THE ROLE OF THE MUSCLE SECRETOME IN HEALTH AND DISEASE

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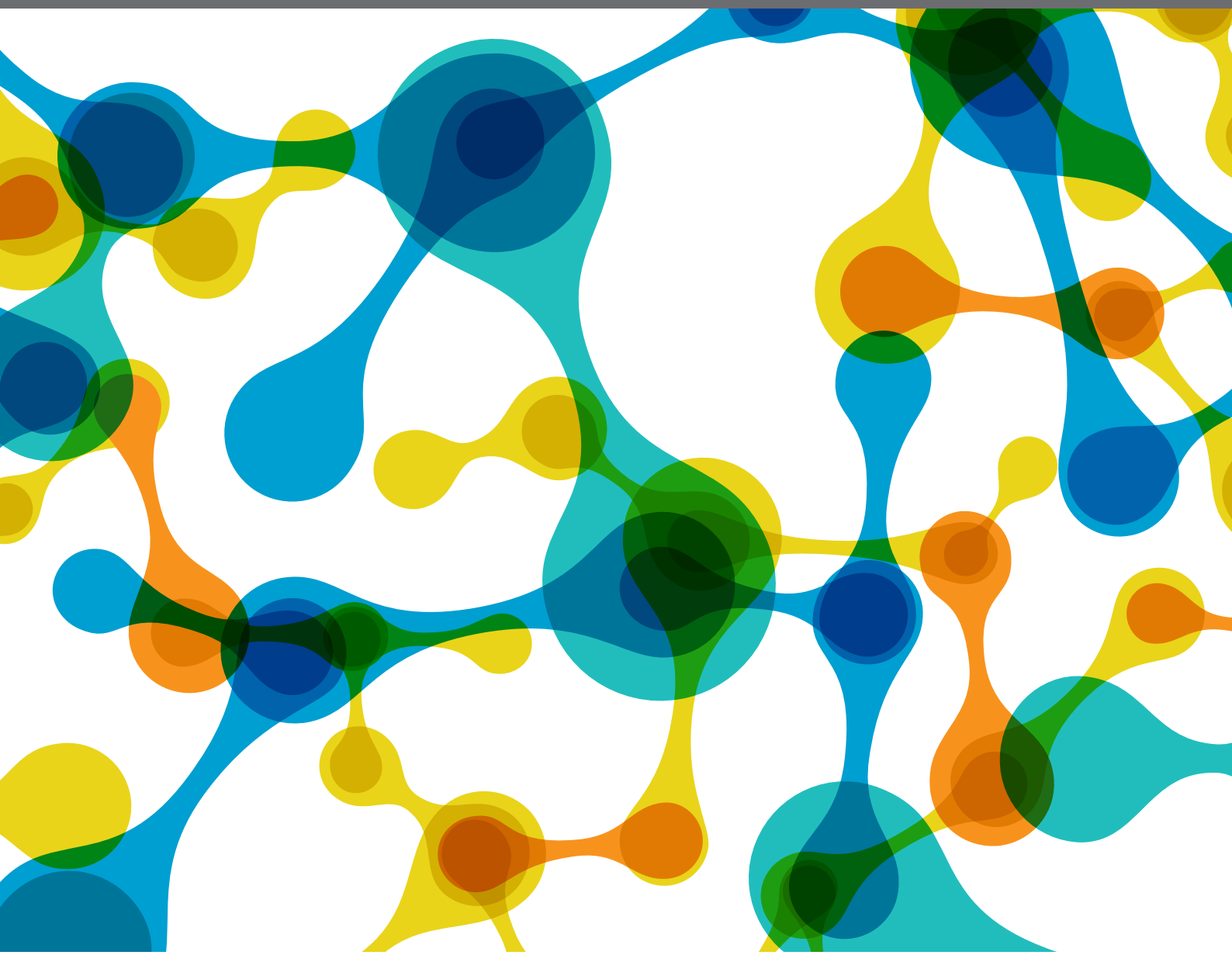

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THE ROLE OF THE MUSCLE SECRETOME IN HEALTH AND DISEASE

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EDITORIAL
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Editorial: The Role of the Muscle Secretome in Health and Disease

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Editorial on the Research Topic

