al., 2019). Obesity is a multifactorial disease, which is characterized by excess adipose tissue (AT) mass. AT is a metabolically active, endocrine organ, playing a central role in immunity, glucose and lipid homeostasis, angiogenesis, coagulation, vascular function, appetite regulation, and body weight control (Coelho et al., 2013). Thus, AT dysfunction is closely associated with an increased risk of cardiometabolic complications (Goossens, 2008, 2017; Rosen and Spiegelman, 2014) and hence mortality (Kopelman, 2000).

AT oxygen tension (pO_2) has been implicated in AT dysfunction in both rodents and humans, as reviewed recently (Lempesis et al., 2020). Although AT hypoxia has been consistently shown in rodent models of obesity, conflicting findings have been reported in humans (Lempesis et al., 2020). We have previously demonstrated higher AT pO_2 in obese compared to lean individuals (Goossens et al., 2011), decreased AT pO_2 following diet-induced weight loss (Vink et al., 2017), and a positive association between AT pO_2 and insulin resistance in humans, independently of adiposity (Goossens et al., 2018). Many *in vitro* studies have been performed to investigate whether exposure to hypoxic environments affects AT glucose and lipid metabolism. Indeed, hypoxia exposure appears to have profound effects on mRNA expression of several genes related to glucose and lipid metabolism in murine and human adipocytes, as reviewed extensively (Trayhurn, 2013; Lempesis et al., 2020). Importantly, the physiological effects of hypoxia are largely dependent upon the severity (mild versus severe), frequency (chronic versus intermittent), and duration (short versus long-term) of exposure (Navarrete-Opazo and Mitchell, 2014; Lempesis et al., 2020).

However, to investigate and better understand the effects of hypoxia exposure on a more functional level, proteomics analysis seems highly valuable. Indeed, previous studies have indicated that the proteomic profile of *3T3-L1* adipocytes during long-term mild hypoxia exposure, at physiological pericellular oxygen concentrations (4% O_2), affects various pathways involved in energy metabolism, suggestive of increased glycolytic metabolism and triacylglycerol synthesis (Weiszenstein et al., 2016). In addition, 24 h exposure to 1% O_2 mainly increased expression of proteins related to the extracellular matrix (ECM) in human adipose stem cells (Riis et al., 2016). These *in vitro* findings suggest that hypoxia may induce ECM remodeling and induce metabolic changes in AT. Importantly, however, human *in vivo* studies that examined the effects of prolonged mild intermittent hypoxia (MIH) exposure on the AT proteome are lacking.

In the present study, we investigated the impact of MIH compared to normoxia exposure on the abdominal subcutaneous AT proteome in overweight and obese men, using untargeted liquid chromatography-mass spectrometry, to elucidate the physiological and functional adaptations in human AT evoked by MIH. Secondly, we explored the associations between MIH-induced alterations in AT protein expression and *in vivo* AT insulin sensitivity.

MATERIALS AND METHODS

Subjects

Twelve overweight and obese (BMI $\geq$28 kg/m^2) male subjects were recruited to participate in the present study. Subjects also needed to be aged between 30 and 65 years and insulin resistant, defined as HOMA-IR index $\geq$2.2. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney malfunction, use of medication known to affect body weight and glucose metabolism, and marked alcohol consumption (>14 alcoholic units/wk). Furthermore, subjects had to be weight stable (weight change <3.0 kg) for at least three months prior to the start of the study. Participants were asked to refrain from drinking alcohol and perform no exercise 24 h prior to the start and during exposure regimen. The study, registered at Netherlands Trial Register (NL7120/NTR7325), was performed according to the Declaration of Helsinki and was approved by the Medical-Ethical Committee of Maastricht University. All subjects gave their written informed consent before participation in the study.

Study Design

The present study was part of a randomized, double-blind, and placebo-controlled cross-over trial designed to investigate the metabolic effects of MIH in humans (van Meijel et al., 2021). Briefly, participants enrolled in this randomized, single-blind, and cross-over study were exposed to normobaric MIH (15% FiO_2) and normobaric normoxia (21% FiO_2) for 7 consecutive days (3 cycles of 2 h/d with 1 h of normoxia exposure between hypoxic cycles), separated by a 3–6 weeks. wash-out period. As described previously, hypoxia exposure was performed in an in-house manufactured airtight clinical room with the capability to accurately adjust oxygen availability at the Metabolic Research Unit Maastricht (Maastricht University, Netherlands). The oxygen level was set and maintained at 15.0 ± 0.2% for the hypoxia exposure regimen. Participants were blinded for the exposure regimen (hypoxia or normoxia; van Meijel et al., 2021). Systemic oxygen saturation was continuously monitored throughout the exposure regimens by finger pulse oximetry (Nellcor N-595 Pulse oximeter, Nellcor). At day 6 of the exposure regimens, AT pO_2 was determined using an optochemical measurement system for continuous monitoring of tissue pO_2, as described previously (Goossens et al., 2011). Both systemic oxygen saturation and AT pO_2 were determined during hypoxia (or normoxia as control) exposure. At day 8, following the 7-day MIH/normoxia exposure regimens, fasting abdominal subcutaneous adipose tissue biopsies were collected, and a two-step hyperinsulinemic-euglycemic clamp was performed to determine AT, hepatic, and peripheral insulin sensitivity under normoxic conditions, as described previously (Reijnders et al., 2016).

Abdominal Subcutaneous Adipose Tissue Biopsy

Fasting abdominal subcutaneous AT biopsies were collected (approximately 1 g) using needle aspiration under local anesthesia (2% lidocaine), 6–8 cm lateral from the umbilicus. After thorough rinsing with sterile saline, visible blood vessels were removed using sterile tweezers. Subsequently, specimens were snap-frozen using liquid nitrogen and stored at −80°C for further analysis. Due to sampling issues, we could not collect enough material following hypoxia exposure in one participant. Therefore, paired adipose tissue biopsies (following both MIH and normoxia exposure) were available for analysis in $n = 11$ individuals.

Protein Isolation and Preparation for LC-MS

Frozen AT (~100 mg) was ground in a mortar with liquid nitrogen. Per microgram of grounded powder, 2 µl of 50 mm ammonium bicarbonate with 5 M urea, was added to dissolve the powder. The solution was freeze-thawed in liquid nitrogen 3 times after which it was vortexed for 5 min. The homogenate was centrifuged at 20,000 g for 30 min at 10°C. The supernatant was carefully collected and protein concentrations were determined with a Bradford-based protein assay (Bio-Rad, Veenendaal, Netherlands).

A total of 100 µg protein in 50 µl 50 mm ammonium bicarbonate (ABC) with 5 M urea was used. 5 µl of DTT solution (20 mm final) was added and incubated at room temperature for 45 min. The proteins were alkylated by adding 6 µl of IAA solution (40 mm final). The reaction was taken place at room temperature for 45 min in the darkness. The alkylation was stopped by adding 10 µl of DTT solution (to consume any unreacted IAA) and incubated at room temperature for 45 min. For the digestion, 2 µg trypsin/lysC was added to the protein and incubated at 37°C for 2 h. 200 µl of 50 mm ABC was added to dilute the urea concentration and further incubate at 37°C for 18 h. The digestion mixture was centrifuged at 2.500 g for 5 min and the supernatant, which contained the peptide mixture, collected for the use of LCMS analysis.

Protein Identification and Quantification Using LC-MS

A nanoflow HPLC instrument (Dionex ultimate 3,000) was coupled online to a Q Exactive (Thermo Scientific) with a nano-electrospray Flex ion source (Proxeon). Each sample was run separately for label free quantification. 5 µl of the peptide mixture was loaded into a C18-reversed phase column (Thermo Scientific, Acclaim PepMap C18 column, 75-µm inner diameter x 15 cm, and 2-µm particle size). The peptides were separated with a 240 min linear gradient of 4–50% in buffer A (100% water with 0.1% TFA) with buffer B (80% acetonitrile and 0.08% formic acid) at a flow rate of 300 nl/min. MS data were acquired using a data-dependent top-10 method, dynamically choosing the most abundant precursor ions from the survey scan (280–1,400 m/z) in positive mode. Survey scans were acquired at a resolution of 70,000 and a maximum injection time of 120 ms. Dynamic exclusion duration was 30 s. Isolation of precursors was performed with a 1.8 m/z window and a maximum injection time of 200 ms. Resolution for HCD spectra was set to 17,500 and the Normalized Collision Energy was 30 eV. The under-fill ratio was defined as 1.0%. The instrument was run with peptide recognition mode enabled, but exclusion of singly charged and charge states of more than five.

Database Search and Quantification

The MS data were searched using Proteome Discoverer 2.2 Sequest HT search engine (Thermo Scientific), against the UniProt human database. The false discovery rate (FDR) was set to 0.01 for proteins and peptides, which had to have a minimum length of 6 amino acids. The precursor mass tolerance was set at 10 ppm and the fragment tolerance at 0.02 Da. One miss-cleavage was tolerated, oxidation of methionine was set as a dynamic modification. Carbamidomethylation of cysteines was set as fixed modifications. Label free quantitation was conducted using the Minora Feature Detector node in the processing step and the Feature Mapper node combined with the Precursor Ions Quantifier node in the consensus step with default settings within Proteome Discoverer 2.2 (Qiao et al., 2019).

Protein Signal Normalization and Adjustment for Blood Protein Contamination

The inter-run variation was normalized using ppm fractional normalization. To reduce the influence of the blood protein contamination on the AT proteome, we retrieved information from the UniProt database and GeneCards to set up a blood protein exclusion list with known proteins exclusively expressed in blood, including all immunoglobulins (Anderson and Anderson, 2002; Stelzer et al., 2016). The amount of signal contributed by these blood proteins in our AT sample were found with a mean value of 76%. We defined valid human AT proteins based on these quality criteria: (1) not exclusively expressed in blood, (2) identification Score Sequest HT ≥ 2.5, 3 present in >50% (≥6 of 11) samples in at least one treatment group. The final signal of a valid human AT protein was the protein relative ppm abundance in a fixed amount (76%) blood contaminated AT sample. This final signal (below referred to as "signal") was used in data analysis (see equation).

$$\text{Final signal}\left[\text{protein } x, \text{sample } i\right] = \frac{\text{Normalized signal}\left[\text{protein } x, \text{sample } i\right]}{\sum \text{norm.signal}\left[\text{sample } i\right] - \sum \text{Blood prot.norm.signal}\left[\text{sample } i\right]} \times 10^6 \times \left(1 - 0.76\right)$$

Statistical Analysis

Only valid human AT proteins were taken into statistical analyses. First, missing values (9.5% of total number of data points) were imputed using random forest algorithm with R missForest package v1.4. Thereafter, data were log2 transformed. The effects of MIH compared to normoxia exposure on abdominal subcutaneous AT protein expression were assessed using two-sided paired Student's t-tests. The fold change was calculated

using the back-transformed means for both conditions. The Benjamini-Hochberg procedure was applied to control for multiple testing, with false discovery rate set at 0.25, and q-values were calculated. Proteins with a *value of* $p < 0.05$ (MIH versus normoxia exposure) were selected for further biological annotation and analysis. Subsequently, these differentially expressed proteins ($p < 0.05$) were imported into Cytoscape plug-in ClueGO v2.5.7 for functional analysis (Bindea et al., 2009), based on GO biological process terms and KEGG pathways dated on 08.05.2020. For this functional analysis, Bonferroni's step-down method was applied, and only functional groups with adjusted value of $p < 0.05$ were considered as significantly affected by MIH compared to normoxia exposure. Spearman's rank correlation analysis was performed to determine correlations between changes in all 123 differentially expressed AT proteins and changes in AT insulin sensitivity [expressed as suppression (%) of FFA plasma concentration upon $10\,mU{\bullet}m^{-2}$ insulin infusion], with subsequent multiple testing correction (Benjamini-Hochberg Procedure). All statistical analyses were performed in R environment, version 3.5, with various packages (stats, missForest, gplots and pheatmap) and SPSS.

RESULTS

Characteristics of the study participants are shown in **Table 1**. All individuals (age range 52–65 yrs) were overweight or obese (BMI $\geq 28\,kg{\bullet}m^{-2}$) and demonstrated a HOMA-IR index ≥ 2.2.

Mild Intermittent Hypoxia Exposure Decreases Adipose Tissue Oxygen Tension

To provide the proof-of-concept in humans that MIH exposure reduces oxygen availability in abdominal subcutaneous AT, we determined both systemic oxygen saturation (finger pulse oximetry, measured at days 1–5) and AT pO_2 (measured at day 6), using a highly accurate, microdialysis-based optochemical measurement system to continuously monitor AT pO_2 *in vivo* in humans (Goossens et al., 2011). As previously reported (van Meijel et al., 2021), MIH exposure reduced systemic oxygen saturation (normoxia: 97.1 ± 0.3 vs. hypoxia: 92.0 ± 0.5%, $p < 0.001$) and decreased AT pO_2 (normoxia: 36.5 ± 1.5 mmHg versus hypoxia: 21.0 ± 2.3 mmHg, $p < 0.001$).

TABLE 1 | Baseline characteristics of male study participants.

	Baseline
Age (y)	61 ± 1
BMI (kg/m^2)	30.8 ± 3.6
Hemoglobin ($mmol{\bullet}l^{-1}$)	9.5 ± 0.5
HbA1c (%)	5.6 ± 0.1
Fasting glucose ($mmol{\bullet}l^{-1}$)	5.7 ± 0.5
2 h-glucose ($mmol{\bullet}l^{-1}$)	6.2 ± 1.3
HOMA-IR	3.7 ± 0.4

Hb, hemoglobin; Hb1Ac, glycated hemoglobin; HOMA-IR, homeostasis model of assessment of insulin resistance; and 2 h-glucose determined during a 75 g oral glucose tolerance test (OGTT). Values are mean ± SEM (n = 11).

Mild Intermittent Hypoxia Exposure Impacts the Adipose Tissue Proteome

We quantified 1,091 accession IDs of 1,074 proteins in the AT samples with HT score > 2.5, which appeared in more than half of the samples in at least one of the conditions (MIH and/or normoxia). Several of these proteins were considered blood-specific (e.g., hemoglobins, serum albumins, and erythrocyte proteins), explained by AT specimen contamination with some blood during sample collection, despite thorough cleaning using sterile saline, as described previously (Vogel et al., 2019). Indeed, 69 identified IDs of 63 unique proteins were blood-specific and contributed to 62–82% of the total protein signal (**Figure 1**). Therefore, we corrected the quantification after removal of these blood-specific proteins (**Supplementary Table 1**). In the resulting 1,022 AT protein IDs for 1,011 proteins, 123 IDs of 123 unique proteins were differentially expressed ($p < 0.05$; **Table 2**; **Supplementary Table 2**) following MIH compared to normoxia exposure (42 upregulated and 81 downregulated), as visualized in a heat map (**Figure 2**). Differential expression of top 5 upregulated/downregulated (based on *value of p*s) proteins per individual is visualized in **Supplementary Figure 1**. Changes of individual proteins did not reach statistical significance after controlling for multiple testing (false discovery rate set at 0.25).

Mild Intermittent Hypoxia Exposure Alters Adipose Tissue Expression of Proteins Involved in Structural and Metabolic Pathways

All 123 differentially expressed proteins were functionally annotated, since MIH-induced changes in specific functional pathways provide more robust information related to functional changes than alterations in single proteins. Thus, 11 functional groups that were enriched in these differentially expressed proteins were identified (**Figure 3**). These functional groups cover 104 representative GO terms and pathways (**Supplementary Table 3**), to which 79 proteins are associated (**Figure 3**). The functional groups cover predominantly structural and metabolic-related GO terms and pathways. MIH-induced AT expression of several proteins related to actin cytoskeleton organization, focal adhesion, and myeloid development, whereas it reduced the expression of proteins related to collagen fibril organization (**Table 3**; **Figure 3**). In addition, MIH reduced several pathways related to oxidoreductase activity, regulation of lipolysis in adipocytes, polysaccharide biosynthetic, and ADP metabolic processes. Moreover, MIH increased proteins involved in bicarbonate transport, iron ion homeostasis, as well as platelet degranulation (**Table 3**; **Figure 3**).

Mild Intermittent Hypoxia-Induced Effects on the Adipose Tissue Proteome Are Related to *in vivo* Adipose Tissue Insulin Sensitivity

Since MIH exposure had a significant impact on several ECM- and cytoskeleton-related proteins, as well as proteins involved in energy metabolism, we next determined the associations

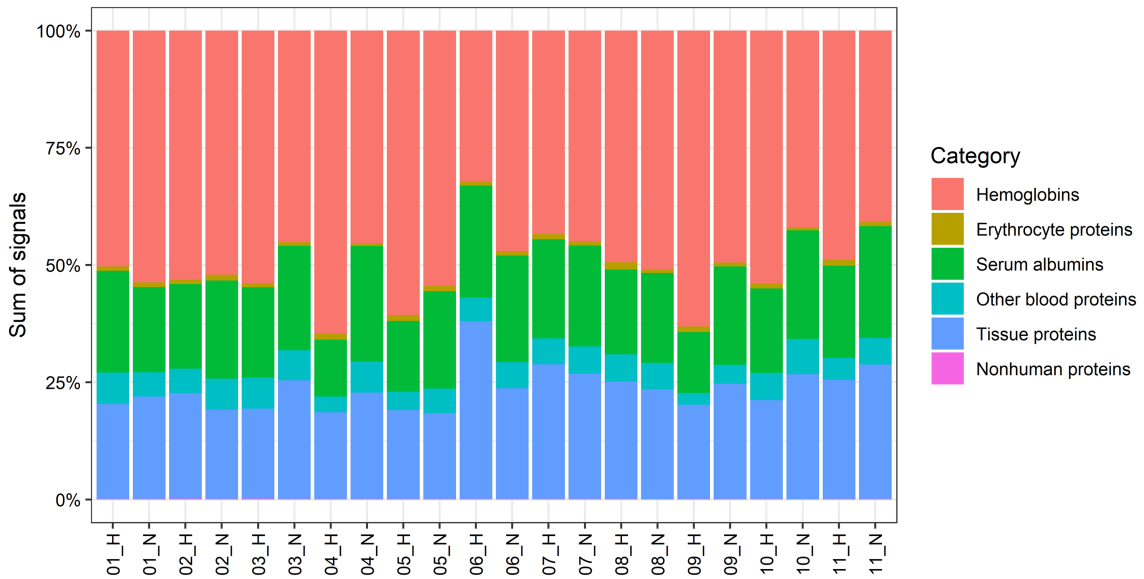

FIGURE 1 | Contribution to the total proteome signals of AT by human abdominal subcutaneous adipose tissue proteins and different groups of blood-specific proteins. Each bar represents an adipose tissue biopsy, with number indicating the respective participant. H; biopsy after mild intermittent hypoxia (MIH) exposure; N; normoxia exposure.

between differentially expressed AT proteins and *in vivo* AT insulin sensitivity. Although AT insulin sensitivity did not significantly change following MIH exposure (data not shown), the increase in tropomodulin-3 (TMOD3) protein expression evoked by MIH exposure was positively associated with the change in AT insulin sensitivity ($r = 0.806$ $p = 0.005$, **Figure 4**). However, after multiple testing correction, this correlation did not remain statistically significant ($q = 0.615$).

DISCUSSION

In the present randomized, single-blind, and cross-over study, we examined for the first time the effects of 7 consecutive days of MIH compared to normoxia exposure on the abdominal subcutaneous AT proteome in overweight and obese men. From the 1,011 AT-specific proteins identified, 123 proteins were differentially expressed following MIH compared to normoxia exposure. Specifically, MIH-induced marked alterations of functional groups, mainly related to oxidoreductase systems, cell-adhesion, actin cytoskeleton and ECM organization, and energy metabolism. Moreover, the MIH-induced increase in TMOD3 expression was significantly related to improved AT insulin sensitivity, suggesting a link between MIH-induced effects on the AT proteome and metabolic changes in human AT. Although we cannot exclude that a single differentially expressed protein might be a false positive finding, the fact that these functional pathways (consisting of many proteins) were significantly upregulated/downregulated after multiple testing correction and with high concordance between its protein members provides strong evidence that MIH exposure alters specific protein pathways within the adipose tissue proteome.

We found that MIH exposure significantly downregulates oxidoreductase activity-related pathways in AT. More specifically, NAD(P)- and NAD(P)H-dependent dehydrogenase activity-related pathways contributed to the changes in this functional group. Out of the 31 proteins associated with oxidoreductase activity, aldehyde dehydrogenase 2 (ALDH2), glycerol-3-phosphate dehydrogenase 1 (GPD1), and 3-hydroxyacyl-CoA dehydrogenase (HADH) were most significantly downregulated. This is in agreement with the MIH-induced reduction in AT gene expression of aldo-keto reductase family 7, member A2 (van Meijel et al., 2021). The expression of ALDH2, which catalyzes the oxidation of aldehydes, has been found to be increased with adiposity (Frohnert et al., 2011). ALDH2 may counteract reactive oxygen species (ROS)-induced lipid aldehyde formation, which has been described in obesity and insulin resistance (Pillon and Soulage, 2012). HADH, a key enzyme involved in fatty acid oxidation, was also downregulated by MIH exposure. In agreement with our findings, it has recently been shown that exposure to severe hypoxia exposure markedly reduced fatty acid oxidation in murine adipocytes (Lu et al., 2016). Of note, hypoxia decreases tissue NAD^+ content and substantially increases the NADH/NAD+ ratio (Yurkov and Safonova, 1976; Eales et al., 2016). Most of these oxidoreductases are dependent upon NAD^+ as an electron acceptor. As such, this may explain reduced expression of these enzymes following prolonged *in vivo* MIH exposure. Notably, an increased NADH/NAD^+ ratio inhibits NAD + -dependent processes (Eales et al., 2016), covering a large number of central metabolic pathways, such as the tricarboxylic acid cycle and fatty acid catabolism (**Supplementary Table 3**). Taken together, our findings suggest

TABLE 2 | Top 20 up- and downregulated proteins by MIH compared to normoxia exposure in overweight and obese individuals.