The Role of the Muscle Secretome in Health and Disease

The study of the mechanisms by which regular exercise improves overall health and influences the activity of tissues, organs, and systems far from the contracting muscle is an extremely attractive field of research. Historically, the first report demonstrating increased circulating cytokines in response to an acute bout of exercise was published in 1983 (Cannon and Kluger, 1983), and the first description of a molecule secreted by skeletal muscle, myostatin, dates from 1997 (McPherron et al., 1997). It was only in 2003 that Pedersen et al. introduced the term "myokines" (Pedersen et al., 2003). Since then, hundreds of papers describing new myokines and several other putative "muscle-derived factors," "muscular cytokines," "exercise factors," or "muscular growth factors" have been published in a rather short period of time. This rapid accumulation of data has prompted extensive investigations of the real role played by the secretory activity of the muscle, such as its role in the adaptation to regular exercise and in the reduction of the risk of mortality and morbidity in trained subjects.

Given the rapid evolution of this research field, it is important to agree on a clear definition of a myokine:

1) A myokine is a peptide or a protein. Molecules other than peptides and proteins secreted by skeletal muscle cells shouldn't be called myokines (i.e., metabolites and miRNAs are not myokines).

2) A myokine is secreted by skeletal muscle cells, either locally (in skeletal muscle interstitium) or in the blood circulation. Therefore, increased circulating levels of a peptide upon muscle activity is not a condition sufficient to establish whether the molecule behaves as a myokine. Indeed, peptides can also be secreted by other organs/tissues. In addition, some myokines can also be released to act locally in the muscle interstitium without entering systemic circulation.

3) A myokine can be secreted independently of muscle contraction. Myokine levels can be positively (e.g., irisin), negatively (e.g., myostatin) or not regulated by muscle contraction.

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Myokines are not the only factors secreted by skeletal muscle during contraction. Since 2010, evidence for exercise-induced skeletal muscle secretion of miRNA and more recently, mitochondrial DNA, is accumulating. A number of metabolites and enzymes are also secreted by contracting skeletal muscles. In the past 5 years, the role of exosomes and extracellular vesicles (EVs) in the transport of some of these factors has also been highlighted.

Many points still need to be clarified to better understand the role played by the muscle secretome. Indeed, many of the factors identified so far in isolated muscle models or in animal models have not yet been replicated in human experiments. In addition, the circulating concentration of these factors measured during and after exercise is extremely variable and their biological role (e.g., irisin) is still topic of heated discussion. We therefore proposed a Frontiers Research Topic to provide a comprehensive update on the current research on the impact of muscle secretome on human health. We have contacted 65 top level authors worldwide, publishing 12 articles that we now bring to your attention.

Ten articles focus on "myokines" or circulating peptides known to be altered in response to exercise and the first five address different aspects of their metabolic roles during exercise. Garneau et al., evaluated the effect of an acute bout of moderate-intensity, continuous cycling exercise on the circulating levels of IL-6, IL-8, IL-10, IL-13, IL-15, secreted protein acidic rich in cysteine (SPARC) and fibroblast growth factor (FGF) 21 in plasma over 24 h. The authors found that the plasma concentration of some of these factors was differentially regulated in non-obese compared to obese women, suggesting that obesity might condition the muscular release of these factors. The mini-review by Laurens et al. discusses, as an integrated perspective, the role of myokines in the regulation of energy metabolism in skeletal muscle itself, white and brown adipose tissues, pancreas, liver, and brain. They also discuss the potential role of impaired myokine secretion in metabolic defects that develop with sedentarity/physical inactivity such as in patients with type 2 diabetes. Ryan et al. obtained primary muscle cells from healthy subjects and patients with type 2 diabetes. These myotubes, set in an experimental model mimicking the inflammatory and metabolic conditions seen *in vivo* in type 2 diabetes, release unknown factors capable of suppressing glucose-induced insulin secretion. This experimental evidence, added to the others presented in the mini-review by Mizgier et al., supports the existence of a skeletal muscle-pancreas crosstalk. Furthermore, it opens up new working hypotheses regarding the possibility that muscle-derived factors could affect insulin secretion both in healthy subjects and in patients with type 2 diabetes. Ma et al. investigated the expression and secretion pattern of the myokine irisin during adipocyte differentiation and the role of endogenous and exogenous irisin on the adipogenic process. Planella-Farrugia et al. designed a clinical trial in elderly subjects evaluated before and after 16 weeks of resistance training. Interestingly, they found that circulating irisin, but not myostatin, constitutes a marker for improved muscular performance in elderly subjects. These data would seem to broaden biological actions of irisin.