Upregulated	Uniprot	Gene	Protein names	Fold change (Hypoxia/ Normoxia)	*value of p*	*q*-value
1	P11166	SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1 (Glucose transporter type 1)	1.81	0.006	0.387
2	P28289	TMOD1	Tropomodulin-1 (Erythrocyte tropomodulin)	1.97	0.006	0.387
3	P16104	H2AX	Histone H2AX	1.29	0.007	0.387
4	O00194	RAB27B	Ras-related protein Rab-27B	2.16	0.008	0.387
5	P07451	CA3	Carbonic anhydrase 3	1.36	0.009	0.387
6	P29972	AQP1	Aquaporin-1 (Water channel protein for red blood cells and kidney proximal tubule)	1.63	0.013	0.387
7	P07384	CAPN1	Calpain-1 catalytic subunit	1.39	0.013	0.387
8	P02730	SLC4A1	Band 3 anion transport protein	1.65	0.015	0.387
9	P22061	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	1.21	0.015	0.387
10	P21333	FLNA	Filamin-A	1.38	0.015	0.387
11	O15511	ARPC5	Actin-related protein 2/3 complex subunit 5	1.30	0.015	0.387
12	P09493	TPM1	Tropomyosin alpha-1 chain	1.21	0.016	0.387
13	P31146	CORO1A	Coronin-1A	1.93	0.018	0.387
14	Q15942	ZYX	Zyxin	1.59	0.018	0.387
15	O60256	PRPSAP2	Phosphoribosyl pyrophosphate synthase-associated protein 2	1.50	0.018	0.387
16	P80188	LCN2	Neutrophil gelatinase-associated lipocalin	2.04	0.018	0.387
17	P48643	CCT5	T-complex protein 1 subunit epsilon	1.15	0.021	0.387
18	O14561	NDUFAB1	Acyl carrier protein, mitochondrial	1.43	0.022	0.387
19	P30626	SRI	Sorcin	1.17	0.022	0.387
20	P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	1.85	0.025	0.387
Downregulated	**Uniprot**	**Gene**	**Protein names**	**Fold change (Hypoxia/ Normoxia)**	***value of p***	***q*-value**
1	P05091	ALDH2	Aldehyde dehydrogenase, mitochondrial	0.81	8.7E-04	0.387
2	Q8WTS1	ABHD5	1-acylglycerol-3-phosphate O-acyltransferase ABHD5 (Lipid droplet-binding protein CGI-58)	0.70	0.003	0.387
3	Q99536	VAT1	Synaptic vesicle membrane protein VAT-1 homolog	0.78	0.004	0.387
4	P06737	PYGL	Glycogen phosphorylase, liver form	0.62	0.006	0.387
5	Q02952	AKAP12	A-kinase anchor protein 12	0.59	0.008	0.387
6	Q16851	UGP2	UTP-glucose-1-phosphate uridylyltransferase	0.65	0.008	0.387
7	P16403	H1-2	Histone H1.2	0.73	0.009	0.387
8	P07099	EPHX1	Epoxide hydrolase 1	0.64	0.014	0.387
9	P16083	NQO2	Ribosyldihydronicotinamide dehydrogenase [quinone]	0.33	0.014	0.387
10	Q9NVD7	PARVA	Alpha-parvin	0.51	0.015	0.387
11	P21695	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	0.56	0.016	0.387
12	P02511	CRYAB	Alpha-crystallin B chain	0.58	0.017	0.387
13	P10301	RRAS	Ras-related protein R-Ras	0.61	0.017	0.387
14	Q16836	HADH	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	0.51	0.018	0.387
15	O60240	PLIN1	Perilipin-1 (Lipid droplet-associated protein)	0.65	0.019	0.387
16	Q9BX66	SORBS1	Sorbin and SH3 domain-containing protein 1	0.53	0.019	0.387
17	Q14112	NID2	Nidogen-2	0.60	0.019	0.387
18	P08294	SOD3	Extracellular superoxide dismutase	0.47	0.020	0.387
19	P36871	PGM1	Phosphoglucomutase-1	0.48	0.020	0.387
20	Q9BX68	HINT2	Histidine triad nucleotide-binding protein 2, mitochondrial	0.46	0.021	0.387

that MIH reduced AT protein expression of oxidoreductases, thereby affecting the direction of redox processes, and may promote alternative pathways to yield energy under hypoxic conditions.

In addition, AT expression of several proteins related to focal adhesion-related processes was increased subsequent to MIH exposure. Focal adhesion between the basement membrane or the extracellular matrix (ECM) and the adipocytes regulates cytoskeletal changes as well as adipocyte differentiation (Kim and Yoo, 2015). In addition, focal adhesions exert a key role in ECM-mediated integrin signaling and may promote adipocyte survival and insulin sensitivity through focal adhesion kinase activity (Luk et al., 2017). Out of the 21 proteins associated with focal adhesion, zyxin (ZYX) was upregulated. ZYX is essential in localizing the focal adhesion sites and stress fibers generated by mechanical cues, thereby regulating the adipocyte cytoskeleton (Zhang et al., 2017). On the other hand, Ras-related (RRAS), which has been demonstrated to markedly enhance focal adhesion formation (Kwong et al., 2003), was most significantly downregulated. In accordance with these MIH-induced changes in proteins related to focal adhesion, we have previously found that MIH decreased the expression

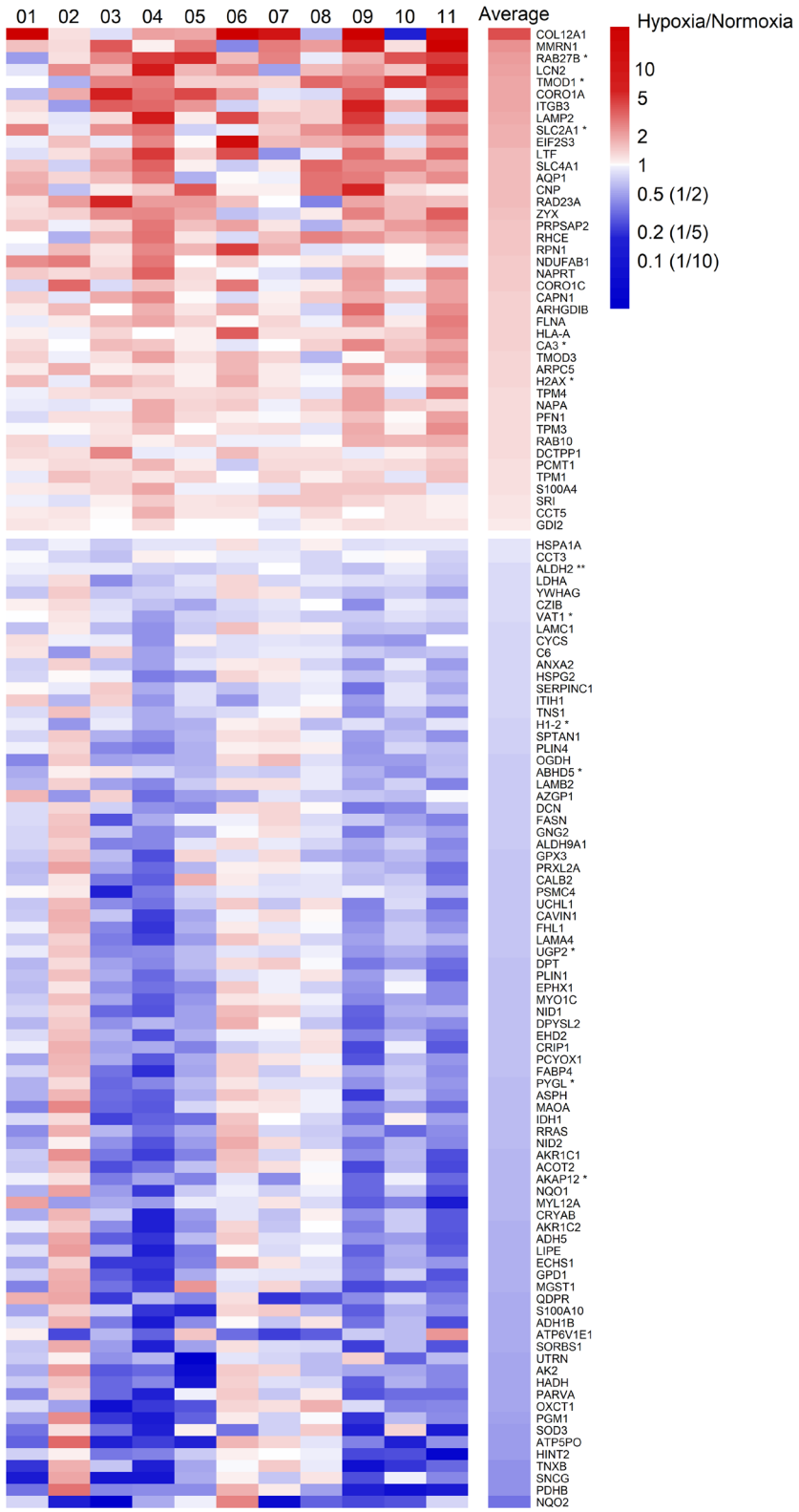

FIGURE 2 | Heatmap of fold changes of differentially expressed proteins at a significance level of $p < 0.05$ in abdominal subcutaneous adipose tissue following MIH compared to normoxia exposure in overweight and obese humans ($n = 11$). These proteins are labeled by gene symbols and are sorted by average fold changes. Each column represents one individual participant labeled by number, and the last column shows the average fold change. The color key is proportional to the log2 transformed fold change. $^{*}p < 0.01$; $^{**}p < 0.001$.

of genes related to focal adhesion in AT(van Meijel et al., 2021). Furthermore, several reports have demonstrated predominant effects of hypoxia on cytoskeletal organization *in vitro*, in particular an increase in actin-stress fibers in various cell types (Vogler et al., 2013; Gilkes et al., 2014).

In line with the above, MIH exposure induced the expression of processes related to actin cytoskeleton organization. Indeed, it has been suggested that actin remodeling is essential during adipocyte maturation, demonstrated by major changes in actin dynamics during

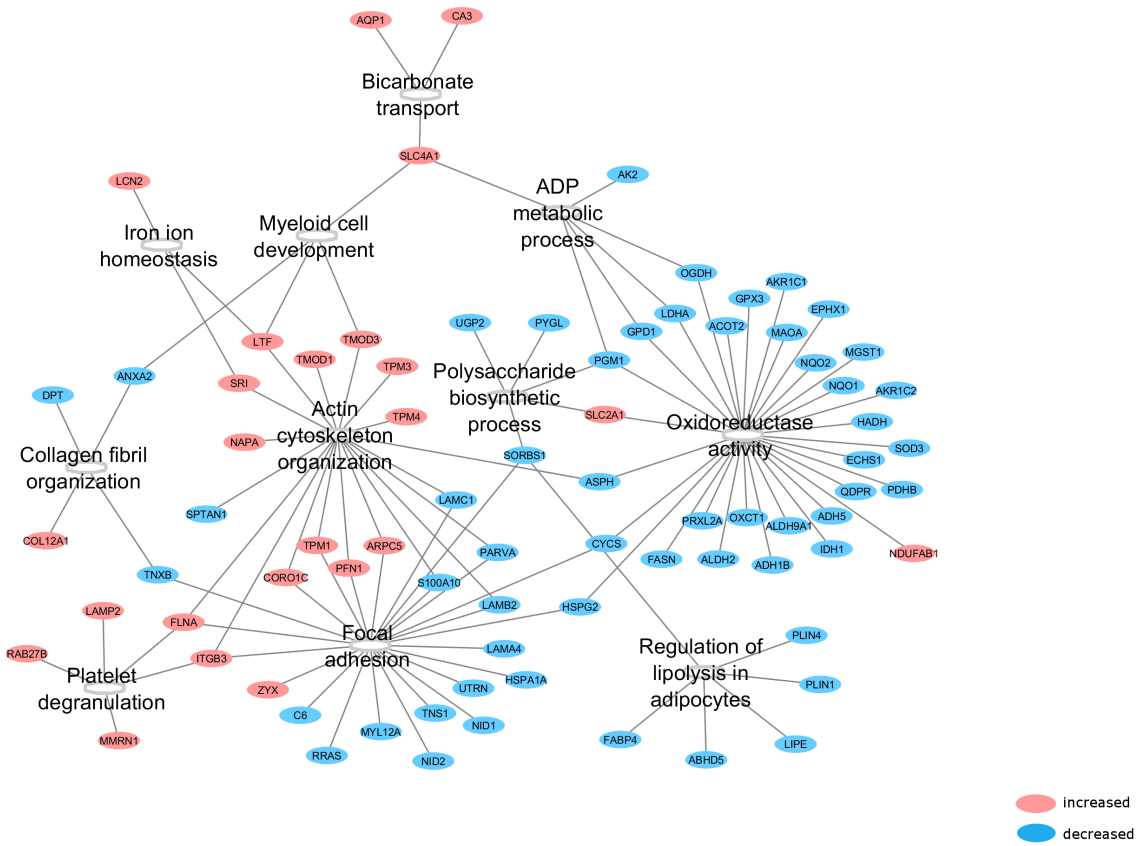

FIGURE 3 | Functional groups affected by MIH exposure. After functional annotation of differentially expressed proteins, 11 functional groups were identified ($p < 0.05$, corrected with Bonferroni step-down procedure) with associated proteins which were altered by MIH exposure. Red encircled proteins represent upregulated, whereas blue encircled proteins represent downregulated proteins subsequent to MIH exposure.

TABLE 3 | Functional groups affected by mild intermittent hypoxia based on representative GO terms and KEGG pathways.

Functional group	Group adjusted *value of p*
Oxidoreductase activity	4.7E-10
Focal adhesion	2.8E-09
Actin cytoskeleton organization	1.0E-06
Collagen fibril organization	0.003
ADP metabolic process	0.005
Myeloid cell development	0.009
Bicarbonate transport	0.010
Platelet degranulation	0.010
Regulation of lipolysis in adipocytes	0.010
Iron ion homeostasis	0.012
Polysaccharide biosynthetic process	0.012

Bonferroni step-down corrected p-values. GO, Gene Ontology; KEGG, Kyoto Encyclopedia for Genes and Genomes.

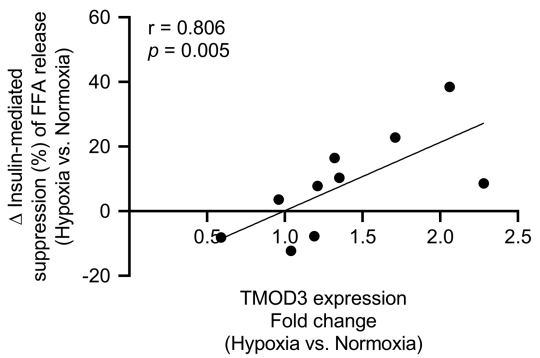

FIGURE 4 | Association between TMOD3 and adipose tissue insulin sensitivity [insulin-mediated suppression of plasma free fatty acids (%)]. The MIH-induced increase in TMOD3 expression was correlated with improved adipose tissue insulin sensitivity ($n = 10$). TMOD3; tropomodulin-3.

adipocyte expansion (Hansson et al., 2019). Furthermore, actin remodeling might enhance insulin signaling and glucose transporter-4 (GLUT4) translocation, thus improving glucose homeostasis (Chiu et al., 2010; Hansson et al., 2019). Here, we found increased AT protein expression of actin-related protein 2/3 complex subunit 5 (ARPC5) and tropomodulin-1 (TMOD1), which are involved in the regulation of actin polymerization and differentiation (Yang et al., 2014). This is in line with recent evidence of MIH-induced upregulation of gene expression of the actin dynamics pathway in human AT(van Meijel et al., 2021). Furthermore, MIH exposure increased TMOD3 protein expression, which is essential for insulin signal transduction (Lim et al., 2015). Interestingly, the MIH-induced increase in AT TMOD3 expression was correlated with improved AT insulin sensitivity, although this correlation did not remain statistically significant after multiple testing correction. In agreement with this, we found that the expression of α-II spectrin (SPTAN1) in AT, which appeared to be higher in subcutaneous adipocytes of obese insulin resistant compared to insulin sensitive individuals (Xie et al., 2016), was reduced after MIH exposure. Collectively, these findings suggest that prolonged exposure to MIH has pronounced effects on dynamics of focal adhesion, as well as AT remodeling, which may contribute to improved AT insulin sensitivity.

Interestingly, we found that collagen fibril organization was reduced by MIH exposure. It has previously been suggested that hypoxia induces AT fibrosis, by increasing collagen type I deposition, resulting in increased stiffness of the ECM (Buechler et al., 2015). The present findings, however, show that MIH reduced the expression of tenascin-X (TNXB) and dermatopontin (DPT), which are both implicated in collagen fibrillogenesis (Minamitani et al., 2004; Okamoto and Fujiwara, 2006). In line with these findings, we have previously found reduced expression of genes involved in assembly of collagen and other multimeric components in AT after MIH exposure (van Meijel et al., 2021). Of note, it has been demonstrated that circulating levels and visceral AT mRNA expression of DPT are increased in obese insulin-resistant individuals (Unamuno et al., 2020). Furthermore, we found 4-fold increased AT protein expression of collagen type XII α 1 chain (COL12A1), which is a non-fibrillar type of collagen involved in adipocyte differentiation, and as such a putative marker of adipogenesis (Tahara et al., 2004). Taken together, our findings indicate that MIH may elicit ECM remodeling and reduce fibrillogenesis in AT, which may enhance the fat storage capacity of adipocytes, allowing safe storage of lipids in abdominal subcutaneous AT (Luk et al., 2017).

Furthermore, AT proteins associated with the regulation of lipolysis in adipocytes appeared to be reduced by MIH. Indeed, we found decreased expression of abhydrolase domain-containing 5 (ABDH5), fatty acid binding protein 4 (FABP4), hormone-sensitive lipase (LIPE) and perilipin-1 (PLIN1), and PLIN4 were found. ABDH5 and PLIN1, which appeared to be most significantly downregulated, exert an important role in lipid catabolism, acting as activator for triacylglycerol hydrolases and coating protein surrounding lipid droplets in adipocytes, respectively (Oberer et al., 2011; Sztalryd and Brasaemle, 2017). It has been reported that long-term hypoxia (5% O_2) reduced ABDH5 and tended to reduce PLIN1 expression in *3T3-L1* adipocytes compared to standard laboratory conditions (21% O_2; Hashimoto et al., 2013). In agreement with our findings, reduced LIPE expression subsequent to long-term hypoxia exposure was also observed in that study (Hashimoto et al., 2013). In addition, it has been demonstrated that chronic hypoxia exposure (3% O_2) may reduce lipid storage and mobilization in human (pre) adipocytes (Mahat et al., 2021). Thus, the present findings, together with results from previous *in vitro* studies, suggest that MIH exposure may reduce lipid turnover in human AT to adapt to the lower O_2 availability. Moreover, this may imply that MIH exposure evokes a shift toward glycolytic metabolism to provide energy for the cells.

Indeed, MIH decreased the expression of several proteins associated with biosynthesis of polysaccharides, and therefore suggests that functional alterations in glycogen metabolism are associated with cellular adaptations to hypoxia, as previously described (Favaro et al., 2012). However, both UDP-glucose pyrophosphorylase 2 (UGP2), phosphoglucomutase 1 (PGM1), and glycogen phosphorylase l (PYGL) enzymes were downregulated subsequent to MIH exposure, suggestive of lower glycogen flux. In adipocytes, it has been demonstrated that hypoxia increases glycogen synthesis, which may induce autophagic flux (Ceperuelo-Mallafre et al., 2016). Furthermore, the present data also show a ~2-fold increase in AT expression of glucose transporter 1 (SLC2A1) protein after MIH exposure, which may imply that hypoxia enhances insulin-independent glucose uptake. Indeed, it has been demonstrated that hypoxic treatment induces insulin-independent glucose transport in various cell types, mainly mediated by SLC2A1 (Wood et al., 2007; Lu et al., 2016). Thus, the present findings suggest that MIH exposure alters glucose homeostasis within AT.

Next, we found that MIH induced several AT proteins associated with iron ion homeostasis. In line with the present results, (hypobaric) hypoxia exposure has previously been implicated in iron homeostasis, affecting the regulation of several enzymes involved in iron absorption, such as repression of hepcidin (Goetze et al., 2013; Shah and Xie, 2014). In addition, bicarbonate transport appeared to be upregulated by MIH, which has also been described previously in tumor hypoxia (McIntyre et al., 2016). The latter may be related to hypoxia-induced lactate production, thereby decreasing interstitial pH (Ye, 2009), which might explain increased bicarbonate transport subsequent to MIH.

Although we have filtered out proteins exclusively abundant in blood in our analysis, the present data analysis still covered some proteins enriched in blood, such as lactotransferrin (LTF), and the altered AT expression of these proteins may be due to blood cells. We found that MIH exposure increased the functional groups "platelet degranulation" and "myeloid cell development," reflecting functional alterations in blood cells. In line, it has been shown that human platelets exposed to 5% O_2 were

characterized by altered phenotype and enhanced activity (Cameron et al., 2018). However, conflicting findings have also been reported in humans. Healthy individuals exposed to short-term severe hypoxia *in vivo* (8% O_2) did not show any differences in platelet activity, and hence blood coagulation (Mantysaari et al., 2011). Therefore, the functional implications of MIH-induced alterations in the expression of proteins associated with platelet degranulation remain to be elucidated.

In conclusion, the present study demonstrates for the first time that human *in vivo* MIH exposure for seven consecutive days affects the abdominal subcutaneous AT proteome in overweight and obese men. Moreover, we found that the increased expression of TMOD3 was associated with improved AT insulin sensitivity following MIH exposure, thereby linking MIH-induced adaptations in the AT proteome to metabolic changes in human AT. Although we cannot exclude that altered expression of certain proteins might be due to false positive findings, the fact that changes in several functional pathways were found after multiple testing correction provides further evidence that MIH exposure impacts the human adipose tissue proteome. Further studies are needed to confirm the present findings, and to elucidate whether adipocytes and/or other cell types present in adipose tissue are responsible for the MIH-induced changes in the human AT proteome that we found in the present study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD029213.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical-Ethical Committee of Maastricht University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RVM, EB, and GG designed the research. RVM and GG performed the sample collection. FB contributed to the sample preparation, data acquisition and the LCMS analysis. FB performed the LCMS analysis. PW and RVM performed the data analysis. RVM wrote the manuscript. PW, FB, EB, EM, and GG revised the manuscript. All authors contributed to data interpretation and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.791588/full#supplementary-material

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Antisense Oligonucleotide Technologies to Combat Obesity and Fatty Liver Disease

Michael F. Keating[1,2,3], *Brian G. Drew*[2,3,4] *and Anna C. Calkin*[1,3,4]*

[1]*Lipid Metabolism and Cardiometabolic Disease Laboratory, Baker Heart and Diabetes Institute, Melbourne, VIC, Australia,* [2]*Molecular Metabolism and Ageing Laboratory, Baker Heart and Diabetes Institute, Melbourne, VIC, Australia,* [3]*Baker Department of Cardiometabolic Disease, University of Melbourne, Parkville, VIC, Australia,* [4]*Central Clinical School, Department of Medicine, Monash University, Melbourne, VIC, Australia*

Synthetic oligonucleotide technologies are DNA or RNA based molecular compounds that are utilized to disrupt gene transcription or translation in target tissues or cells. Optimally, oligonucleotides are 10–30 base pairs in length, and mediate target gene suppression through directed sequence homology with messenger RNA (mRNA), leading to mRNA degradation. Examples of specific oligonucleotide technologies include antisense oligonucleotides (ASO), short hairpin RNAs (shRNA), and small interfering RNAs (siRNA). *In vitro* and *in vivo* studies that model obesity related disorders have demonstrated that oligonucleotide technologies can be implemented to improve the metabolism of cells and tissues, exemplified by improvements in fat utilization and hepatic insulin signaling, respectively. Oligonucleotide therapy has also been associated with reductions in lipid accumulation in both the liver and adipose tissue in models of diet-induced obesity. Recent advances in oligonucleotide technologies include the addition of chemical modifications such as N-acetylgalactosamine (GalNAc) conjugates that have been successful at achieving affinity for the liver, in turn improving specificity, and thus reducing off target effects. However, some challenges are still yet to be overcome relating to hepatic injury and off-target effects that have been reported with some compounds, including ASOs. In summary, oligonucleotide-based therapies are an effective tool to elucidate mechanistic insights into metabolic pathways and provide an attractive avenue for translational research into the clinic.

Keywords: antisense oligonucleotides, lipid metabolism, obesity, fatty liver disease, metabolism

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Sahar Keshvari,
University of Queensland, Australia

Reviewed by:
Ling Yang,
Temple University,
United States
Brian Neal Finck,
Washington University in St. Louis,
United States

***Correspondence:**
Anna C. Calkin
anna.calkin@baker.edu.au

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INTRODUCTION

Since the discovery and isolation of DNA in the mid-20th century, the central dogma of molecular biology has been the unidirectional transcription of DNA to RNA, followed by translation into protein (Crick, 1970). Over time, our understanding of this process has been further refined to reflect additional and more nuanced molecular mechanisms that influence these processes. Examples include the discovery of exons and introns, DNA CpG methylation, histone acetylation, non-coding RNAs, response and repressor elements, and alternative splice sites, to name a few. These greater mechanistic insights have enabled the development of a

class of therapeutics broadly known as synthetic oligonucleotides or simply, oligo therapies. Synthetic oligonucleotide agents are short sequences of chemically modified DNA or RNA nucleotides that are utilized to alter transcript abundance or translation in target tissues or specific cell populations. Pertinent examples of these molecular tools include antisense oligonucleotides (ASOs), short interfering RNA (siRNA), and short hairpin RNA (shRNA). Collectively, these oligo technologies have been successfully utilized as molecular research tools as well as therapeutic agents in cellular, preclinical, and clinical studies. Herein, we will focus on the utilization of ASOs in the setting of obesity and fatty liver disease as well as discuss the current limitations and challenges encountered with their usage for such a purpose.

OBESITY AND NON-ALCOHOLIC FATTY LIVER DISEASE

Based on current global trends in the rate of obesity, approximately 50% of the world will be overweight or obese by 2030 (Smith and Smith, 2016). Obesity is defined clinically by a BMI over 30 kg/m^2 (Jensen et al., 2014) and is metabolically characterized by the excessive expansion of adipose tissue. White adipose tissue (WAT) is the primary storage site for lipid. However, once saturated, WAT becomes ineffective at storing lipids, resulting in adipocyte dysregulation, systemic dyslipidemia, and deposition of lipids in non-adipose tissues, promoting lipotoxicity. Indeed, these perturbations to lipid homeostasis drive a chronic low-grade inflammatory milieu both in adipocytes themselves as well as in numerous metabolic tissues including the liver (Calder et al., 2011). Consequently, this can promote the development of insulin resistance (Eckel et al., 2005; McQuaid et al., 2011), a significant risk factor for metabolic syndrome. A major clinical consequence of chronic obesity is the development of non-alcoholic fatty liver disease (NAFLD). The links between adipose tissue and the development of NAFLD are well described in both preclincial and clinical studies, where associations have been demonstrated between NAFLD and unhealthy and dysfunctional adipose tissue, including inflammation, insulin resistance, and enhanced collagen synthesis, known as fibrogenesis (du Plessis et al., 2015; Beals et al., 2021). NAFLD is characterized by the accumulation of ectopic lipid in the liver, predominantly triacyclglycerols, known as hepatic steatosis. Simple steatosis and early stage NAFLD can often be a benign condition for a significant period of time, however, can progress rapidly without obvious signs or symptoms (Vernon et al., 2011). Left untreated, NAFLD can progress to a more pathological state in a subset of individuals, known as non-alcoholic steatohepatitis (NASH).

A well established metabolic defect that contributes to dyslipidemia in the setting of high dietary fat intake, is the upregulation of the *de novo* lipogenesis (DNL) pathway (Kawano and Cohen, 2013). The DNL pathway is responsible for the endogenous production of fatty acids (FAs) from acetyl-CoA substrates. These FAs can then be incorporated into numerous lipid species, including sphingolipids, glycerophospholipids, and neutral lipids such as triacylglycerols and cholesterol esters. Increased flux through the DNL pathway can result in the production and accumulation of toxic lipid species, compounding the already lipotoxic cellular environment (Kawano and Cohen, 2013). Obesity and its associated co-morbidities present significant challenges to the healthcare system, highlighting an unmet clinical need for therapeutic interventions to treat these conditions. Current treatment strategies for reducing obesity are met with significant challenges including poor long-term compliance with lifestyle intervention, and several unwanted side effects associated with pharmacological treatments. Thus, the use of oligo therapeutics such as ASOs provides an attractive alternate approach, particularly given their amenability to chemical modification and broad therapeutic treatment window, as discussed below.

ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides are short, single stranded nucleic acid sequences that are between 10 and 30 base pairs in length (Stein, 2001). ASOs are typically administered *via* injection into either the subcutaneous adipose tissue or peritoneal cavity for *in vivo* preclinical studies. Upon administration, they have a broad tissue distribution and bioavailability, although they primarily accumulate in the liver (first pass metabolism) and in the kidney (excretion; Geary et al., 2015). Notably, given that ASOs do not cross the blood brain barrier, intrathecal injection is required to target a gene of interest (GOI) in the central nervous system (Geary et al., 2015). ASOs are internalized from the systemic circulation *via* endocytosis, however, this process can be augmented substantially *via* the addition of chemical modifications, such as those described below. Upon entry to the cell, ASOs can be deposited into the cytoplasm or sequestered to the nucleus. In both instances, ASOs are designed to bind *via* Watson and Crick hybridization, to a complementary messenger RNA (mRNA) sequence. The resulting mRNA/ASO heteroduplex is then targeted by RNase H1, which mediates their decay (Bennett et al., 2017). RNase H1-dependent degradation results in a reduction in functional mRNA abundance and thus translation of the target mRNA. Current generation ASOs employ a gapmer configuration, an engineered design element whereby the central ASO sequence is homologous to the target sequence and is flanked by stabilizing nucleotides to improve longevity of the ASO. Beyond traditional antisense activity, ASOs can function as an exon skipping agent, whereby ASOs mediate the removal of a mutant or deleterious exon sequence in pre-mRNA that can enable partial rescue of protein function. For example, in Duchenne Muscular Dystrophy (DMD), a hereditary muscle wasting disorder driven primarily by loss of the protein dystrophin, researchers have utilized ASOs to partially restore protein expression of dystrophin (Lim et al., 2017). Briefly, individuals with DMD often carry mutations that disrupt or modify the open-reading frame on exon 51 leading to improper dystrophin protein production (Aartsma-Rus et al., 2009). ASOs directed against exon 51 have thus been shown to clinically improve locomotion in these individuals (Lim et al., 2017).

Considerable work has gone into improving the pharmacokinetics and pharmacodynamics of ASOs. This has been driven by both the need to address the inherent issue of "naked" DNA or RNA degradation, mediated by ubiquitously expressed endonucleases in mammalian organisms, as well to limit off target tissue expression and toxicity. Structural modifications to "native" or unmodified ASOs can be broadly characterized into three distinct groups: nucleic acid modifications (NAMs), backbone modifications (BMs), and macromolecule conjugations. The most common NAMs include locked nucleic acid (LNA) and 2′,4′-constrained 2′-*O*-ethyl bridged nucleic acid (cEt). Substitution for a bridging LNA or cEt nucleic acids improves hybridization and mismatch discrimination for a given sequence (Seth et al., 2010). Common BMs include phosphorothioate (PS) and 2′-O-methoxyethyl (2′-MOE). These backbone modifications increase stability by inhibiting endonuclease degradation of the ASO, although 2′-MOE ASOs have been shown to reduce the affinity for RNase H1 activity (Cook, 1993; Roberts et al., 2020). Recently, Anderson et al. (2021) suggested that next generation ASO chemistries utilizing mesyl-phosphoramidate linkages may address limitations with current generation ASOs. Lastly, a recent addition to the ASO chemistry arsenal is the conjugation of N-acetylgalactosamine (GalNAc) with a native or modified ASO. The GalNAc moiety has previously been shown to facilitate the delivery of non-glycoproteins to the liver (Rogers and Kornfeld, 1971). More recently, it was demonstrated that GalNAc moiety has high affinity for the asialoglycoprotein receptor (ASGPR), which is expressed almost exclusively on hepatocytes (Ashwell and Harford, 1982). The addition of the GalNAc conjugate to ASOs improves their affinity for the liver by ~10-fold compared to native ASOs, while maintaining a toxicity profile that is comparative to native ASOs, as assessed by liver function tests such as plasma alanine transaminase (ALT) levels (Prakash et al., 2014). Currently, published data demonstrating the generation of ASOs that have engineered affinity for adipocytes is lacking.

UTILIZATION OF ASOs IN THE SETTING OF OBESITY AND NON-ALCOHOLIC FATTY LIVER DISEASE

A greater understanding of the key regulators and pathogenic processes involved in the development and progression of obesity and NAFLD, and ways in which to target them with ASOs has progressed over time. Early ASO studies focused on targeting components of the DNL pathway such as stearoyl–CoA desaturase-1 (SCD-1; Brown et al., 2008). Although *Scd-1*-ASO administration was associated with an attenuation of diet-induced obesity, increased atherosclerotic lesion deposition was observed in these mice compared to control-ASO treated mice. However, targeting other components of the DNL pathway and alternate lipid regulators has yielded more beneficial metabolic effects, as outlined below. Indeed, ASOs have been utilized to improve a range of common pathogenic features associated with obesity and NAFLD, including the dysregulation of lipid metabolism (Yu et al., 2008), lipid droplet dysfunction (Imai et al., 2012; Langhi et al., 2017), improper hepatic lipid partitioning (Nuñez-Durán et al., 2018; Cansby et al., 2019; Caputo et al., 2021), tissue remodeling and fibrosis (Yu et al., 2013; Kobayashi et al., 2020), and adipokine flux (Tan et al., 2015). Here, we have focused on a few select ASO studies that demonstrate effects on obesity and fatty liver disease in preclinical models (**Figure 1**). These examples describe the utilization of unconjugated (native) ASOs, which lack tissue specificity and thus have broad tissue distribution, silencing target gene expression in multiple tissues including in liver and adipose tissue.

Recently, our group utilized a native ASO to validate a novel regulator of hepatic lipid metabolism (Parker et al., 2019). Silencing of this protein, proteasome 26S subunit non-ATPase 9 (PSMD9), was associated with numerous beneficial metabolic effects. Specifically, we demonstrated, in two mouse strains (C57BL/6J and DBA/2J) fed a western diet, that *Psmd9*-ASO treated mice exhibited a significant reduction in liver steatosis compared to their control-ASO treated counterparts, as indicated by reduced hepatic lipid deposition, specifically diacylglycerol and triacylglycerol species, and hepatocyte ballooning as assessed by histology. Plasma lipid abundance was also significantly reduced, suggesting that overall lipid burden of *Psmd9*-ASO treated mice was attenuated. The significant downregulation of key proteins of the DNL pathway in the livers of *Psmd9*-ASO mice likely contributed to this effect. This is of therapeutic importance given that the DNL pathway is significantly upregulated in individuals with obesity and NAFLD (Diraison et al., 2003). However, greater mechanistic and physiological

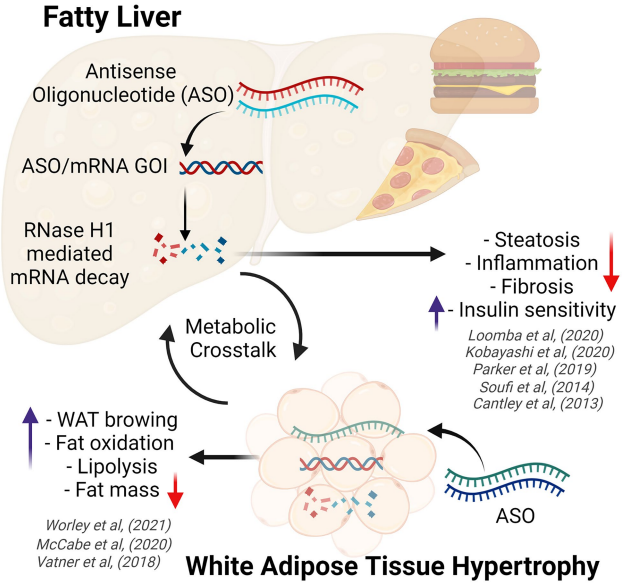

FIGURE 1 | Diagram demonstrating the effects of obesity on white adipose tissue and the liver (fatty liver disease), highlighting key metabolic benefits conferred by antisense oligonucleotide therapy. ASO, antisense oligonucleotides; GOI, gene of interest; WAT, white adipose tissue. Created with BioRender.com.

insight into the role of PSMD9 in the setting of NAFLD is required.

Another example of the use of ASOs in the setting of metabolic syndrome is the targeting of angiopoietin-like 8 (ANGPTL8). ANGPTL8 regulates the expression of lipoprotein lipase (LPL), a protein critical for the lipolysis and trafficking of lipids in mammalian systems (Quagliarini et al., 2012). Mice received weekly intraperitoneal injections of *Angptl8*-ASO, with concurrent feeding of a high fat diet for 3 weeks. *Angptl8*-ASO mice exhibited a significant reduction in liver steatosis and improved insulin sensitivity compared to control-ASO treated mice (Vatner et al., 2018). Hepatic injury was not assessed in mice, but in a cohort of *Angptl8*-ASO treated rats, which also exhibited reduced hepatic steatosis and improved insulin sensitivity, no increase in plasma aspartate transaminase (AST) or ALT levels was observed, a well known side effect of ASO administration (Burel et al., 2016), which is discussed further below. This short study highlights the effectiveness of *Angptl8*-ASO to improve key metabolic readouts that support the maintenance of lipid homeostasis and combat diet-induced NAFLD. However, given the chronic nature of NAFLD, longer term preclinical studies would be required to assess the effectiveness of *Angptl8*-ASO in this setting to understand its capacity to prevent subsequent conditions such as NASH.

Monoacylglycerol acyltransferase 1 (MGAT1), encoded by *Mogat1*, catalyzes the formation of mono- to diacylglycerols, in the lipid synthesis pathway. Inhibition of MGAT1 expression was previously demonstrated to attenuate lipid accumulation induced by oleic acid treatment in HepG2 cells (Yu et al., 2015). Administration of *Mogat1*-ASOs to *ob/ob* mice and wild type mice fed a high fat diet was associated with improved glucose tolerance in the absence of a reduction in hepatic diacylglycerol or triacylglycerol abundance compared to control-ASO treated mice (Hall et al., 2014). Enhanced hepatic insulin signaling was also observed in DIO mice treated with *Mogat1*-ASO, however, no change in body weight was seen. Soufi et al. (2014) administered ASOs against *Mogat1* to C57BL/6J mice fed a modified high fat, high cholesterol diet, that promotes hepatic injury and inflammation. They also demonstrated that mice exhibited improved glucose tolerance and hepatic insulin signaling (Soufi et al., 2014). Moreover, *Mogat1*-ASO treated mice exhibited a significant reduction in weight gain compared to control-ASO treated mice. Epididymal WAT mass and hepatic triacylglycerol abundance were also significantly reduced in *Mogat1*-ASO mice, although no improvements in liver inflammation or injury were observed, suggesting that targeting *Mogat1* may provide metabolic benefit in early liver disease. Further studies by this group examined the effect of *Mogat1*-ASOs in *Mogat1* whole body knockout mice (MOKO) fed a high fat diet and demonstrated improved glucose tolerance in these mice (Lutkewitte et al., 2021). These findings recapitulate their abovementioned findings with *Mogat1*-ASO, however, they suggest that *Mogat1*-ASOs may improve glucose homeostasis independent of *Mogat1* knockdown. A significant induction of type I interferon responsive genes, *Oasl1*, *Ifit1*, and *Ifit2* was seen in *Mogat1*-ASO treated MOKO mice, with a similar trend observed in *Mogat1*-ASO treated wild type mice, consistent with the findings of McCabe et al. (2020), as discussed below. However, co-administration of a type I interferon alpha/beta receptor-1 (IFNAR1) neutralizing antibody to wild type did not impact on the improvements in glucose tolerance seen with the *Mogat1*-ASO, suggesting that this pathway was not responsible for the effect on glucose handling.

Diacylglycerol O-Acyltransferase 2 (DGAT2) catalyzes the final step in the triacylglycerol synthesis pathway. Targeting of DGAT2 in preclinical dietary models has yielded mixed results. Researchers have observed improvements in hepatic steatosis with a marked reduction in liver triacylglycerol content with *Dgat2*-ASO administration in mice fed a high fat diet (Yu et al., 2005). Likewise, Yamaguchi et al. (2007) observed that in *db/db* mice fed a methionine choline deficient diet, a model of NASH, *Dgat2*-ASO administration was associated with improved liver steatosis. Despite this, the authors reported an increase in hepatic free FAs, fibrosis, and an upregulation of oxidative stress markers in *Dgat2*-ASO treated *db/db* mice. *Dgat2*-ASO administration was also associated with elevated ALT levels, indicating hepatic injury. One possible explanation for these discrepancies is a difference in the severity of the diet and genetic models utilized. More severe models can be associated with irreversible damage to cellular machinery and metabolic processes rendering treatments such as *Dgat2*-ASO, which appears to impact on steatosis alone, ineffective. These contrasting studies highlight a key consideration when developing ASOs for therapeutic utility, as timing of ASO administration is likely to be a critical factor impacting on their efficacy.

Overall, these preclinical studies establish strong evidence that targeting lipid regulatory pathways in the liver with ASOs can have beneficial effects on hepatic steatosis and subsequent pathological processes. However, the variability and limitations of currently available preclinical models and the severity of their phenotype, need to be considered in the context of the translational pipeline.

CHALLENGES AND LIMITATIONS IN ASO STUDIES

Given that the liver is a major site of ASO uptake, ASO-mediated hepatic toxicity remains a significant challenge (Burel et al., 2016). Many studies that have engaged ASO technology to reduce target gene expression, have reported on the impact of these therapeutics on liver function through the measurement of ALT, AST, and/or gamma-glutamyltransferase (GGT). Elevated levels of these liver enzymes can vary widely in response to ASO treatment. For example, ASOs have previously been shown to induce a severe inflammatory response in some cases (Burel et al., 2012). However, plasma levels of ALT and AST can be difficult to interpret as these markers can be elevated in models of general liver dysfunction, thus readouts can be confounded by improvements in liver function due to ASO-mediated actions, as seen with ASOs targeting *Stk25* (Cansby et al., 2019). Nephrotoxicity has also been observed with *in vivo* LNA-ASO usage (Moisan et al., 2017). Using a

human kidney cell model, researchers demonstrated that epidermal growth factor (EGF) was an effective *in vitro* biomarker of LNA-ASO induced kidney injury.

Off-target effects present a substantial challenge to ASO based approaches, as highlighted by studies that administered *Mogat1*-ASOs (Lutkewitte et al., 2021). Induction of the type I interferon response observed in this study, was similarly reported in a recent publication by McCabe et al. (2020). Here, authors used multiple ASOs sequences to silence tetratricopeptide repeat protein 39B (TTC39B) in mice, resulting in protection against diet-induced obesity. Unexpectedly, *Ttc39b*-ASOs also elicited a type I interferon response in gonadal WAT (gWAT), likely originating from resident adipose tissue macrophages. The authors demonstrated independently *via* RNAseq and quantitative PCR that the genes *Ifit1*, *Ifit3*, and *Oasl1* were significantly elevated. In contrast to the *Mogat1*-ASO studies by Lutkewitte et al. (2021), McCabe et al. (2020) demonstrated that the effect on body weight gain was abolished in IFNAR1 knockout mice, indicating that this was an IFNAR1-dependent effect. However, when authors used liver-targeted GalNAc-ASOs, targeting hepatocytes, this response was not observed. An important caveat to these studies is whether these finding are due to different ASO chemistries, the lack of targeting in adipose tissue with GalNAc-ASOs, or whether sequence specificity contributes to this phenomenon, the latter of which has been explored in some detail. Indeed, the evidence for sequence specific toxicity in ASOs arises from studies in mice utilizing over 70 distinct ASOs sequences (Burdick et al., 2014). Researchers identified 58 permutations of 2–5 base pairs that were hepatotoxic (Burdick et al., 2014). Specifically, TGC and TCC motifs were shown to be strongly associated with toxicity as assessed by AST and ALT levels, and this was attributed to aberrant ASO binding to critical hepatic proteins that regulate cell cycling and apoptosis (Burdick et al., 2014).