Two articles expand on the current view of the muscular proteome. Kurgan et al. assessed the influence of high-intensity interval exercise on serum proteome of healthy males. After maximal exercise, several novel exercise-regulated proteoforms with a broad range of effects were identified. Kluess, with her mini review, introduces Dipeptidyl peptidase IV (DPP-IV) as a novel myokine that participates in the control of muscle blood flow.

The last two articles focusing on myokines address two unique aspects: the first, by Hutchinson et al., examines the profile of exercise-induced peptides in pregnant and non-pregnant women after an acute bout of moderate-intensity exercise. For the first time, the authors observed that circulating levels of FGF21, EPO, and IL-15 significantly increased in response to the acute exercise in the pregnant group only, opening the debate on myokines and pregnancy. In the second of two, Sanchis Gomar et al. review the role of the neuromuscular electrical stimulation as an emerging effective physical exercise substitute for myokine induction.

Finally, two papers focused on extracellular vesicles released by skeletal muscle. Rome et al. extensively review the role of skeletal muscle EVs in muscle physiology and in the development of metabolic diseases whereas Bittel and Jaiswal focused on EVs released by myofibers and other cells in the injured muscle, having a role in specific reparative and regenerative processes.

Due to the increasing amount of newly identified molecular species secreted by muscle (e.g., nucleic acids vs. peptides vs. vesicles), the concept of muscle secretome is constantly evolving from initially being almost a synonym of myokine to encompass a broader catalog of molecules. By proposing this Research Topic, our initial goal was to attract manuscripts focusing on the role of the whole muscle secretome in the physiological and metabolic adaptations to exercise, with a focus on the synergistic/additive effects of different muscle-derived factors. Ultimately, despite the significant body of work collected, we only managed to scratch the surface of this rapidly evolving and interesting field. Future studies will bring us closer to unraveling the function of the whole-muscle secretome in health and diseases.

AUTHOR CONTRIBUTIONS

CA, EL, and DDR conceived, wrote, and approved the editorial for publication. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Changes to the Human Serum Proteome in Response to High Intensity Interval Exercise: A Sequential Top-Down Proteomic Analysis

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Exercise has been shown to improve health status and prevent chronic diseases. In contrast, overtraining can lead to maladaptation and detrimental health outcomes. These outcomes appear to be mediated in part by released peptides and, potentially, alterations in protein abundances and their modified forms, termed proteoforms. Proteoform biomarkers that either predict the beneficial effects of exercise or indicate (mal)adaptation are yet to be elucidated. Thus, we assessed the influence of high-intensity interval exercise (HIIE) on the human serum proteome to identify novel exercise-regulated proteoforms. To this end, a top-down proteomics approach was used, whereby two-dimensional gel electrophoresis was used to resolve and differentially profile intact proteoforms, followed by protein identification via liquid chromatography-tandem mass spectrometry. Blood was collected from six young-adult healthy males, pre-exercise and 5 min and 1 h post-exercise. Exercise consisted of a maximal cycle ergometer test followed by 8 min × 1 min high-intensity intervals at 90% W_{max}, with 1 min non-active recovery between intervals. Twenty resolved serum proteoforms changed significantly in abundance at 5 min and/or 1 h post-HIIE, including apolipoproteins, serpins (protease inhibitors), and immune system proteins, known to have broad anti-inflammatory and antioxidant effects, involvement in lipid clearance, and cardio-/neuro-protective effects. This initial screening for potential biomarkers indicates that a top-down analytical proteomic approach may prove useful in further characterizing the response to exercise and in understanding the molecular mechanisms that lead to health benefits, as well as identifying novel biomarkers for exercise (mal)adaptation.

Keywords: exerkines, inflammation, exercise intensity, HIIE, biomarkers, proteoforms, top-down proteomics, two-dimensional gel electrophoresis

INTRODUCTION

It is well-documented that regular exercise is associated with numerous health benefits (Smorawinski et al., 2000; Warburton et al., 2006). Mechanisms that lead to the numerous health benefits associated with exercise training may be stimulated through multiple bouts of acute (single-session) exercise, i.e., high-intensity interval exercise (HIIE), that occurs over a time-period of training (Hojman et al., 2018). Specifically, it is hypothesized that HIIE elicits alterations to protein function or content in blood, contributing to the numerous multi-system health benefits observed with continued training. However, excessive training can also lead to an apparent catabolic and systemic inflammatory state (i.e., mal-adaptation) (Kurgan et al., 2018).