Lastly, practical considerations are also worth noting with regard to oligonucleotide therapy for conditions such as obesity. For example, ASOs are administered *via* injection, which can be undesirable for patients that experience needle phobia, a condition that affects close to 30% of the general adult population (McLenon and Rogers, 2019). There are also cost considerations. ASO therapies generally cost significantly more than oral medications. For example, Spinraza®, an ASO used in the treatment of spinal muscular atrophy, had a price indication of US$750,000 for the first year of treatment in 2016 (Maharshi and Hasan, 2017). These price regimens may therefore preclude the widespread use of ASOs. There may also be resistance from patients due to the perceived risk of genetic manipulation as seen with COVID-19 mRNA vaccines (Hotez et al., 2021). These downsides should, however, be balanced against the potential benefits of ASO therapeutics, which have a long-term efficacy upward of several months in humans, and thus optimized treatment might require injections only 2–3 times per year, significantly cutting costs and negating the need for daily treatment.

FUTURE PERSPECTIVES

Antisense oligonucleotides represent a promising avenue as selective agents to ameliorate the adverse effects associated with the dysregulation of lipid homeostasis in the setting of obesity and its complications. Equally though, challenges exist in designing long-term, high-quality studies to assess their effectiveness in preclinical models that more accurately exhibit key metabolic features and reflect the disease progression observed in obesity and NAFLD. A greater understanding of the off-target effects associated with specific ASO sequences will be required. Finally, regression studies are critical to assessing the ability of ASOs to reverse established disease. This is particularly relevant in the clinical setting, as it represents an important steppingstone for the translation of ASOs to the clinic.

AUTHOR CONTRIBUTIONS

MK, BD, and AC contributed to conception and design of the review and wrote and edited sections of the manuscript. MK wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of Interest: BD and AC have patents for the use of ASOs in obesity and fatty liver and have material transfer agreements with Ionis Pharmaceuticals relating to projects that use ASO technology.

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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REVIEW
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Adipose-Derived Extracellular Vesicles: Systemic Messengers and Metabolic Regulators in Health and Disease

Simon T. Bond[1,2,3]*, *Anna C. Calkin*[1,2,3] and *Brian G. Drew*[1,2,3]*

[1]Baker Heart and Diabetes Institute, Melbourne, VIC, Australia, [2]Central Clinical School, Monash University, Melbourne, VIC, Australia, [3]Baker Department of Cardiometabolic Health, University of Melbourne, Melbourne, VIC, Australia

Adipose tissue is comprised of a heterogeneous population of cells that co-operate to perform diverse physiological roles including endocrine-related functions. The endocrine role of adipose tissue enables it to communicate nutritional and health cues to other organs, such as the liver, muscle, and brain, in order to regulate appetite and whole body metabolism. Adipose tissue dysfunction, which is often observed in obesity, is associated with changes in the adipose secretome, which can subsequently contribute to disease pathology. Indeed, secreted bioactive factors released from adipose tissue contribute to metabolic homeostasis and likely play a causal role in disease; however, what constitutes the entirety of the adipose tissue secretome is still poorly understood. Recent advances in nanotechnology have advanced this field substantially and have led to the identification of small, secreted particles known as extracellular vesicles (EVs). These small nano-sized lipid envelopes are released by most cell types and are capable of systemically delivering bioactive molecules, such as nucleic acids, proteins, and lipids. EVs interact with target cells to deliver specific cargo that can then elicit effects in various tissues throughout the body. Adipose tissue has recently been shown to secrete EVs that can communicate with the periphery to maintain metabolic homeostasis, or under certain pathological conditions, drive disease. In this review, we discuss the current landscape of adipose tissue-derived EVs, with a focus on their role in the regulation of metabolic homeostasis and disease pathology.

Keywords: adipose tissue, extracellular vescicles, exosome, metabolic homeostasis, white adipose tissue, brown adipose tissue, adipose tissue secretome

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***Correspondence:**
Simon T. Bond
simon.bond@baker.edu.au
Brian G. Drew
brian.drew@baker.edu.au

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INTRODUCTION

Adipose tissue and its secreted factors play an important role in maintaining metabolic homeostasis. However, dysfunctional adipose tissue, which is often observed in obesity, can contribute to the development of obesity-related complications (Rosen and Spiegelman, 2006). Traditionally, white adipose tissue is described as a fat storage site and brown adipose tissue as a thermal regulator; however, adipose tissue is now known as one of the largest endocrine organs (Coelho et al., 2013). Indeed, it is only within the last fifty years that adipose tissue has been recognised as

having endocrine capacity, when it was shown to secrete unknown factors that influenced appetite. These factors, now known as the hormones adiponectin and leptin, are well recognised factors secreted from adipose tissue. Leptin was one of the first adipose secreted factor discovered to play a role in appetite suppression, where humans and animals deficient in leptin exhibited hyperphagia and early onset obesity, highlighting the important endocrine role that adipose tissue plays in maintaining metabolic homeostasis (Zhang et al., 1994; Halaas et al., 1995; Pelleymounter et al., 1995). Since those initial discoveries, it has been demonstrated that adipose tissue modulates many systemic biological functions by releasing a vast array of secreted factors which constitute the adipose secretome, including hormones, free fatty acids, lipids, cytokines, nucleic acids, and more recently, extracellular vesicles.

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are small lipid bound particles, released from almost all eukaryotic cells, that contain biological cargo including fatty acids, proteins, and nucleic acids, all of which can be transferred from the parent cell to a neighbouring or distant recipient cell (Keller et al., 2006; Yáñez-Mó et al., 2015; Shah et al., 2018; van Niel et al., 2018). Since their discovery, EVs have been shown to play a role in regulating a diverse range of physiological functions, which is mostly dictated by the composition of the secreted EVs (Mathieu et al., 2019). In turn, the composition of EVs is determined by the cell of origin (parent cell), which is further governed by the immediate characteristics or metabolic state of that parent cell.

There has been conjecture over the classification of EVs, as there are currently no known specific markers that define each vesicle subtype. Subsequently, there is at present, no internationally accepted definition of EVs. To date, EVs have generally been classified by size (**Figure 1**) and include the following subtypes: exosomes (~40–100 nm), microvesicles (~100–1,000 nm), and apoptotic bodies (~1,000–5,000 nm; Gao et al., 2017; van Niel et al., 2018). Due to the fact that exosomes and microvesicles can be similar in their biochemical properties and also overlap in size, it can often be challenging to distinguish between this highly heterogeneous population of small vesicles, hence the general term "small EVs" covers both exosomes and small microvesicles (Kowal et al., 2016; Crewe et al., 2018). However, exosomes are distinct from microvesicles and apoptotic bodies in the way they are generated and subsequently secreted. Exosomes are generated *via* an endocytic pathway, while microvesicles and apoptotic bodies are formed directly *via* blebbing of the plasma membrane (**Figure 1**; Borges et al., 2013). Further research is required to better understand the assembly, molecular architecture, and loading of EVs, including the underlying mechanisms which govern these pathways.

ADIPOSE TISSUE EXTRACELLULAR VESICLES

There are two distinct types of adipose tissue found in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). Further to this, adipose tissue is comprised of diverse cell populations that includes adipocytes, adipose-derived stem cells (ADSC), pre-adipocytes (adipocyte progenitors), lymphocytes, macrophages, myeloid cells, pericytes, fibroblasts, endothelial cells, and smooth muscle cells, which all play a role in maintaining and regulating metabolism and immune functions(Gimble et al., 2013; Gao et al., 2017). In addition, there are three distinct types of adipocytes, which have different primary functions: white adipocytes (storage), brown adipocytes (thermogenesis), and beige adipocytes (that can have thermogenic properties similar to brown adipocytes). The three types of adipocytes can communicate with each other in order to respond to metabolic demands and environmental stimuli, including *via* the release of EVs (Zhou et al., 2021).

EVs provide mammalian adipose tissue with an additional mode of endocrine and paracrine functional capability, with the majority of the adipose tissue secretome associated with adipocyte-derived EVs (Thomou et al., 2017; Hartwig et al., 2019). Indeed, adipose tissue-derived EVs have been shown to regulate cellular processes in both local and distant tissues, and are now considered important modulators of metabolism(Thomou et al., 2017; Ji and Guo, 2019). All three types of adipocytes (white, brown, & beige) and ADSCs have all been shown to release EVs (Ogawa et al., 2010; Chen et al., 2016; Gao et al., 2017; Connolly et al., 2018; Crewe and Scherer, 2021). These different types of adipocytes can secrete EVs with varying cargo, which likely target various cell types to differentially regulate systemic functions. Furthermore, the production and specific cargo of adipose-derived EVs have been shown to be altered under metabolic stresses, such as obesity and type 2 diabetes, and can precipitate or exacerbate disease (Eguchi et al., 2016; Gao et al., 2017; Thomou et al., 2017; Shah et al., 2018; Jafari et al., 2021). These findings suggest that EVs have the potential to serve as diagnostic biomarkers of adipose tissue health and metabolic disease (Eguchi et al., 2016; Gao et al., 2017; Thomou et al., 2017; Shah et al., 2018; Jafari et al., 2021). Accordingly, investigating the fundamental mechanisms that regulate adipose-derived EVs in health and disease could yield novel insights with regard to metabolic regulation and disease progression, and may provide new diagnostic and therapeutic opportunities.

White Adipose Tissue Extracellular Vesicles

White adipose tissue (WAT) is the primary tissue for fat storage and release in mammals but as previously mentioned, also plays an important role in maintaining whole body metabolism. WAT-secreted EVs have garnered significant interest in recent times, particularly with regard to their ability to carry miRNAs to other tissues and regulate transcriptional pathways in target cells (Thomou et al., 2017). Although previous findings have mostly focused on the miRNA harboured within WAT EVs, new studies are now investigating the biological composition of WAT EVs in greater detail, as well as whole body responses to WAT EVs.

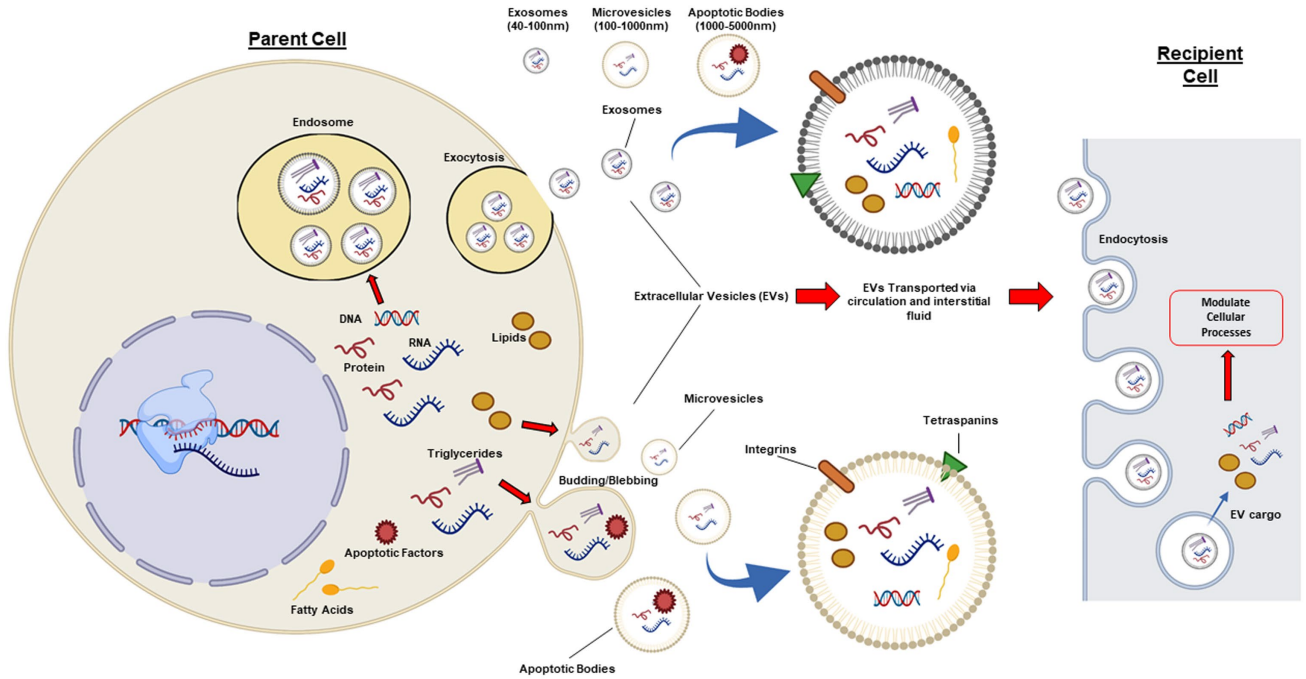

FIGURE 1 | Cellular pathways involved in extracellular vesicle biogenesis. Extracellular vesicles (EVs) are a heterogeneous population of lipid bound vesicles that are released by the majority of mammalian cell types into the extracellular space and circulation. EVs consist of three main subtypes: exosomes (40–100 nm); microvesicles (100–1,000 nm); and apoptotic bodies (1,000–5,000 nm), which differ in their size, content, biogenesis, and release. Exosomes are generated and released *via* a pathway involving exocytosis, whereas microvesicles and apoptotic bodies are released *via* a budding/blebbing pathway. EV cargo includes nucleic acids, proteins, and lipids, which are highly regulated by the health status of the parent cell. Once in the extracellular space or circulation, EVs can target adjacent cells and peripheral tissues. EVs have common surface markers (tetraspanins: CD63, CD9, CD81, and CD82) and can have specific surface markers for cell targeting, such as integrins. EVs can elicit effects in recipient cells *via* cell receptor interactions or *via* absorption and release of biological cargo into the cytoplasm of the recipient cell. Figure created with BioRender.com.

Although our understanding of WAT EV composition, release, and function is still in its infancy, ongoing research is slowly piecing together an intricate and highly regulated signalling network. This includes the transfer of biological cargo as a means of communication within adipose tissue, between adipocytes and other adipose-resident cell types such as endothelial cells and macrophages, as well as to peripheral tissues such as the brain and liver. A significant finding in regard to WAT EV communication within adipose tissue depots was the discovery that adipose tissue-resident endothelial cells communicated and delivered active biomolecules *via* EVs to adipocytes (Crewe et al., 2018). This was elegantly demonstrated by Crewe et al. (2018) in adipose-specific Cav1 KO mice, which unexpectedly still exhibited an abundance of Cav1 in white adipocytes where the gene had been genetically ablated. This study demonstrated that white adipocytes communicate with resident endothelial cells *via* an EV mechanism, which initiates the trafficking of Cav1 from neighbouring endothelial cells to adipocytes (Crewe et al., 2018). Such findings highlight the importance of EVs to communicate between cells, which can facilitate the transfer of enzymes and other important biological cargo from one cell type to another, which is critically necessary if a recipient cell type is depleted or cannot produce a given protein/substrate themselves. Interestingly, Crewe et al. further identified that WAT EV secretion was regulated by the metabolic status of the animal. Specifically, they demonstrated that fasting increased EV secretion *via* a glucagon-stimulated pathway, a process that was almost completely blocked in genetic and diet-induced mouse models of obesity (Crewe et al., 2018). This demonstrates that adipose tissue EVs alter their function in response to changes in systemic nutrient availability. In addition to this, Crewe et al. (2021) have demonstrated that the cargo of WAT-derived EVs is altered in response to whole body nutritional status. Notably, this study and many others that have investigated the proteomic cargo of WAT-derived EVs, have observed the presence of mitochondrial proteins (Lee et al., 2015; Durcin et al., 2017; Crewe et al., 2018; Hartwig et al., 2019; Clement et al., 2020). Indeed, WAT EVs package various aspects of mitochondrial cargo based on nutritional state. For example, WAT EVs from fasted mice contain elevated levels of electron transport chain proteins and reduced levels of mitochondrial fatty acid oxidation proteins (Crewe et al., 2018). In the setting of obesity, stressed white adipocytes release EVs that contain functional but oxidatively damaged mitochondrial components (Crewe et al., 2021). Together, these findings further support the notion that the generation of EVs, and their composition, is regulated by whole body nutritional state. The ability of WAT to selectively sort EV cargo based on nutritional or pathological state has significant implications regarding tissue cross-talk and disease,

which could provide new avenues for the treatment of obesity and metabolic disease.

In addition to the exchange of EVs with endothelial cells, WAT EVs can also communicate with other-resident cell types located within the stromal vascular fraction of adipose tissue. WAT EVs reciprocally communicate with adipose tissue macrophages (ATMs) to regulate inflammatory pathways and insulin sensitivity, and vice versa (Kranendonk et al., 2014). Specifically, WAT EVs can stimulate the differentiation of monocytes into macrophages and promote inflammatory actions leading to insulin resistance (Kranendonk et al., 2014). Moreover, when ATM EVs isolated from healthy or obese mice were administered *in vivo*, they had profound systemic effects. When EVs from ATMs of obese mice were administered to lean, healthy mice, this induced insulin resistance in adipocytes, myocytes, and hepatocytes (Ying et al., 2017). Conversely, EVs isolated from ATMs of healthy mice improved insulin sensitivity when administered to obese mice (Ying et al., 2017; Liu et al., 2019). Similar effects were observed with EVs derived from white adipose tissue of genetically obese *ob/ob* mice, which also led to insulin resistance when administered to healthy mice. This phenomenon was reduced in TLR4 KO mice, suggesting that WAT EVs communicate with immune cells *via* this receptor to impact on insulin sensitivity and in turn, alter glucose metabolism (Deng et al., 2009; Gao et al., 2017). The relationship between white adipocytes and ATMs has a strong influence on metabolic homeostasis, adipose tissue function, and disease progression. Thus, the communication between adipocytes and ATMs *via* EVs requires further investigation to reveal the processes that regulate this reciprocal communication and to understand the conditions that lead to different physiological and pathological outcomes.

Visceral and Subcutaneous White Adipose Tissue Extracellular Vesicles

Another factor that influences WAT EV composition and their consequent systemic effects, is the region or adipose depot from which the EVs are derived. The abundance of each different adipose tissue depot confers a different degree of cardiometabolic risk, with visceral adipose tissue (VAT) being associated with increased risk, and subcutaneous adipose tissue (SAT) associated with reduced risk (The ADIPOGen Consortium et al., 2015). The mechanisms precipitating this difference between VAT and SAT are not fully understood, but the varying effects of WAT-derived EVs from the different depots could play a role. For instance, EVs from VAT have been implicated in insulin resistance in human liver and muscle cells (Kranendonk et al., 2014). A recent key study that characterised human-derived VAT and SAT EVs isolated from morbidly obese individuals, identified 574 proteins in VAT-derived EVs, and 401 proteins in SAT-derived EVs (Camino et al., 2021). Of the 574 proteins identified in VAT, only 50% overlapped with EVs isolated from SAT (Camino et al., 2021). In addition, VAT EVs contained more obesity-related adipokines and were enriched for proteins implicated in adipose tissue inflammation and insulin resistance, demonstrating that the diversity in EV composition can be dependent upon their adipose depot of origin (Camino et al., 2021). Interestingly, the different composition of EVs from VAT and SAT could partially explain how VAT and SAT confer distinct disease risk profiles, which will be discussed in the following section.

An important consideration of EV functionality is their ability to cross the blood brain barrier. Indeed, it has been demonstrated that VAT EVs can cross the blood brain barrier and deliver cargo to the hypothalamus, where VAT EVs from obese mice were shown to increase food intake and body weight in chow fed mice (Gao et al., 2020). This was suggested to be a result of the transfer of miRNA and lncRNA packaged into VAT EVs, which were transferred to POMC neurons in the hypothalamus, subsequently increasing metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) expression, and in turn activating mTORC1 signalling (Gao et al., 2020). This same mechanism has previously been shown to reduce food intake, with high-fat fed mice that were administered VAT EVs from lean mice demonstrating reduced appetite and weight gain *via* reduced mTORC1 signalling in the hypothalamus (Gao et al., 2020). Adipose EVs can also impact myocardial signalling. Specifically, EVs isolated from primary mouse epididymal adipocytes (VAT) treated with high glucose and palmitate, then injected intramyocardially 48 h prior to myocardial ischemia/reperfusion (MI/R), exacerbated MI/R injury in a mouse model of diabetes (Gan et al., 2020). This effect was attributed to the microRNA, miR-130b-3p, which was elevated in EVs from diabetic epididymal adipocytes, as well as with the suppression of cardioprotective and anti-apoptotic pathways (Gan et al., 2020). Conversely, Crewe et al. (2021) demonstrated that EVs shed from stressed adipocytes in obese mice contained components of mitochondria that had been damaged by oxidative stress. These EVs targeted cardiac tissue, where a burst of ROS was observed, which preconditioned the heart against ischemia/reperfusion injury by stimulating antioxidant signalling pathways (Crewe et al., 2021). This study demonstrated that not only can adipose EVs transport functional mitochondrial particles between tissues, but also that cargo which seemingly appeared to be toxic may instead be a "primer" to confer protection to other tissues.

Further to this effect on cardiac tissues, VAT EVs shed from pericardial (pFat) and epicardial (eFat) adipose tissues, which surround the heart, can also influence heart function (Shaihov-Teper et al., 2021). In unhealthy individuals, pFat and eFAT EVs can exacerbate existing conditions, leading to further complications. Specifically, eFat EVs from individuals with atrial fibrillation were enriched with proteins linked to distinct pro-inflammatory, pro-fibrotic, hypertrophic, and pro-arrhythmic pathways, likely contributing to the development of atrial myopathy and fibrillation (Shaihov-Teper et al., 2021). Moreover, EVs from pericardial fat (pFat) of obese mice promoted pathological vascular remodelling (Li et al., 2019). In obese mice, pFat that exhibited chronic low-grade inflammation, along with adipocyte hypertrophy and pro-inflammatory macrophage infiltration, secreted EVs which contained elevated levels of miR-221-3p (Li et al., 2019). miR-221-3p has previously been found to promote white adipose

tissue inflammation and impair insulin sensitivity (Peng et al., 2018). In mouse cardiac tissue, pFat EVs containing miR-221-3p, stimulated the proliferation and migration of vascular smooth muscle cells, and vascular dysfunction in the femoral artery by suppression of contractile genes in the arterial wall (Li et al., 2019). Thus, EVs shed from VAT depots under disease conditions, such as obesity, can have detrimental systemic effects that contribute to disease. Moreover, they have led to significant interest into the relatively understudied epicardial and pericardial adipose tissues, to understand how EVs from these depots may contribute to cardiovascular diseases.

Together, these studies demonstrate a role for WAT-derived EVs to exchange cellular material between different cell types, both within adipose tissue depots and in peripheral tissues, that can initiate physiological and pathological responses. However, to date, the tissues that are targeted and regulated by WAT EVs are not fully understood. In order to understand these processes in detail, a comprehensive characterisation of WAT EVs is required. In particular, analyses to determine the composition of WAT EVs and to identify the cells targeted by WAT EVs under different conditions is required, including the subsequent effects elicited in recipient cells targeted by WAT EVs.

Brown Adipose Tissue Extracellular Vesicles

Brown adipose tissue (BAT) is a highly metabolically active form of adipose tissue that plays a key role in thermogenesis and maintaining metabolic health (Simcox et al., 2017). To date, the secretory properties of BAT have received little attention; however, recent studies have demonstrated that BAT does indeed mediate important endocrine functions (Keller et al., 2006; Shah et al., 2018). Although BAT adipokines (batokines) have been identified, a comprehensive understanding of batokines and the BAT secretome, including BAT EVs, is limited. Human BAT has indeed been shown to secrete EVs; however, the role of these BAT-derived EVs is poorly understood (Chen et al., 2016). Importantly, the activation of BAT results in significant changes to the number of EVs released into the circulation. For example, human BAT exhibits a 9-fold increase in the secretion of EVs following cold-induced thermogenesis, one of the primary functions of BAT, suggesting that EV release is likely to be critical to this BAT activity (Chen et al., 2016; Thomou et al., 2017). It remains unclear why adrenergic stimulation increases BAT EV secretion, and even more so, details of the molecular characteristics and physiological role of this pathway and of these EVs (Chen et al., 2016).

In light of these findings, there has been renewed interest into BAT, in particular BAT EVs and their therapeutic potential to mimic activated BAT for the treatment of obesity. For example, BAT EVs released from *ex vivo* sections of mouse BAT tissue elicited beneficial effects in high-fat diet fed mice, by improving glucose tolerance and hepatic steatosis (Zhou et al., 2020). While supportive of a beneficial outcome, further work needs to be performed to repeat these studies at thermoneutrality and to determine the composition and target tissues/cells of these BAT EVs (Zhou et al., 2020). In addition, the components of BAT EVs that were responsible for the beneficial health effects observed are still unknown. To address this, a study by Scheele et al. investigated the proteome of conditioned media from human primary BAT-like adipocytes and identified and validated the protein, ependymin-related protein 1 (EPDR1), as a novel batokine (Deshmukh et al., 2019). EPDR1 promoted the differentiation of pre-adipocytes into brown or beige adipocytes (Deshmukh et al., 2019). When recombinant EPDR1 was subcutaneously injected into mice, it led to beneficial metabolic effects, such as increased energy expenditure, likely through programming of pre-adipocytes to differentiate into thermogenic adipocytes (Deshmukh et al., 2019). Conversely, reduced oxygen consumption and physical activity were observed in EPDR1 KO mice (Deshmukh et al., 2019). To confirm the role of EPDR1 as a batokine and in adipose tissue biology, it would be pertinent to investigate this further using tissue-specific animal models, such as EPDR1 overexpression or deletion specifically in WAT or BAT. Furthermore, it remains to be determined whether EPDR1 is packaged into BAT EVs, although from the secreted proteins, this study identified; a 97% overlap with Vesiclepedia and an 80% overlap with ExoCarta suggesting that the majority of secreted BAT proteins is indeed packaged into EVs (Deshmukh et al., 2019).

Although there is a lack of knowledge in regard to the composition of BAT EVs, similar to EVs released by WAT, BAT EVs have been shown to regulate the function of other tissues, such as the liver, through the transport of miRNA (Thomou et al., 2017). Specifically, BAT EVs can regulate fibroblast growth factor 21 (FGF21) levels in the liver *via* packaged miRNAs originating from BAT (Thomou et al., 2017). This was demonstrated in adipose-specific (white and brown) Dicer KO mice, which lack the machinery to correctly process miRNAs, leading to adipose tissue devoid of functional miRNA and a subsequent decline in the levels of circulating exosomal miRNAs (Thomou et al., 2017). Dicer KO mice developed aspects of lipodystrophy, BAT whitening, and insulin resistance (Thomou et al., 2017). This phenotype was rescued *via* the transplantation of wild-type mouse BAT into Dicer KO mice, which restored the levels of circulating exosomal miRNA, leading to improved glucose tolerance and reduced fibroblast growth factor-21 (FGF21) in liver and serum (Thomou et al., 2017). Such findings demonstrate that BAT EVs constitute an important source of exosomal miRNAs in the circulation.

These novel findings further enforce the important role of BAT, and BAT EVs, in the maintenance of metabolic health. As a result, BAT EVs are now emerging as an exciting and novel therapeutic modality for the treatment of metabolic disease. BAT EVs could improve complications associated with obesity and impaired metabolism and may offer a realistic therapeutic avenue to harness the metabolic benefits of activated BAT. This is exemplified by the fact that BAT activators have failed to translate to the clinic, as BAT is often reduced or dysfunctional in obesity. In addition, the tissue-specific delivery of small chemical activators remains a significant challenge. Moving forward, rather than activating BAT, it could be plausible

to isolate EVs *ex vivo* from healthy activated BAT and administer these to obese individuals to mimic the beneficial effects of BAT activation. BAT EVs could thus act to recapitulate the metabolic benefits of activating healthy BAT in the setting of obesity, by targeting and modulating other metabolic tissues to increase energy expenditure and browning of WAT.

Beige Adipocyte Extracellular Vesicles

Beige (or brite) adipocytes are an intermediate adipocyte subtype, that are dispersed throughout adipose tissues, and display thermogenic properties (Wu et al., 2012). Beige adipocytes are proposed to have a high cellular plasticity, which provides the ability of the beige adipocyte to switch from white adipocyte-like functions to brown adipocyte-like functions as required, thus making them distinct from both white and brown adipocytes. Beige adipocytes have been identified in adult humans and have been shown to release EVs (Wu et al., 2012; Chen et al., 2016). Similar to BAT EVs, beige adipocytes increase the release of EVs when activated (Chen et al., 2016). For example, in response to cAMP, there was an 11-fold increase in EV release from beige adipocytes, which was not observed in white or brown adipocytes (Chen et al., 2016). EVs released from these activated beige adipocytes contained factors that were protective against diabetes, which when administered to primary white adipocytes, increased insulin sensitivity, and insulin-stimulated glucose uptake (Su et al., 2018).

Currently, beige adipocyte EVs remain largely under-investigated, but similar to BAT-derived EVs, hold significant therapeutic potential for the treatment of obesity. Of specific interest is the possibility that EVs released from beige and brown adipocytes might perpetuate further beiging and activation of BAT pathways in WAT and adipocyte progenitors. However, several limitations remain regarding the interrogation of beige adipocytes, particularly regarding the difficulty in distinguishing beige cells from white and brown adipocytes and in addition, obtaining sufficient quantities of beige adipocytes. However, new *in vitro* techniques using human-induced pluripotent stem cells (iPSC) have yielded promising results, yet the study of beige adipocyte EVs *in vivo* remains challenging (Su et al., 2018).

Adipose-Derived Stem Cell Extracellular Vesicles

Adipose tissue is a rich source of mesenchymal stem cells (MSCs), commonly referred to as adipose-derived stem cells (ADSCs). Much like other cell types, new insights have revealed that ADSCs secrete EVs that exert protective effects in several disease settings, with the ability to regulate adipocyte progenitor differentiation and adipocyte function (Zhao et al., 2018; Li et al., 2020; An et al., 2021). Specifically, ADSC-derived EVs can attenuate the progression of obesity-related complications *via* effects on WAT inflammation, systemic insulin resistance, dyslipidemia, and hepatic steatosis in diet-induced mouse models of obesity (Zhao et al., 2018). Improvements in these parameters were attributed to activation of M2 (pro-resolving) macrophage polarisation and beiging of WAT (Zhao et al., 2018). These findings have since been recapitulated by EVs isolated from human ADSCs during either white or beige adipogenic differentiation (Jung et al., 2020). EVs isolated from differentiating ADSCs induced cell reprogramming by promoting the differentiation of independent ADSCs into either white or beige adipocytes, respectively (Jung et al., 2020). Moreover, ADSC EVs released during beige adipogenic differentiation were administered to high-fat diet fed mice, which was associated with an amelioration of hepatic steatosis and glucose tolerance, thought to be mediated by miRNAs within the EVs (Jung et al., 2020).

Currently, the characterisation and therapeutic potential of ADSC-derived EVs from different adipose depots, and their regulation in different disease pathologies, needs to be further explored. Future studies could utilise the therapeutic potential of ADSC EVs to complement, or even replace, current stem cell therapies. The ability to easily isolate human ADSC and culture them allows researchers to readily isolate EVs from ADSCs exposed to various treatments for characterisation and assessment of their physiological effects.

ADIPOSE TISSUE EXTRACELLULAR VESICLES IN DISEASE

Research to date has demonstrated that EVs isolated from resident cells of healthy adipose tissue including adipocytes, macrophages, and stem cells have the ability to improve metabolic complications (**Figure 2**). Conversely, there is sufficient evidence to demonstrate that EVs released from unhealthy adipose tissue, such as in the setting of obesity, can contribute to disease pathologies. Metabolic diseases are inherently complex conditions, which is partly explained by the multiple tissues involved and the intricate cross-talk that occurs between these tissues under various pathological and environmental conditions. Understanding the roles that EVs play in this complex interplay, particularly in regard to adipose tissue, may help us to uncover novel pathological drivers of disease.

Although EVs are released from cells in a constitutive manner, pathophysiological stimuli can modulate EV biogenesis and release. Furthermore, protein and miRNA packaging into EVs can be selective under conditions of physiological change or pathological insults, thus mirroring the microenvironment in the parent cell (Keller et al., 2006; Shah et al., 2018; Mathieu et al., 2019). Garcia-Martin et al. (2021) recently described a mechanism by which cells actively sort miRNA for cellular retention or packaging into EVs *via* miRNA motifs. These findings can provide clues to assist with identifying the tissue of origin for circulating EVs, as well as provide novel approaches for RNA-mediated therapies. However, little is known about how EV cargo is regulated in response to disease. In the setting of obesity, an increase in circulating adipose-derived EVs is observed, where they are implicated in the development of obesity-associated metabolic disorders including insulin resistance and type 2 diabetes (Gao et al., 2017; Kwan et al., 2021). Some of the pathways that are regulated by adipose-derived EVs in obesity include the stimulation of monocyte

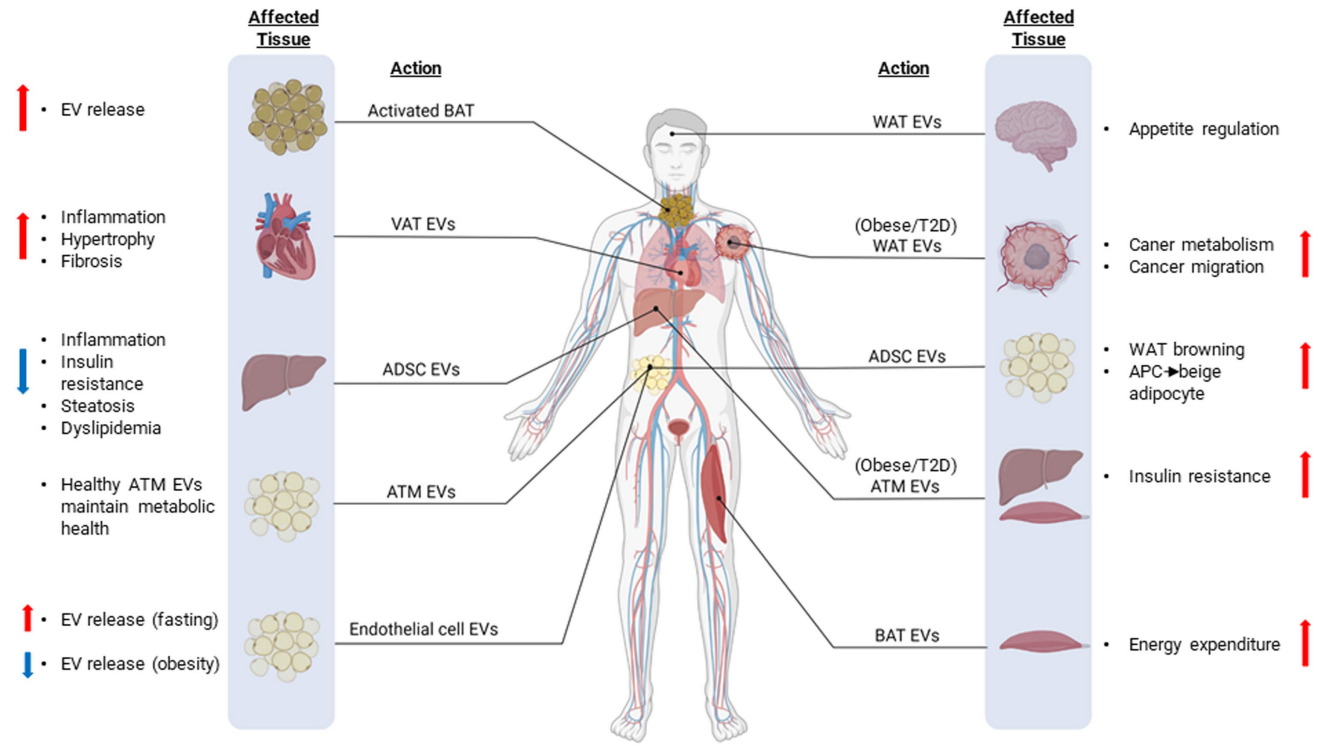

FIGURE 2 | Overview of adipose-derived small extracellular vesicles in health and disease. Extracellular vesicles (EVs) derived and released from adipose tissues can modulate physiological processes in peripheral tissues. In healthy individuals, adipose EVs may play an important endocrine and paracrine role to maintain metabolic homeostasis through reciprocal communication with peripheral tissues and other cell types residing in adipose tissue depots. EVs shed from unhealthy adipose tissue, which often occurs in diseases such as obesity and type 2 diabetes, can exacerbate or drive pathologies associated with disease complications. VAT, Visceral adipose tissue; EVs, Extracellular vesicle; ATM, Adipose tissue macrophage; WAT, White adipose tissue; BAT, Brown adipose tissue; APC, Adipocyte progenitor cell; ADSC, Adipose-derived stem cell; and T2D, Type 2 Diabetes. Figure created with BioRender.com.

differentiation and macrophage activation, upregulation of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and inflammation and dysregulation of the transforming growth factor-beta (TGF-β) pathway which progresses the development of fatty liver disease (Deng et al., 2009; Koeck et al., 2014). Many of these factors have been shown to be regulated through transcriptional reprogramming *via* the transfer of miRNAs that are packaged into adipose-derived EVs. This is observed in EVs isolated from VAT of obese individuals, which contributed to hepatic and skeletal muscle insulin resistance mediated by adipocyte-derived miR-27a inhibition of Protein kinase B (Akt) phosphorylation and peroxisome proliferator activated receptor alpha (PPARα) expression (Kranendonk et al., 2014; Yu et al., 2018).

In addition to the negative metabolic roles of adipose-derived EVs described in the setting of metabolic syndrome, there is now sufficient evidence that EVs shed from unhealthy adipose tissue can also exacerbate other conditions, such as cancer (**Figure 2**). Adipose EVs released under certain conditions, such as in the setting of obesity or type 2 diabetes, can regulate and sustain the high energy demands of cancer cells (Jafari et al., 2021). This is facilitated by the transfer of nucleic acids which can upregulate metabolism in cancer cells, or by the transfer of metabolic substrates and machinery (Lazar et al., 2016; Clement et al., 2020; Jafari et al., 2021). White adipocytes have been shown to package fatty acids and fatty acid oxidation enzymes into EVs for delivery to tumours, providing cancer cells with the necessary substrates and machinery to facilitate fatty acid oxidation (Lazar et al., 2016; Clement et al., 2020). Importantly, EVs shed from unhealthy adipose tissue can increase the aggressiveness of cancers by not only enhancing metabolism, but also by facilitating the migration of cancer cells *via* induction of the epithelial-to-mesenchymal transition process (Lazar et al., 2016; Jafari et al., 2021). This raises the possibility of targeting adipose EVs as a potential treatment for cancer. In addition, adipose EVs may become reliable markers of cancer severity or progression. While current research relating to adipose-derived EVs is promising in regard to disease development and progression, the mechanisms and key mediators of these processes remain poorly understood.

Therapeutic Considerations

Extracellular vesicles are emerging as an exciting new tool with diagnostic and therapeutic potential for a variety of diseases including metabolic diseases and cancer (Ghafouri-Fard et al., 2021). The stability of EVs compared with other therapeutics is a favourable aspect where for instance, EVs isolated from ADSCs are easily stored for long periods, compared with more traditional ADSC therapeutics. In addition, EV dosage can be easily controlled, and to date, there is no evidence of rejection by the recipient's immune system (An et al., 2021). Further to this, with the potential for targeted delivery of EV-encapsulated cargo, recent technological advances are leading to large scale commercial production of EVs (Whitford and Guterstam, 2019). This includes the production of "näive" or native EVs (naturally produced by cells) and also the production of engineered EVs loaded with specific cargo. Even though there are currently no FDA approved EV therapeutics, several pharmaceutical companies and institutions have now commenced clinical trials using EVs (Fuster-Matanzo et al., 2015; Chen et al., 2020). Clinical trials are currently utilising EVs from a variety of sources and for a diverse range of purposes, such as cancer treatment, wound healing, disease biomarkers, and vaccines, which to date, have been well tolerated, and are yielding positive results (Fuster-Matanzo et al., 2015; Santos and Almeida, 2021). Given this, many companies are now investing in new technologies so that EV production can be readily up scaled for commercial purposes (Chen et al., 2020; Santos and Almeida, 2021).

Other therapeutic opportunities could include the identification of factors that inhibit the biogenesis, release, or delivery and function of EVs released from unhealthy adipose tissue. By targeting these pathways, it could be possible to prevent the detrimental effects mediated by unhealthy adipose tissue EVs. Further investigation into the fundamental biology of adipose tissue-derived EVs, in health and disease, will help to progress the translation of clinical applications which can leverage off the mechanisms that underpin adipose EV biology.

CONCLUSION

New research into EV biology has provided us a better understanding of how adipose tissue-derived EVs can modulate metabolism within adipose tissue and other peripheral organs. With technical advances in isolation, purification, and characterisation of EVs, we are gaining a clearer understanding of the roles of adipose-derived EVs in both health and disease. It is now well established that the communication between adipose tissue and other organs *via* secreted factors, including those in EVs, is essential for preserving metabolic health. However, many questions still remain in this field, including the need to understand how these particles communicate with each other and other tissues. In particular, what are the differences in EV composition and function from the various adipocytes and the different adipose regions? In addition to this, it is unclear how recipient cells distinguish between the EVs they receive and co-ordinate their biological responses. In order to answer these questions, it is essential to develop appropriate laboratory tools to investigate adipose EVs. To this end, we need to understand whether there are differences in EV composition between primary and immortalised cell types, and whether they faithfully recapitulate adipose tissue EVs *in vivo*. It is possible that novel animal models could be developed to interrogate the role of EVs in a tissue-specific manner, which would allow better clarity around the quantity, destination, and effects of EVs from specific tissues. Overall, while the EV field is an exciting and emerging area, significantly more work is necessary to understand the fundamental biology of EV function before we can ultimately harness their potential for therapeutic utility.

AUTHOR CONTRIBUTIONS

BD and SB directed the concepts in the review and wrote the manuscript. AC contributed to discussion and provided the critical review. All authors contributed to the article and approved the submitted version.

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Weighing in on Adipogenesis

*Elizabeth R. Nunn, Abhijit B. Shinde and Elma Zaganjor**

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, United States

Obesity is a growing health concern worldwide because of its contribution to metabolic syndrome, type II diabetes, insulin resistance (IR), and numerous cancers. In obesity, white adipose tissue (WAT) expands through two mechanisms: increase in adipocyte cell number by precursor cell differentiation through the process of adipogenesis (hyperplasia) and increase in existing mature adipocyte cell size (hypertrophy). While hypertrophy is associated with the negative effects of obesity on metabolic health, such as inflammation and lipotoxicity, adipogenesis prevents obesity-mediated metabolic decline. Moreover, in metabolically healthy obesity adipogenesis is increased. Thus, it is vital to understand the mechanistic basis for adipose expansion to inform novel therapeutic approaches to mitigate the dysfunction of this tissue and associated diseases. In this mini-review, we summarize recent studies on the regulation of adipogenesis and provide a perspective on targeting adipogenesis as a potential therapeutic avenue for metabolic disorders.

Keywords: epigenetics, mitochondria, metabolism, adipose, adipogenesis

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Prashant Rajbhandari, Icahn School of Medicine at Mount Sinai, United States

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Liang Guo, Shanghai University of Sport, China
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****Correspondence:***
Elma Zaganjor
elma.zaganjor@vanderbilt.edu

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INTRODUCTION

Although adiposity is linked to numerous diseases, adipocytes are critical for maintaining healthy systemic metabolism. Adipocytes perform important functions: coordinating energy balance, releasing nutrients in times of fasting, and storing nutrients in times of surplus. WAT is an endocrine organ that controls appetite, fertility, body temperature, glucose homeostasis, and insulin sensitivity by secreting hormones (adipokines), cytokines, and lipids. Adipose serves physical functions, such as cushioning organs like the heart, kidneys, ovaries, and extremities that experience high levels of stress, like the palms and heels. WAT expands through hyperplasia, an increase in adipocyte cell number through precursor cell differentiation *via* a process termed adipogenesis, and hypertrophy, an increase in existing, mature adipocyte cell size. Hypertrophy is accompanied by inflammation, lipotoxicity, and ectopic lipid accumulation, which may lead to IR. In contrast, adipogenesis prevents obesity-mediated metabolic decline. Genetically engineered mouse models which exhibit increased adipogenesis in the subcutaneous adipose depot are metabolically healthy despite increased adiposity (Kim et al., 2007; Kusminski et al., 2012). Therefore, inducing adipogenesis and limiting hypertrophy could present a therapeutic strategy for combating metabolic diseases induced by overnutrition. What are the molecular mechanisms that promote adipogenesis? Various signaling pathways and transcriptional and epigenetic regulators of adipogenesis have been elucidated. However, how cellular metabolism mechanistically regulates adipogenesis is only now being evaluated. In this mini-review, we summarize the literature on the role of cellular metabolism in adipogenesis and discuss the potential implications for the treatment of obesity-mediated disorders.

SIGNALING PATHWAYS IN ADIPOGENESIS

Hormones and ligands regulate adipogenesis by modulating signaling pathways. Insulin engages the insulin receptor, activating the signaling cascade consisting of insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K), AKT, and the mechanistic target of rapamycin complex 1 (mTORC1) to promote glucose uptake into differentiating preadipocytes. Glucocorticoids bind glucocorticoid receptor (GCR) to stimulate transcription *via* CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ), enhancing insulin signaling required for adipogenesis (Ambele et al., 2020). The bone morphogenic proteins (BMPs), specifically BMP2 and BMP4, promote adipogenesis in part through SMAD4-mediated upregulation of PPARγ (Zamani and Brown, 2011). The hedgehog signaling pathway opposes adipogenesis through interference of BMP signaling (Suh et al., 2006; Garten et al., 2012). Canonical Wnt signaling suppresses adipogenesis through the stabilization of β-catenin that represses the PPARγ-C/EBPα complex (Kanazawa et al., 2005; Rosen and MacDougald, 2006). Other pathways, such as TGFβ, FGF, ERK/MAPK, p38/MAPK, and Notch signaling, can be pro- or anti-adipogenic depending on the experimental system, specific ligands used, and cell type, as well as the developmental or differentiation stage (Bost et al., 2005; Aouadi et al., 2006; Rosen and MacDougald, 2006; Zamani and Brown, 2011).

TRANSCRIPTIONAL CONTROL OF ADIPOGENESIS

PPARγ is one of the key transcriptional regulators of adipogenesis. Although early mechanistic studies investigating the function of PPARγ in adipogenesis were performed using *in vitro* model systems (Tontonoz et al., 1994; Rosen et al., 1999), the loss of WAT in PPARγ-deficient mouse models such as chimeric PPARγ-null (Rosen et al., 1999), tetraploid rescued (Barak et al., 1999), and adipocyte-specific PPARγ knockouts (KOs) (Wang et al., 2013a) provide evidence for its role in adipogenesis *in vivo*. The action of PPARγ promotes the transcriptional activity of another adipogenic regulator, co-activator CCAAT/enhancer-binding protein-α (C/EBPα) (Wu et al., 1999). When expressed ectopically, C/EBPα induces adipogenesis in fibroblasts (Freytag et al., 1994). However, C/EBPα-deficient fibroblasts can only undergo partial adipogenesis, displaying several defects, such as decreased lipid accumulation and IR (Wu et al., 1999). The terminal step of embryonic adipogenesis is independent of C/EBPα, possibly due to redundancy with other C/EBP family members, whereas it is essential for various WAT adipogenic conditions during adulthood (Wang et al., 2015). Moreover, PPARγ can induce adipogenesis in the absence of C/EBPα, but C/EBPα fails to do so in PPARγ-deficient fibroblasts (Rosen et al., 2002). These observations suggest that C/EBPα and PPARγ work cooperatively in a single pathway, although PPARγ plays a central effector role in adipogenesis. C/EBPβ and C/EBPδ are expressed earlier during induction of the adipogenic program in 3T3-L1 cells, and they promote the expression of C/EBPα during the final phase of adipogenesis (Cao et al., 1991; Yeh et al., 1995). Additionally, mice lacking both C/EBPβ and C/EBPδ showed a dramatic volume reduction of epididymal WAT (eWAT) (Tanaka et al., 1997). Apart from these main factors, several other transcriptional regulators of adipogenesis have been identified and are reviewed in-depth elsewhere (Farmer, 2006; Rosen and Spiegelman, 2014; Lee et al., 2019).

METABOLIC REGULATION OF ADIPOGENESIS

Given that adipocytes are a reservoir for lipid stores and sense nutrient state to regulate organismal energy balance, metabolites themselves are significant regulators of adipogenesis. Glucose availability dictates adipocyte maturation, such that GLUT4-mediated glucose uptake promotes adipogenesis through a number of distinct mechanisms (Kahn and Flier, 2000). First, glucose contributes to nicotinamide adenine dinucleotide phosphate (NADPH) synthesis through the pentose phosphate pathway. As NADPH is a required cofactor for lipogenesis, glucose directly mediates adipocyte differentiation through this pathway (Jackson et al., 2017). Another functional use of glucose is in acetyl-coenzyme A (acetyl-CoA) production. Preadipocytes derive acetyl-CoA for *de novo* lipogenesis from glucose, with the rate-limiting step in this pathway being catalyzed by pyruvate dehydrogenase (PDH). Upon differentiation, adipocytes decrease glucose contribution to lipogenic acetyl-CoA. To supplement this carbon pool, the catabolism of branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, is another source of lipogenic acetyl-CoA that promotes adipogenesis (Green et al., 2016). Mitochondrial BCAA catabolism involves transamination of BCAAs by the branched-chain amino transferase (BCAT), generating branched-chain α-keto acids (BCKAs). BCKAs are oxidized by the BCAA dehydrogenase (BCKDH) complex. During the remainder of oxidation BCAA carbons are either lost as CO_2 or contribute carbons to the tricarboxylic acid (TCA) cycle as succinyl-CoA or acetyl-CoA (Neinast et al., 2019a). In adipogenesis, a mitochondrial sirtuin, SIRT4, promotes leucine catabolism by increasing the activity of methylcrotonyl-CoA carboxylase (MCCC1), an enzyme that catalyzes the carboxylation of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA (Anderson et al., 2017; Zaganjor et al., 2021). The induction of leucine catabolism occurs early in the process of differentiation and further promotes PPARγ function (Zaganjor et al., 2021). Adipogenesis reduces the contribution of glutamine carbon to fatty acids and instead promotes *de novo* glutamine synthesis (Green et al., 2016). Moreover, knockdown of glutaminase (Gls), which promotes glutaminolysis by deamination of glutamine to glutamate, stimulates adipogenic fate in skeletal stem cells (Yu et al., 2019). Therefore, glutamine oxidation appears inhibitory to adipogenesis, although the mechanism remains elusive. Because almost all the aforementioned nutrient oxidation pathways, other than the pentose phosphate pathway, occur in the mitochondria,

these studies demonstrate a vital contribution of mitochondrial function in adipogenesis (**Figure 1**).

MITOCHONDRIAL FUNCTION IN ADIPOCYTE DIFFERENTIATION

Adipogenesis involves a 20- to 30-fold increase in the concentration of mitochondrial proteins as determined by both proteomics and electron microscopy (Wilson-Fritch et al., 2003). Mechanistically, the transcriptional program consisting of cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB), PPARγ, C/EBPα, estrogen-related receptor α (ERRα), and PPARγ co-activator 1 (PGC-1) are engaged to support mitochondrial biogenesis. Mitochondrial respiratory capacity increases with differentiation, suggesting that mitochondria are biochemically altered (Wilson-Fritch et al., 2003). Successful adipogenesis relies on mitochondrial biogenesis.

Which mitochondrial functions support differentiation? Beyond nutrient oxidation within the mitochondria to generate acetyl-CoA, the mitochondrial electron transport chain (ETC) is a source of reactive oxygen species (ROS) that can also modulate differentiation. ROS can regulate mitochondrial dynamics, increasing mitochondrial fission in bone marrow mesenchymal stem cells (BMSCs) to promote adipogenesis (Guo et al., 2020). While mitochondrial-targeted antioxidants block adipogenesis (Tormos et al., 2011), constitutively high levels of ROS have an inhibitory effect on adipogenesis through the induction of a transcriptional repressor C/EBP homologous protein 10 (CHOP-10) (Carriere et al., 2004; Furukawa et al., 2004). The toxic elevation of ROS also causes structural cellular damage to mitochondrial proteins, lipids, and DNA, including damage to the ETC, which results in depletion of ATP and nicotinamide adenine dinucleotide (NAD) (Rolo et al., 2012). In human clinical trials, antioxidants were not successful in preventing risks of type II diabetes (T2D), possibly because of the beneficial effects of ROS on adipogenesis (Castro et al., 2016). Gaining insight into the threshold of ROS that has beneficial, pro-adipogenic effects may be critical in treating metabolic dysfunction.

EPIGENETIC CONTROL OF ADIPOGENESIS

Epigenetic modifications including acetylation, methylation, phosphorylation, and ribosylation are regulated by several

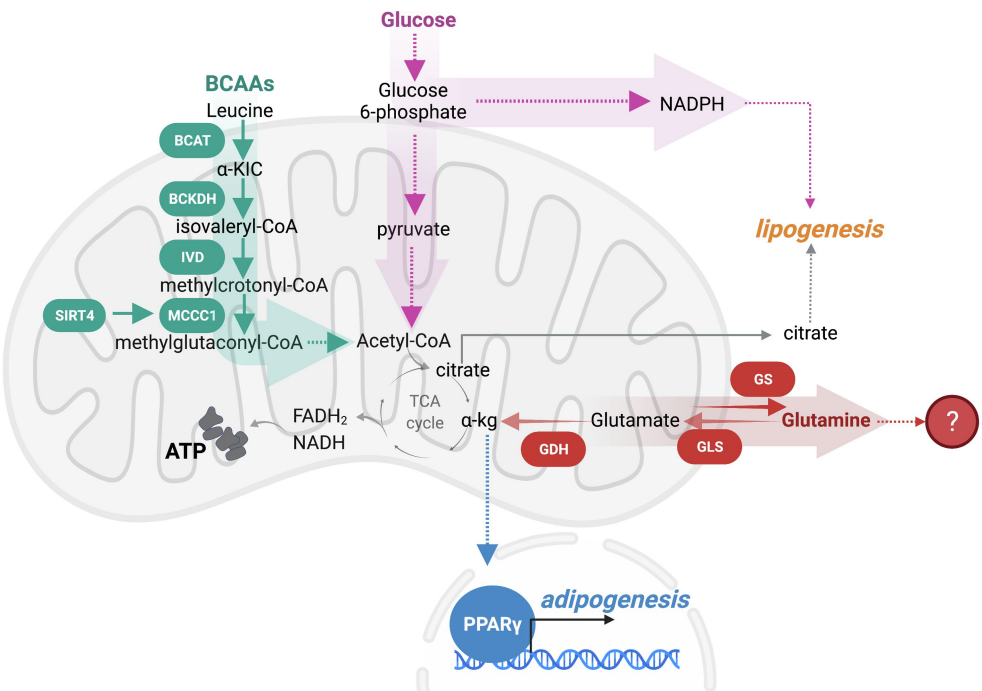

FIGURE 1 | Nutrient oxidation in the mitochondria modulates adipogenesis. Branched-chain amino acids (BCAAs) and glucose catabolism within the mitochondria produce ATP and metabolic intermediates that promote adipogenesis. As such, leucine catabolism is an early regulator of adipogenesis that is initiated by a BCAA transaminase (BCAT) generating α-ketoisocaproic acid (a-KIC). a-KIC is irreversibly oxidized by the BCAA dehydrogenase (BCKDH) complex forming isovaleryl-CoA. Isovaleryl-CoA dehydrogenase (IVD) converts isovaleryl-CoA to methylcrotonyl-CoA. Sirtuin SIRT4 induction of methylcrotonyl-CoA carboxylase (MCCC1) promotes the carboxylation of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA, which can be further oxidized to produce acetyl-CoA. BCAA catabolism promotes the PPARγ-mediated transcriptional adipogenic program. While a portion of glucose is oxidized to generate acetyl-CoA, some glucose is diverted away from the mitochondrial oxidation to the pentose phosphate pathway (PPP) to support the production of nicotinamide adenine dinucleotide phosphate (NADPH), a required cofactor for lipogenesis. Glutaminolysis appears to oppose adipogenesis, although the mechanistic understanding of fuel switching that supports adipogenesis is limited.

different classes of enzymes that connect the energetic status of the cell to changes in the epigenetic landscape. Epigenetic regulators may have positive or negative effects on adipogenesis and include histone acetyltransferases (HATs) and deacetylases (HDACs), histone, and DNA methyltransferases (HMTs and DNMTs), and demethylases that include ten-eleven translocation DNA demethylases (TETs), and histone demethylases, such as JmjC domain-containing histone demethylases (JMJDs) and histone lysine demethylase 1 (LSD1) (Musri et al., 2010; Chatterjee et al., 2011; Lee and Ge, 2014) (**Figure 2**). The epigenetic landscape is complex and multi-faceted, as mono-, di-, and tri-methylation can occur at both histone and DNA methylation marks, and acetylation, phosphorylation, and ribosylation are just a few of the dynamic modifications that can be added and removed by enzymatic regulators (Lee et al., 2019). Epigenetic patterns are altered as preadipocytes commit to this lineage and differentiate into mature adipocytes, and these changes can silence or promote the expression of particular genes as necessary. For example, histone H3K9 di-methylation *via* G9a in the promoter region of *Pparγ* represses its expression and subsequently blocks differentiation (Wang et al., 2013b). LSD1 decreases H3K9 di-methylation while maintaining H3K4 di-methylation at the *Cebpa* promoter to promote adipogenesis (Musri et al., 2010). LSD1 can also demethylate H3K9 to support adipogenesis through PPARγ activity (Jang et al., 2017). Furthermore, a specific bivalent chromatin signature H3K4/H3K9me3 keeps the expression of *Pparγ* and *Cebpa* at low levels, allowing preadipocytes to remain primed for differentiation (Matsumura et al., 2015). This complex array of histone and DNA modifications that are added and removed by epigenetic regulators rely on metabolites generated within the mitochondria.

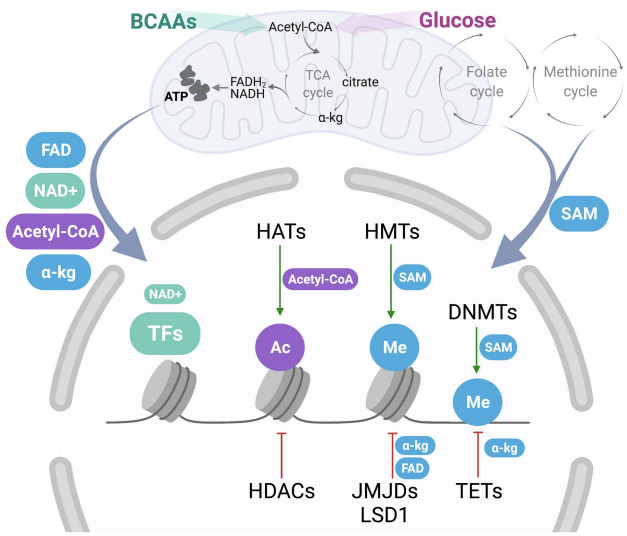

FIGURE 2 | Communication between mitochondria-derived metabolites and nuclear epigenetic regulators. One-carbon metabolism, consisting of folate and methionine cycle, generates S-adenosylmethionine (SAM), a metabolite contributing to epigenetic regulation. Oxidation of fuels, such as BCAAs and glucose, coupled to the activity of the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) produce important metabolic byproducts, including flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD+), acetyl-coenzyme A (acetyl-CoA), and α-ketoglutarate (a-kg). Once exported out of the mitochondria, these metabolites may contribute to cytosolic signaling, or act as cofactors and donor groups to epigenetic regulators in the nucleus, including histone acetyltransferases (HATs) and deacetylases (HDACs), histone and DNA methyltransferases (HMTs and DNMTs), and demethylases that include ten-eleven translocation DNA demethylases (TETs), and histone demethylases, such as JmjC domain-containing histone demethylases (JMJDs) and histone lysine demethylase 1 (LSD1), as well as other nuclear transcription factors (TFs). HATs add acetyl groups (Ac, in purple) to histones using acetyl-CoA as a substrate, while HDACs remove these groups. HMTs utilize SAM as a donor of a methyl group (Me, in blue) to histones, while methyl groups are removed by JMJDs, which utilize a-kg, or LSD1, which couples with FAD synthesis. Similarly, DMNTs utilize SAM for DNA methylation, while a-kg is a cofactor used by TETs for DNA demethylation. Additionally, NAD+ acts as an important cofactor for nuclear TFs and other regulatory enzymes, including PARP-1 and sirtuins.

METABOLIC REGULATION OF EPIGENETICS IN ADIPOGENESIS

Metabolic intermediates and cofactors, including acetyl-CoA, α-ketoglutarate, and NAD+, are derived in the mitochondria through nutrient oxidation or the TCA cycle, where they can then act as cofactors to mitochondrial enzymes or be exported into the cytosol. The TCA cycle produces metabolites that contribute to epigenetic modification (**Figure 2**). For example, mutations to the TCA cycle enzyme isocitrate dehydrogenase (IDH) and production of 2-hydroxyglutarate (2HG) decrease α-ketoglutarate levels. The loss of α-ketoglutarate contributes to the decreased activity of lysine-specific demethylase 4C (KDM4C), causing an increase in H3K9 methylation that inhibits adipogenesis (Lu et al., 2012). In the following sections, we will discuss research that has illuminated how important metabolites contribute to epigenetic regulation in adipogenesis.

Acetyl-CoA

Acetyl-CoA is a component of central carbon metabolism that provides carbon for lipogenesis. Beyond this role, acetyl-CoA is a donor group for histone acetylation *via* the activities of HATs (Shi and Tu, 2015). Within the mitochondria, acetyl-CoA is generated through β-oxidation of fatty acids, from pyruvate, and *via* branched-chain amino acid catabolism, and is reported to exit mitochondria through several mechanisms, including *via* the carnitine/acylcarnitine translocase and *via* SLC25A1 as citrate (Madiraju et al., 2009; Zaidi et al., 2012). Within the cytosol, the activity of ATP-citrate lyase (ACLY) converts citrate to acetyl-CoA and oxaloacetate. Cytosolic acetyl-CoA can take part in lipogenesis, steroidogenesis, or become a building block for glutamine, proline, and arginine (Pietrocola et al., 2015). Acetyl-CoA can also be regenerated from citrate in the nucleus by ACLY, driving a global increase in histone acetylation in 3T3-L1 cells (Wellen et al., 2009). When acetyl-CoA is depleted following siRNA-mediated silencing of ACLY or following glucose restriction, histone acetylation levels are decreased,

and adipocyte differentiation is inhibited (Wellen et al., 2009). However, some compensation has been observed as the loss of ACLY stimulates the expression of acyl-CoA synthetase short-chain family member 2 (ACSS2), which can generate acetyl-CoA from acetate (Zhao et al., 2016). High-fat diet leads to a decrease in whole-tissue acetyl-CoA levels in murine WAT and a subsequent decrease in H3K23ac, showing a correlation between diet, acetyl-CoA levels, and histone acetylation (Carrer et al., 2017). Elucidating the influence of diet on metabolite levels and histone alterations could have important implications for understanding obesity and associated diseases.

NAD+

Metabolic pathways including the TCA cycle, ETC, fatty acid oxidation, and glycolysis influence the redox state of the cell through NAD+ metabolism. NAD+ is a small molecule that can be synthesized from precursors including nicotinamide (NAM), nicotinic acid (NA), and nicotinamide riboside (NR); through the *de novo* synthesis pathway from tryptophan; and through NAD+ salvage (Katsyuba et al., 2020). NAD+ acts as both a cofactor in redox reactions and a substrate for enzymes like poly [ADP-ribose] polymerases (PARPs) and sirtuins, which act as deacylases in the mitochondria, nucleus, and cytoplasm (Katsyuba et al., 2020). NAD+ is thought to exist in distinct mitochondrial, cytosolic, and nuclear compartments due to the presence of distinct nicotinamide mononucleotide adenylyltransferase (NMNAT) isoforms in these cellular locations (Berger et al., 2005; Nikiforov et al., 2011). NAD+ usage in these compartments causes fluctuations in nicotinamide mononucleotide (NMN) and NAD+ availability that have differing effects on epigenetic events that influence adipogenesis. Within the mitochondrial compartment, increased NAD+ production *via* increased flux through the TCA cycle is required to induce adipogenesis in 3T3-L1 cells (Okabe et al., 2020). Induction of adipogenesis increases the expression of cytosolic NMNAT2 resulting in elevated cytosolic NAD+ (Ryu et al., 2018). This activity of NMNAT2 decreases the availability of NMN for nuclear NMNAT1 and leads to a decline in nuclear NAD+. As nuclear NAD+ drops, PARP-1 activity decreases, leading to a subsequent reduction in its ADP-ribosylation of CEBPα. Loss of this modification increases the CEBPα proadipogenic transcriptional program (Ryu et al., 2018). Furthermore, NMNAT1 activity and synthesis of NAD+ direct PARP-1 ability to PARylate aspartate and glutamate residues on histones (Huang et al., 2020). These findings illustrate the temporal and compartment-specific influence of NAD+ metabolism on the cellular epigenetic state that modulates adipogenesis.

Compartment-specific alterations in NAD+ levels may also impact sirtuin activity. Increased mitochondrial NAD+ may promote the activity of SIRT4, an inducer of adipogenesis, while decreased nuclear NAD+ may decrease the activity of SIRT1, a negative regulator of adipogenesis (Mayoral et al., 2015; Zaganjor et al., 2021). NAD+ compartmentalization and availability may fine-tune adipogenesis *via* multiple independent mechanisms. SLC25A51 has been recently identified as a mitochondrial NAD+ transporter (Girardi et al., 2020; Kory et al., 2020; Luongo et al., 2020), which may present an opportunity to modulate NAD+ compartmentalization and cell differentiation.

SAM, FAD, and α-Ketoglutarate

Other metabolites have been shown or suggested to influence epigenetic regulation, but their roles are not as well-defined. One such metabolite is S-adenosylmethionine (SAM). SAM acts as a major methyl donor and is utilized by histone lysine methyltransferases (HKMTs) as a cofactor, where the donation of a methyl group yields S-adenosylhomocysteine (SAH) (Wiese and Bannister, 2020). This metabolite is derived by S-adenosyl methionine transferase (MAT) by the catabolism of methionine and ATP or one-carbon metabolism (Wiese and Bannister, 2020). The addition of SAM to culture media induces differentiation in 3T3-L1 cells, although the precise mechanism has not been elucidated (Liu et al., 2013).

Another metabolite that participates in epigenetic regulation is flavin adenine dinucleotide (FAD). FAD is a cofactor utilized in redox reactions by demethylases, such as LSD1 and, once reduced, can be re-oxidized by molecular oxygen (Berger and Sassone-Corsi, 2016). Through coupling with FAD synthesis, LSD1 epigenetically regulates the expression of energy expenditure genes through the removal of mono- and di-methylation of H3K4, and knockdown of LSD1 results in activation of mitochondrial respiration and lipolysis in mature adipocytes (Hino et al., 2012). FAD levels increase both in differentiating 3T3-L1 preadipocytes as well as in mature 3T3-L1 cells following palmitate exposure to stimulate lipid storage and suppress PGC-1α (Hino et al., 2012). Alternatively, the knockdown of FAD-dependent LSD1 in differentiating 3T3-L1 cells leads to a significant decrease in lipid accumulation and expression of adipogenic regulator *Cebpa* (Musri et al., 2010). These findings suggest that FAD-dependent enzymes directly impact epigenetic regulation in adipocytes, indicating that FAD itself is a significant metabolite modulating adipogenesis.

Similarly, α-ketoglutarate acts as a cofactor for histone demethylases, as well as for DNA demethylation by TETs (Carey et al., 2015). Okabe et al. report that an increase in α-ketoglutarate is likely responsible for demethylation of H3K9me3 at the *Pparγ* promoter, supporting adipogenesis (Okabe et al., 2020). These studies support the model that metabolites relay nutrient state information to alter gene expression and cell fate.

THERAPEUTIC INTERVENTION

Adipogenesis is important in systemic metabolic health and increasing adipogenesis may profoundly improve outcomes for patients with obesity-related diseases, such as diabetes. Mutations in genes that regulate lipid droplet formation lead to lipodystrophy, a condition characterized by severe IR and dyslipidemia (Magre et al., 2001; Agarwal et al., 2002; Ghaben and Scherer, 2019). Inhibition of adipogenesis through the depletion of PPARγ in the progenitor population results in pathological WAT expansion in mice (Shao et al., 2018).

Collectively, these studies suggest the protective effects of adipogenesis and warrant the search for therapeutic interventions that promote the process. Thiazolidinediones (TZDs), synthetic PPARγ activators, although promising in their ability to promote adipogenesis, adipose tissue beiging, insulin sensitivity and reduced inflammation, also carry reported cardiac and osteoporosis risks (Ahmadian et al., 2013). Evaluating metabolic targets in their ability to induce adipogenesis in the context of diet-induced obesity may have therapeutic benefits. Notably, BCAAs accumulate in diabetic patients and BCAA catabolism in adipose tissue is dysfunctional in diabetic mouse models (Felig et al., 1969; Newgard et al., 2009; Neinast et al., 2019b). Identifying small molecules that potentiate this pathway may improve adipogenesis and overall health. Similarly, high-fat diet downregulates ACLY in WAT, alters histone acetylation, and disrupts lipid storage (Carrer et al., 2017). Future studies are necessary to evaluate the impact of maintaining ACLY levels on adipogenesis and metabolic health after a dietary challenge. Recent technological advances in mass spectrometry, compartmentalized metabolomics and general growth of the field will allow for discoveries on new regulators of adipose development (Chen et al., 2017; Collins et al., 2021). Elucidating the metabolic drivers of adipogenesis may lead to novel approaches to mitigate obesity-mediated disorders.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Ether Lipids in Obesity: From Cells to Population Studies

Yvette L. Schooneveldt[1,2], Sudip Paul[1,3], Anna C. Calkin[1,2,3] and Peter J. Meikle[1,2,3]**

[1] Baker Heart and Diabetes Institute, Melbourne, VIC, Australia, [2] Central Clinical School, Faculty of Medicine, Nursing & Health Sciences, Monash University, Melbourne, VIC, Australia, [3] Baker Department of Cardiometabolic Health, University of Melbourne, Parkville, VIC, Australia

Ether lipids are a unique class of glycero- and glycerophospho-lipid that carry an ether or vinyl ether linked fatty alcohol at the *sn*-1 position of the glycerol backbone. These specialised lipids are important endogenous anti-oxidants with additional roles in regulating membrane fluidity and dynamics, intracellular signalling, immunomodulation and cholesterol metabolism. Lipidomic profiling of human population cohorts has identified new associations between reduced circulatory plasmalogen levels, an abundant and biologically active sub-class of ether lipids, with obesity and body-mass index. These findings align with the growing body of work exploring novel roles for ether lipids within adipose tissue. In this regard, ether lipids have now been linked to facilitating lipid droplet formation, regulating thermogenesis and mediating beiging of white adipose tissue in early life. This review will assess recent findings in both population studies and studies using cell and animal models to delineate the functional and protective roles of ether lipids in the setting of obesity. We will also discuss the therapeutic potential of ether lipid supplementation to attenuate diet-induced obesity.

Keywords: plasmalogens, adipose tissue, obesity, alkylglycerols, ether lipids

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Sahar Keshvari,
University of Queensland, Australia

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Sumaira Z. Hasnain,
University of Queensland, Australia

****Correspondence:***
Anna C. Calkin
anna.calkin@baker.edu.au
Peter J. Meikle
peter.meikle@baker.edu.au

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ETHER LIPIDS

Structure and Biological Functions

Ether lipids are a unique class of peroxisome-derived glycero- and glycerophospho-lipid. They carry an ether or vinyl ether linked fatty alcohol at the *sn*-1 position, and an ester linked fatty acid either at the *sn*-2 position (ether phospholipids), or at both the *sn*-2 and *sn*-3 positions (ether glycerolipids). This is contrary to conventional glycerol-based lipids that have acyl chains attached by an ester linkage at the *sn*-1 position (**Figure 1**). To date, ether analogs of triacyclglycerols [mono-alkyl-diacylglycerols, TG(O)] and various phospholipid classes, including phosphatidylethanolamine [alkyl-phosphatidylethanolamine, PE(O)], phosphatidylcholine [alkyl-phosphatidylcholine, PC(O)], phosphatidylinositol [alkyl-phosphatidylinositol, PI(O)] and phosphatidylserine [alkyl-phosphatidylserine, PS(O)] have been reported (Nagan and Zoeller, 2001; Ivanova et al., 2012; Nagy et al., 2012; Ma et al., 2017).

Ether lipids are highly abundant molecules that account for around 20% of the total phospholipid content in mammalian cells (Nagan and Zoeller, 2001; Paul et al., 2019). They make up a significant component of subcellular membranes, including the membranes of the nucleus, endoplasmic reticulum (ER), post-Golgi network and mitochondria (Nagan and Zoeller, 2001; Honsho et al., 2008). Importantly, these lipids contain varying structural and physico-chemical properties, including different head groups and fatty acyl chains. These features give rise

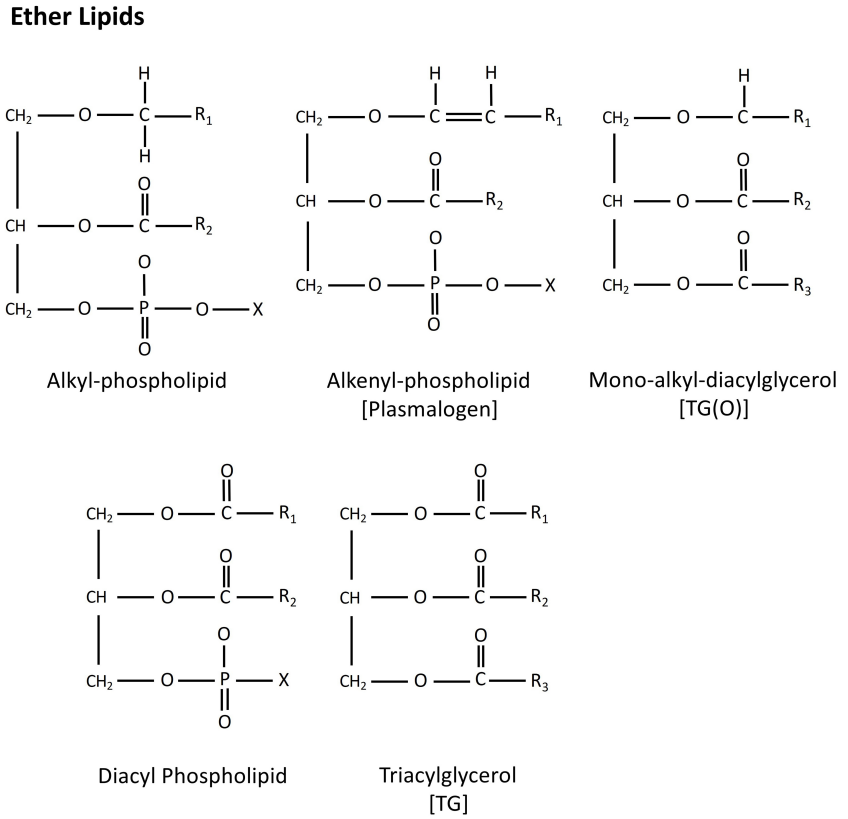

FIGURE 1 | Chemical structure of alkyl-, alkenyl- and mono-alkyl-ether lipids. Diacyl phospholipid and triacylglycerol exhibits typical structure of a glycerol-lipids.

to disparities between their distribution and function amongst tissues. High levels of ether lipids have been detected in the brain, heart, kidney, skeletal muscle and certain immune cells, including neutrophils and macrophages, whilst low levels have been reported in the liver (Lohner, 1996; Lee, 1998; Farooqui and Horrocks, 2001; Braverman and Moser, 2012). As the liver is considered a primary site for ether lipid synthesis, it has been suggested that its' low ether lipid content is due to subsequent transport of ether lipids to other tissues via lipoproteins (Vance, 1990).

Plasmalogens are a subset of ether glycerophospholipids that bear a *cis* double bond adjacent to the ether linkage, forming a "vinyl-ether linkage" (Nagan and Zoeller, 2001; Braverman and Moser, 2012). In mammalian cells, plasmalogens are considered the most abundant and biologically active class of ether lipids, primarily consisting of palmitic (16:0), stearic (18:0) or oleic (18:1) alkenyl chains at the *sn*-1 position, and polyunsaturated fatty acids (PUFA), such as linoleic acid (18:2), arachidonic acid (20:4; AA) or docosahexaenoic acid (22:6; DHA) are typically at the *sn*-2 position (Gross, 1984; Wallner and Schmitz, 2011; Braverman and Moser, 2012).

Plasmalogens were first identified in 1924, however, it was only recently that they received attention, as studies demonstrated their capabilities as potent anti-oxidants (Feulgen and Voit, 1924; Zoeller et al., 1988, 1999). The enhanced electron density and position of the vinyl-ether linkage makes it a primary target for a variety of oxidants (Engelmann, 2004). Subsequent cleavage of the vinyl-ether linkage provides additional downstream benefits, as it prevents the oxidation of PUFAs and protects unsaturated membrane lipids. This is because plasmalogen oxidation products are unable to initiate further lipid peroxidation (Khaselev and Murphy, 1999; Murphy, 2001; Engelmann, 2004). Due to their high PUFA content at the *sn*-2 position, plasmalogens are also considered key storage depots of PUFAs. These PUFAs can be cleaved and metabolised into potent second messenger molecules, such as protectins and resolvins, to induce anti-inflammatory and anti-apoptotic effects (Ford and Gross, 1989; Schwab et al., 2007; Gaposchkin et al., 2008). Later work has since described additional roles for plasmalogens, including, but not limited to, their involvement in membrane fluidity and dynamics, intracellular signalling, immunomodulation and cholesterol metabolism (Han and Gross, 1990; Mandel et al., 1998; Farooqui and Horrocks, 2001; Gorgas et al., 2006; Lessig and Fuchs, 2009; Wallner et al., 2014; Honsho et al., 2015; Rubio et al., 2018).

Ether Lipid Synthesis

Ether lipid synthesis is a well characterised process that involves multiple enzymes within the peroxisome and ER (**Figure 2**). The pathway begins in the peroxisome with the esterification

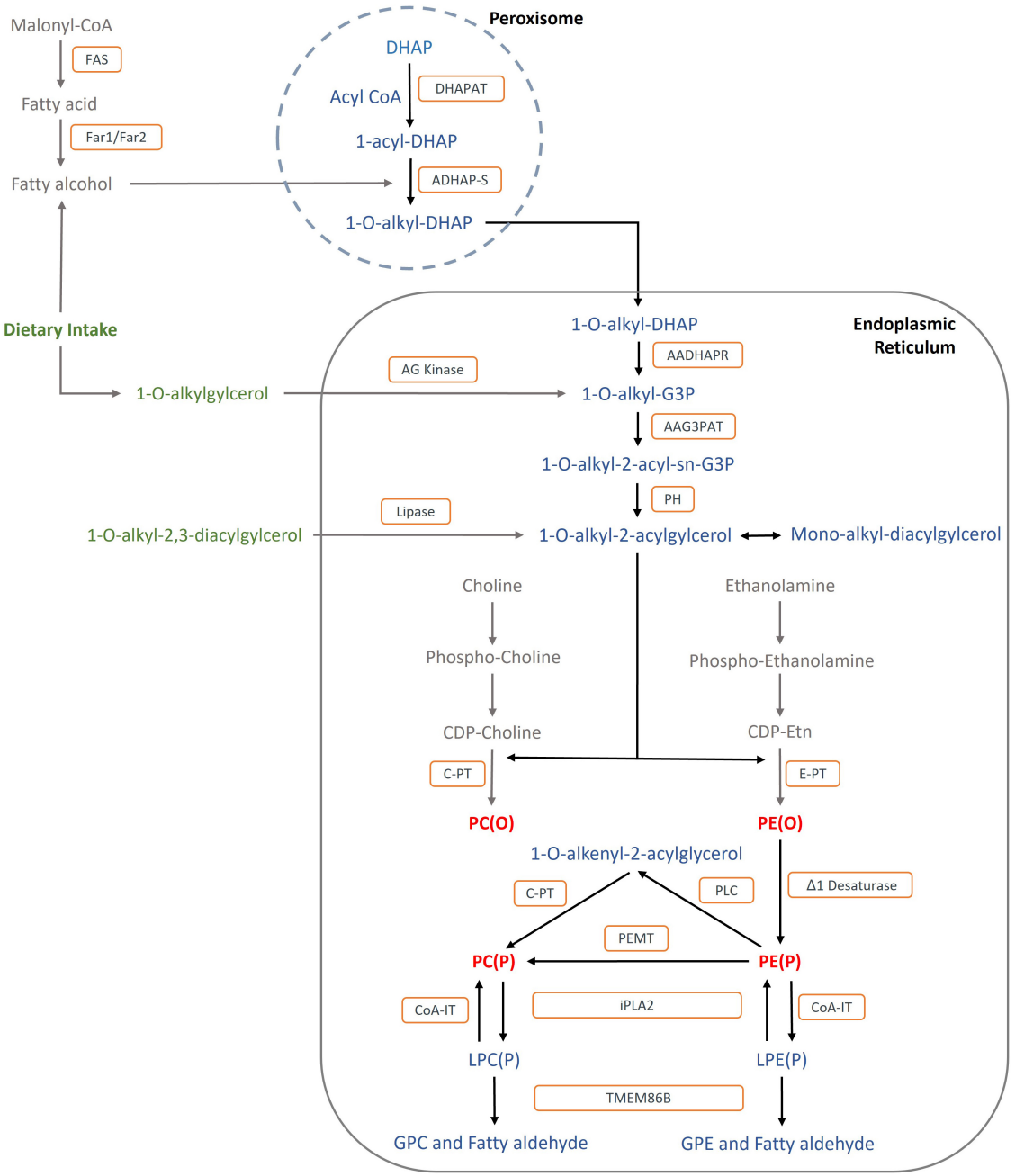

FIGURE 2 | Biosynthetic and catabolic pathways of ether lipids: The formation of fatty alcohol by FAR1 and FAR2 in the peroxisome is the rate-limiting step: Metabolites are shown in blue and red: DHAP, dihydroxyacetonephosphate; G3P; glycerol-3- phosphate GPC, glycerophospho-choline; GPE, glycerophospho-ethanolamine; PC(O), Alkyl-phosphatidylcholine; PE(O), Alkyl-phosphatidylethanolamine; PC(P), PC plasmalogen, PE(P), PE Plasmalogen; LPC(P), Lyso-PC Plasmalogen; LPE(P), Lyso_PE Plasmalogen. Enzymes are shown in orange squares: DHAPAT, DHAP acyltransferase; ADHAP-S, alkyl- dihydroxyacetone phosphate synthase; AADHAPR, acyl/alkyl dihydroxyacetone phosphate reductase; AAG3PAT, acyl/alkyl-glycero-3- phosphate acyltransferase; PH, phosphohydrolase; AG kinase, alkylglycerol kinase; $\delta\delta$1 Desaturase, plasmanyl-ethanolamine delta1-desaturase; C-PT, choline phosphotransferase; E-PT, ethanolamine phosphotransferase; PEMT, phosphatidylethanolamine N-methyltransferase; PLC, phospholipase C; CoA-IT, coenzyme A-independent transacylase; i-phospholipase A2, calcium independent phospholipase A2; TMEM86B, lysoplasmalogenase; FAR1, fatty acyl-CoA reductase 1; FAR2, fatty acyl-CoA reductase 2.

of dihydroxyacetone phosphate (DHAP) (Nagan and Zoeller, 2001). After the replacement of the acyl-chain for an alkyl-chain at the *sn*-1 position, 1-alkyl DHAP crosses to the cytosolic side of the ER where it enters the biosynthetic pathway of diacyl-phospholipids (Lee, 1998). Plasmalogens are the major end product of the biosynthetic pathway, however, platelet-activating factor (1-alkyl-2-acetyl glycerophosphoryl-choline, PAF), and the lipid moiety of distinct glycosyl-phosphatidylinositol anchored

proteins are also synthesised. Paul *et al.* have reviewed the ether lipid biosynthetic pathway in more detail (Paul et al., 2019).

Ether Lipids in Obesity

Ether lipids have been implicated in neurodegenerative disorders, cardiovascular disease (CVD), metabolic disease and some genetic disorders (Gould and Valle, 2000; Goodenowe et al., 2007; Pietiläinen et al., 2007; Graessler et al., 2009; Meikle et al., 2011). This review will focus on obesity, as it is now considered a major health burden and contributes to a range of pathologies, including CVD, insulin resistance, type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). Indeed, lipidomic studies of large human cohorts have identified decreased levels of circulating ether lipids to be associated with numerous features of metabolic diseases (Pietiläinen et al., 2007; Graessler et al., 2009; Meikle et al., 2011, 2013; Weir et al., 2013; Beyene et al., 2020).

An early lipidomic study, analysing plasma samples from monozygotic twins discordant for obesity, demonstrated that obesity was associated with increased levels of lyso-phospholipid species, which possess some pro-inflammatory effects, and a concurrent decrease in ether lipids, independent of genetic factors (Pietiläinen et al., 2007). Another study, analysing the plasma lipidome of over 1,000 individuals from Mexican-American families, observed similar results. Several ether lipid species were negatively associated with body mass index, independent of age, sex, systolic blood pressure, 2 h post-load glucose plasma levels and smoking status (Kulkarni et al., 2013). Reduced ether lipids have also been implicated in hypertension, NAFLD, pre-diabetes, T2D and ageing (Puri et al., 2009; Meikle et al., 2013; Weir et al., 2013; Paul et al., 2019).

Peroxisomes are membrane bound organelles that perform multiple functions, including ether lipid synthesis, reactive oxygen species (ROS) metabolism, fatty acid oxidation and cholesterol transport (Cipolla and Irfan, 2017). Deficiencies in the peroxisomal membrane protein Pex11a, involved in peroxisome maintenance and proliferation, reduced plasmalogen levels and caused dyslipidemia and obesity in mice (Chen et al., 2018). Additional preclinical studies have made similar observations, linking peroxisomal dysfunction, characterised by reduced levels of plasmalogens, with various metabolic pathologies including dyslipidemia, obesity, NAFLD and T2D (Cipolla and Irfan, 2017; Park et al., 2019b). Whilst the mechanisms underlying these associations remain unclear, it has been postulated that a reduction in ether lipids contributes to the disease pathology through multiple pathways, including the disruption of cellular membranes, increased oxidative stress, ER stress and inflammation. These mechanisms have been reviewed previously, and whilst they offer some insights, it is becoming increasingly apparent that the physiological roles of ether lipids are likely to be specific to a given tissue. Accordingly, investigators have begun to explore the composition of lipids in adipose tissues and subsequently uncovered novel roles for plasmalogens in the regulation of adiposity.

IN VITRO AND *IN VIVO* STUDIES

Lipidomic Profiling of Adipose Tissue

Adipose tissue plays a central role in regulating energy metabolism and homeostasis. There are three distinct types of adipose tissue; white (WAT), brown (BAT) and beige adipose tissue. WAT is comprised of uniocular lipid droplets and is involved in storing excess energy in the form of neutral lipids, such as triacylglycerols (TG), that can be remobilised in times of energy deficiency (Leiria and Tseng, 2020). Conversely, BAT has multilocular lipid droplets and is highly metabolically active, driving non-shivering thermogenesis through the oxidation of fatty acids to generate heat (Park et al., 2019a). Beige adipocytes occur as clusters within WAT depots and are inducible, often in response to prolonged cold exposure (Leiria and Tseng, 2020). As a result, beige adipocytes are able to develop a BAT-like phenotype, giving rise to their mixed BAT and WAT functions as well as multilocular/unilocular morphology. Importantly, brown and beige adipocytes are enriched in mitochondria and express uncoupling protein 1 (UCP1), a mitochondrial membrane protein that dissociates oxidative phosphorylation from ATP production (Fedorenko et al., 2012). Lipidomic profiling of the different adipose tissues and depots has provided enormous insight into their unique composition and function.

Hoene et al. (2014) comprehensively examined the lipidome of BAT and subcutaneous WAT (SAT) in male and female mice. They demonstrated a pronounced difference in the lipid profiles of the adipose tissues, as well as a distinct sex-dependent difference in BAT. More specifically, they observed that phospholipid classes, including phosphatidylethanolamine (PE) and phosphatidylcholine (PC) ether lipids, were elevated in BAT compared to SAT (Hoene et al., 2014). Conversely, TG and diacylglycerol (DG) species were higher in SAT compared to BAT (Hoene et al., 2014). These differences likely reflect the metabolic activity and function of each tissue, as phospholipids regulate UCP1 within mitochondrial inner membranes of thermogenic BAT cells, whilst TG species are typically stored in WAT (Leiria and Tseng, 2020). Despite the lower levels of ether lipids in SAT compared to BAT, lipidomic analysis of human WAT reported that plasmalogens are still highly abundant relative to other lipid species (Lange et al., 2021). Findings showed that PE plasmalogens were the most abundant species containing PUFA as well as the fourth most abundant lipid class overall. Interestingly, PE plasmalogen levels were around 3 times higher than PE-diacyl species (Lange et al., 2021). Interestingly, another study that conducted lipidomic analysis on primary brown, white and beige adipocytes *in vitro* revealed contrary results (Schweizer et al., 2019). White adipocytes exhibited higher amounts of PE and PC ether lipids as well as lipid species containing long-chain PUFA compared to the beige and brown adipocytes. The authors suggested that this increase may reflect a protective adaptation of white adipocytes to mitigate the elevated production of ROS in the obese mice. In addition, the same group reported that brown adipocytes had a significant increase in cardiolipins (CL) compared to white and beige adipocytes (Schweizer et al., 2019). As CL are major constituents of

mitochondrial membranes, these findings likely reflect the increased abundance of mitochondria within brown adipocytes compared to white adipocytes. The lipidomic profiles of brown and beige adipocytes were considered comparable (Schweizer et al., 2019).

Whilst our understanding of the adipose lipidome remains limited, particularly in relation to ether lipids, research has begun to explore the apparent remodelling of adipose tissue in the setting of obesity. Multiple studies have now comprehensively analysed the lipidomic signatures of adipose tissue from obese and lean individuals (Pietiläinen et al., 2011; Lange et al., 2021). One study conducted by Pietiläinen et al. (2011) revealed unique changes in the composition of ether lipids in twin pairs discordant for obesity (Pietiläinen et al., 2011). Their results demonstrated that the adipose tissue of the obese twins presented with increased levels of PUFA-containing ether lipids, and a proportional decrease in phospholipids containing shorter and more saturated fatty acids, compared to that of the lean twins (Pietiläinen et al., 2011). As the ether lipid and diacyl-phospholipid biosynthetic pathways are linked, these findings suggest that there is a preferential flux through the ether lipid pathway, and a concurrent decrease through the diacyl-phospholipid pathway. This shift between pathways may be linked to the important role of plasmalogens in facilitating membrane remodelling of enlarged adipocytes during obesity (Pietiläinen et al., 2011; Lange et al., 2021).

Lipidomic analyses of SAT and visceral adipose tissue (VAT) from lean and obese individuals offered greater insights into the composition of adipose tissue ether lipids in obese individuals (Lange et al., 2021). Specifically, the study revealed that higher amounts of PC plasmalogens with long-chain PUFA were characteristic of obese SAT depots, whilst PE plasmalogen species accumulated in obese VAT depots. Similar findings were also observed in women with insulin resistance, which is a hallmark of obesity (Wentworth et al., 2016). Wentworth et al. (2016) reported that PC ether lipids were more abundant in the SAT depots of women with insulin resistance compared to the VAT depots. Together, these findings demonstrate that the differences in lipid composition appear to be highly depot specific. SAT is considered a more metabolically healthy adipose tissue, in part due to its ability to undergo browning, and therefore has the potential to develop a BAT-like phenotype (Chechi et al., 2018). Conversely, VAT is known to be a major risk factor for cardiometabolic disease and has been linked to hyperglycaemia, hyperinsulinemia, hypertriglyceridaemia and impaired glucose tolerance (Ibrahim, 2010). Interestingly, PE and PC phospholipids have specific and opposing effects on membrane stability, as PE lipids promote membrane rigidity whilst PC lipids maintain membrane fluidity (Harayama and Riezman, 2018). It has been suggested that the expansion of adipose tissue associated with obesity triggers remodelling of membrane phospholipids in an effort to maintain membrane composition and function (Lange et al., 2021). However, the specific role of PE and PC plasmalogen species in this context remains elusive. Furthermore, it is unclear whether these findings reflect increased endogenous synthesis of ether lipids in WAT or uptake of ether lipids from the circulation.

Following on from their early work, Pietiläinen et al. (2011) performed lipidomic analysis on the SAT of healthy and morbidly obese weight-discordant twins (Pietiläinen et al., 2011). Surprisingly, they demonstrated that unlike in the obese twins, remodelling of PUFA-plasmalogens was considerably reduced in the morbidly obese twins. These findings suggest that the protective adaptations induced by the onset of obesity are lost as adiposity increases. One plausible explanation involves the enzyme calcium independent phospholipase A2 (iPLA2), which hydrolyses plasmalogens into lyso-plasmalogens (Paul et al., 2019). Importantly, iPLA2 is known to be elevated in the setting of obesity and has been shown to contribute to diet-induced weight gain, adipocyte hypertrophy and insulin resistance, via changes in fatty acid oxidation and mitochondrial content *in vivo* (Garces et al., 2010; Mancuso et al., 2010). Based on the current literature, it is likely that iPLA2 levels increase during obesity which contributes to the reduction in plasmalogen levels. This would result in a subsequent decrease in mitochondrial content and function, as well as exacerbate diet-induced obesity. Furthermore, deacylation of plasmalogens results in the release of PUFAs at the *sn*-2 position. PUFAs are highly susceptible to lipid peroxidation, resulting in the formation of toxic lipid peroxyl radicals and hydroperoxides (Antonio et al., 2014). One example of this is oxidation of AA. AA can be oxidised into precursors of multiple eicosanoids, including thromboxane, prostaglandins, and leukotrienes, which have potent inflammatory functions (Farooqui et al., 1995; Marzo, 1995). Elevated levels of these lipid mediators could contribute to the systemic inflammation associated with morbid obesity. Indeed, this rapid break down of plasmalogen species via iPLA2 would likely exceed the rate of endogenous plasmalogen synthesis, resulting in the overall decrease of PUFA-containing plasmalogens that was observed. It is important to note that it remains unclear whether iPLA2 is plasmalogen specific, or it hydrolyses other structurally similar lipids concurrently. Furthermore, due to the complex nature of lipid metabolism, the regulatory mechanisms responsible for this obesity-induced lipid dysregulation are likely to involve multiple intrinsic and overlapping pathways.

Functional Roles of Ether Lipids in Adipose Tissue

Studies by Brites et al. (2011) and Park et al. (2019b), have been critical in developing our understanding of the functional roles of ether lipids, particularly plasmalogens, within adipocytes. Using *in vivo* and *in vitro* models of impaired peroxisomal function to induce plasmalogen deficiency, Brites et al. (2011) revealed a crucial role for plasmalogens in facilitating lipid droplet formation. Mice deficient in plasmalogens, via knockout of the peroxisomal factor 7 (Pex7), presented with extremely reduced epididymal, inguinal, retroperitoneal and subscapular WAT depots, whilst the brown adipocytes had abnormally small lipid droplets (Brites et al., 2011). Interestingly, dietary supplementation with the plasmalogen precursor alkylglycerols, rescued plasmalogen levels and normalised the size and number of lipid droplets in both the BAT and WAT of the plasmalogen deficient mice (Brites et al., 2011). These results suggest a role

for plasmalogens in lipid droplet formation and maintenance. Consistent with this, additional studies demonstrated that plasmalogen-deficient mouse embryonic fibroblasts (MEFs) had fewer and smaller lipid droplets when compared to control MEFs (Brites et al., 2011). Similarly, treatment with alkylglycerols restored the number and volume of lipid droplets, further supporting a role for plasmalogens in the regulation of lipid droplet homeostasis.

Studies have demonstrated that lipid droplets also mediate ER stress (Hapala et al., 2012). ER stress is a common feature of obesity that results in the disruption of protein folding and synthesis (Basseri and Richard, 2011). Researchers have shown that an increase in lipid droplet biogenesis, often during obesity, induces ER stress. The primary mechanisms thought to drive this association was a combination of reduced phospholipid synthesis and an up-regulation of TG synthesis (Basseri and Richard, 2011). As plasmalogens have been implicated in lipid droplet formation, it is likely that the known reduction of plasmalogens caused by obesity in directly contributes to the progression of ER stress. This is supported by recent work by Ogawa and colleagues who discuss a potential link between elevated ER stress, mitochondrial dysfunction and inflammation with reduced levels of PE plasmalogens in patients with bipolar disorder (Ogawa et al., 2020). Additionally, an *in vivo* study utilising peroxisome-deficient *Pex2* knockout mice, demonstrated that functional peroxisomes are critical for the prevention of chronic ER stress (Kovacs et al., 2009). Whilst the study did not link peroxisomal function with plasmalogen levels directly, combining these results with their known role as potent anti-oxidants suggests a protective role of plasmalogens against oxidative stressors. Further exploration into the specific role of ether lipids in the setting of ER stress is required.

Recently, peroxisomal lipid metabolism, and subsequent ether lipid synthesis, has been shown to facilitate thermogenesis via the regulation of mitochondrial dynamics. Inhibition of peroxisome biogenesis via the WAT-specific deletion of the peroxisomal biogenesis factor Pex16 *in vivo*, decreased mitochondrial DNA content and impaired mitochondrial function in brown and beige adipocytes (Park et al., 2019b). As a result, the knockout mice presented with severe cold intolerance and reduced thermogenesis. Furthermore, when placed on a high fat diet, the knockout mice had significantly increased fat mass and body weight compared to control mice on the same diet, demonstrating diet-induced obesity. Subsequent dietary supplementation of alkylglycerols was able to restore plasmalogen levels, mitochondrial morphology and cold sensitivity in these mice (Park et al., 2019b). Together, these findings suggest that peroxisomal synthesis of ether lipids is important for regulating mitochondrial dynamics and thermogenesis. Using an alternative model of plasmalogen deficiency, via knockdown of the endogenous ether lipid synthesis enzyme glyceronephosphate O-acyltransferase (GNPAT), researchers observed similar impairments in mitochondrial fission and oxygen consumption in BAT stromal vascular fraction (SVF) *in vitro* (Park et al., 2019b). These findings are particularly intriguing as attenuation of *Gnpat* inhibits ether lipid synthesis but does not impede peroxisomal function. This suggests that the observed mitochondrial dysfunction may occur in response to reduced ether lipids, rather than peroxisomal dysfunction *per se*.

The underlying mechanisms by which ether lipids regulate thermogenesis are starting to become more clear, as a study has now demonstrated that mitochondrial membrane lipids, including plasmalogens, mediate thermogenesis through crosstalk between organelles, including the nucleus and peroxisomes (Jiménez-Rojo and Riezman, 2019). Park et al. (2019b) demonstrated that thermogenic stimuli increased peroxisome proliferation via activation of PRDM16 (PR domain containing 16). This subsequently increased plasmalogen content within the mitochondria, which promoted mitochondrial fission and potentiated free fatty acid (FFA)-induced uncoupling and energy expenditure in BAT (Park et al., 2019a). The known role of the vinyl-ether linkage to foster non-lamellar lipid structures and regulate membrane dynamics supports this role for plasmalogens. However, the extent to which plasmalogens mediate mitochondrial fission and morphology remains unclear. It has been speculated that it may involve effects on mitochondrial localisation and/or activity of fission factors (Park et al., 2019a).

PUFAs have been shown to promote thermogenesis via cell signalling (Sadurskis et al., 1995; Fan et al., 2019). As plasmalogens are rich in PUFAs, this may be an additional mechanism by which they promote thermogenesis in BAT. An *in vivo* study exploring the effect of PUFAs on non-shivering thermogenesis demonstrated that mice on a high PUFA diet exhibited an improved thermogenic capacity of BAT compared to mice fed a diet with standard fat content (Sadurskis et al., 1995). Interestingly, these effects appear to be specific to omega-3 PUFA, such as eicosapentaenoic acid (EPA) and DHA. EPA has been shown to promote BAT differentiation, increase UCP1 gene expression and decrease adiposity in mice (Ghandour et al., 2018). Surprisingly, omega-6 PUFA, such as AA, inhibit the conversion of white to beige adipocytes and favour obesity (Pisani et al., 2017). As plasmalogens carry both omega-3 and omega-6 PUFA at the *sn*-2 position, understanding their composition in BAT and WAT may offer further insights into their functional roles in these tissues.

Ether Lipids in Infant Adipose Tissue

A recent study has identified a unique role of ether lipids in breast milk in the prevention of obesity in early life (Yu et al., 2019). Breast milk is a rich source of lipids and is essential for adipose tissue physiology (Rosen and Spiegelman, 2014; Koletzko, 2016). TG(O)'s are the primary ether lipid in breast milk and have been shown to not only facilitate the development of beige adipocytes in neonatal mice, but also impede white adipocyte accumulation (Yu et al., 2019). In this study, investigators increased the alkylglycerol intake of neonatal mice by 20% and observed an increase in mitochondrial content, UCP1 transcription and beiging area of the SAT of the treated mice. Conversely, adipocyte size and TG content was markedly reduced in the treated mice when compared to the control mice that did not receive alkylglycerol supplementation. This effect of alkylglycerols on

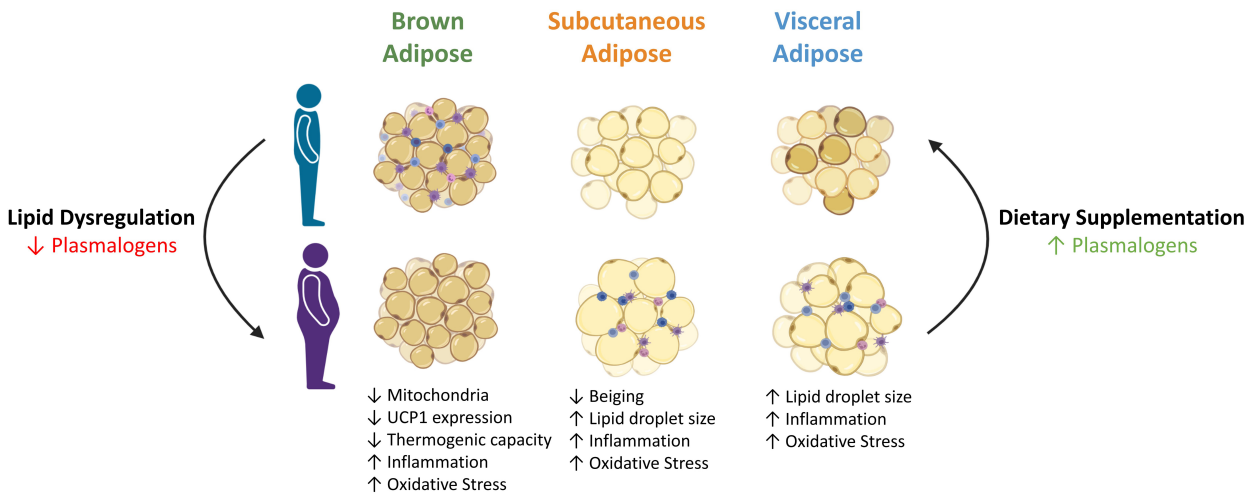

FIGURE 3 | Possible role of plasmalogens in adipose tissue metabolism and function in the setting of obesity. Obesity is characterised by enlarged adipocytes and severe lipid dysregulation, including reduced plasmalogens. Decreased plasmalogen levels impedes the normal function and size of adipocytes, further exacerbating the metabolic complications associated with obesity. Conversely, increasing plasmalogen levels via dietary supplementation appears to revert the effects of obesity on adipocytes by restoring their size and function. UCP1, uncoupling protein 1. Created with Biorender.com (2021).

infant adipocytes was proposed to occur via adipose tissue macrophage signalling. Adipose tissue macrophages (ATMs) are important for lipid and energy metabolism as well as mitochondrial function in adipocytes (Li et al., 2020). This study demonstrated that the alkylglycerols were metabolised by ATMs, triggering an increase in PAF levels and the subsequent release of IL-6 (Yu et al., 2019). IL-6 is an interleukin that activates the transcription of adipocyte STAT3, which facilitates beige adipocyte development (Yu et al., 2019). Interestingly, a lack of alkylglycerol intake during infancy led to a premature loss of beige adipocytes and an increase in fat accumulation (Yu et al., 2019). Whilst further exploration is required, this novel study highlights the importance of breast milk alkylglycerols in promoting healthy adipose tissue development in early life.

Later in life, ATMs perform contrasting roles, as they maintain metabolic homeostasis, but also contribute to the aetiology of obesity through non-resolving inflammation (Morgan et al., 2021). Progressive lipid accumulation within macrophages drives a switch between the polarization of anti-inflammatory M2 ATMs to pro-inflammatory M1 ATMs (Prieur et al., 2011). In the setting of obesity, M2 ATMs are critical for the removal of necrotic-like adipocytes, as well as facilitating lipid storage in WAT (Cinti et al., 2005; Cox et al., 2021). A recent study revealed that treatment of M1 and M2 bone marrow-derived macrophages (BMDM) with exogenous fatty acids caused an increase in TG and cholesterol ester (CE) species in M1 macrophages, whilst a greater increase in PE and PC ether lipids was observed in M2 macrophages (Morgan et al., 2021). Mechanistically, M2 macrophages express higher levels of *Gnpat* and *Far1*, the enzymes involved in the first and rate limiting steps of endogenous ether lipid synthesis, respectively (Jha et al., 2015). Together, these findings suggest that ether lipids may play an important role in promoting the anti-inflammatory phenotype of M2 macrophages. Furthermore, the anti-oxidant properties of the vinyl-ether linkage may be a key mechanism by which plasmalogens reduce inflammation and oxidative stress, thereby negating some of the effects of obesity. Based on these findings it is evident that promoting M2-like ATMs may be important for reducing the influx of obesity-associated inflammatory cytokines and mediators, driven by M1-like ATMs (Zeyda et al., 2007).

THERAPEUTIC POTENTIAL OF ETHER LIPIDS FOR ATTENUATING OBESITY

As the protective effects of ether lipids have become more clear, considerable work has begun to explore the potential therapeutic effects of modulating ether lipid levels to attenuate obesity and its subsequent complications. There are two major approaches for modulating endogenous ether lipid levels: 1) genetic modulation of enzymes involved in the ether lipid metabolism and 2) supplementation with their metabolic precursors, such as alkylglycerols (1-O-alkylglycerol or 1-O-alkyl-2,3- diacylglycerol) (**Figure 2**).

Utilising a *Gnpat* knockout mouse model to induce plasmalogen deficiency, Jang et al. (2017) demonstrated that upon feeding of a high fat diet, mice were more susceptible to hepatic lipid accumulation, adipose tissue inflammation and high fat diet-induced insulin resistance compared to wild type mice (Jang et al., 2017). In contrast, alkylglycerol supplementation has been shown to increase plasmalogen levels in cells, animals and humans, resulting in the suppression of some features of metabolic diseases (Das et al., 1992; Brites et al., 2011). A pivotal study using alkylglycerol supplementation in a mouse model of diet-induced obesity and insulin resistance demonstrated that 8 weeks of alkylglycerol treatment decreased body weight, serum TG, cholesterol and fasting insulin levels (Zhang et al., 2013). It is possible that the reduction in body

weight may be linked to increased lipolysis of WAT in an attempt to release more FFA to facilitate thermogenesis. An *in vitro* study has also shown that alkylglycerols are effective at reducing oxidative stress, a hallmark of obesity and subsequent metabolic diseases (Zoeller et al., 2002). Cultured human pulmonary arterial endothelial cells (PAEC) were supplemented with alkylglycerols for 6 days and exhibited a two-fold increase in plasmalogen levels. Importantly, the PAEC were protected against hypoxia and other stressors linked to reactive oxygen species (Zoeller et al., 2002).

Shark liver oil is a natural product rich in alkylglycerols. A recent preliminary clinical study assessed the ability of shark liver oil supplementation to modulate plasma and immune cell plasmalogen levels in overweight or obese men (Paul et al., 2021b). The study reported significant changes in the levels of multiple ether lipid species in plasma and circulating white blood cells, including 59% and 15% increases in PE plasmalogens in the plasma and white blood cells, respectively (relative to total PC levels). Furthermore, total cholesterol, TG levels and the inflammatory marker C-reactive protein all decreased (Paul et al., 2021b). These results support the concept that shark liver oil enriches plasma and cellular plasmalogens to provide protection against obesity-related dyslipidaemia and inflammation.

A recent study examined the impact of supplementation of an alkylglycerol mix on the plasma and various tissues, including the liver, VAT and skeletal muscle of mice *in vivo* (Paul et al., 2021a). After 1 to 12 weeks of the mixed alkylglycerol treatment, PE and PC ether lipids, including plasmalogens, progressively increased in the VAT. These results demonstrate the ability of dietary alkylglycerols to penetrate the adipose tissue and successfully incorporate into the ether lipid biosynthetic pathway (Paul et al., 2021a). As discussed previously, dietary supplementation with alkylglycerols rescued adipocyte morphology and reduced diet-induced obesity in mice with plasmalogen deficiencies (**Figure 3**) (Brites et al., 2011). Whilst obesity has been the focal point for this review, it is important to note that alkylglycerols have also proven effective in the treatment of NAFLD, genetic peroxisomal disorders and CVD (Das et al., 1992; Wood et al., 2011; Rasmiena et al., 2015; Parri et al., 2016; Jang et al., 2017). Furthermore, natural plasmalogens such as scallop-purified and chick-skin PE plasmalogens have been successful at increasing plasmalogen levels in human and animal studies (Maki et al., 2009; Tandy et al., 2009; Mawatari et al., 2012, 2020).

In summary, ether lipids are important biological molecules with functional roles within adipose tissue. Numerous studies now demonstrate a clear relationship between circulating ether lipids and obesity, as reduced plasmalogen levels are apparent in obese individuals and facilitate diet-induced obesity *in vivo*. Combining these studies further suggests that the increased oxidative stress and inflammation associated with obesity promotes the dysregulation of ether lipids in adipose tissue. As lipid metabolism is highly complex, it remains unclear whether the reduced ether lipids drive obesity, or obesity drives the observed decrease in ether lipids. Importantly, this work highlights potential functional roles for ether lipids in the protection against diet-induced obesity. Thus, increasing plasmalogen levels may be beneficial in the attenuation of obesity and its complications via the promotion of thermogenesis, antioxidant effects and cellular signalling to reduce inflammation.

AUTHOR CONTRIBUTIONS

YLS: writing—original draft preparation. YLS, SP, ACC, and PJM: writing—manuscript revision. SP, ACC, and PJM: supervision. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: PJM is inventor on a patent; WO 2021/007623 A1; Title: Compositions for maintaining or modulating mixtures of ether lipid molecules in a tissue of a human subject.

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