In order to better elucidate mechanisms underlying beneficial and/or detrimental effects of exercise, alterations to the skeletal muscle proteome (Petriz et al., 2017) [which may influence/be influenced by the plasma/serum proteome(s)], plasma peptidome (Parker et al., 2017; Santos-Parker et al., 2018), serum metabolome (Nieman et al., 2013; Pechlivanis et al., 2013), and plasma extracellular vesicle content (Whitham et al., 2018) have been investigated. Recently, a cross-sectional assessment of the effects of high and low levels of physical activity on the plasma proteome utilizing an aptamer-based SOMAscan proteomic assay has also been reported (Santos-Parker et al., 2018). Collectively, these have found large perturbations in bioactive peptides, metabolic pathways (e.g., glycolysis), cell cycle regulatory proteins, and proteins related to immunity. However, untargeted and comprehensive assessments of plasma and/or serum proteomes in response to HIIE – inclusive of gene, splice, and post-translationally modified variants (i.e., proteoforms), which are influenced by metabolic needs and health/disease state (Jungblut et al., 2008; Thiede et al., 2013; Coorssen and Yergey, 2015) – are yet to be carried out.

Exercise-regulated changes in the abundance of targeted proteins within serum have been found to profoundly effect cell proliferation and differentiation *in vitro* (e.g., anti-cancer effects) (Hojman et al., 2018). Increased serum collagen leading to increased tensile strength in engineered ligaments has also been observed (West et al., 2015). Many of the observed health benefits to exercise have been attributed to a select number of myokines, including IL-6 (Pedersen, 2012), IGF-1 (Hamrick et al., 2010), BDNF (Pedersen et al., 2009), and irisin (Bostrom et al., 2012; Pedersen and Febbraio, 2012). Alterations with positive effects on the immune response, including increased plasma and serum IL-6 and natural killer cell (NK) tumor infiltration (Pedersen et al., 2016), as well as inhibition of toll-like receptors (TLR), recruitment of M1 macrophages and $CD8^+$ T lymphocytes (reviewed in: Lancaster and Febbraio, 2014), and NK gene expression and microRNA changes associated with cancer and cell communication (e.g., p53 signaling pathway) (Radom-Aizik et al., 2013), are also reported. Thus, here we assessed the immediate and delayed impacts of HIIE on the human serum proteome to further elucidate underlying molecular mechanisms and identify novel candidate biomarkers (e.g., exercise regulated factors/exerkines) for the therapeutic and disease-preventative effects of exercise.

To assess intact proteoforms rather than simply amino acid sequences, two-dimensional gel electrophoresis (2DE), coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) for subsequent protein identification, was utilized to differentially profile the proteome of whole serum, preceding, immediately following, and an hour following HIIE. This top-down approach, which currently offers the most reproducible, comprehensive resolution and quantitative detection of intact proteoforms (Oliveira et al., 2014; Coorssen and Yergey, 2015; Zhan et al., 2018), revealed several changes following HIIE which may, with further investigation, improve our understanding of and predictions for health outcomes of exercise.

MATERIALS AND METHODS

Materials

Where applicable, consumables were of electrophoresis grade or higher. Vacutainer® SST (serum-separator tubes) and 21G butterfly needles were from BD (Franklin Lakes, NJ, United States). ReadyStrip™ immobilized pH gradient (IPG) strips (17 cm, pH 3–10 non-linear), Bio-Lyte carrier ampholytes (pH 3-10, pH 4-6), and 2-D SDS-PAGE Standards were from Bio-Rad Laboratories (Hercules, CA, United States). AEBSF, agarose I, bovine serum albumin (BSA), CHAPS, dithiothreitol (DTT), leupeptin, mineral oil, and TG-SDS buffer concentrate were from Amresco (Solon, OH, United States). Acetic acid was from Anachemia (Montreal, Quebec); sodium dodecyl sulfate (SDS) was from J. T. Baker Chemical Co. (Phillipsburg, NJ, United States); mass spectrometry-grade trypsin was from G-Biosciences (St. Louis, MO, United States); Coomassie Brilliant Blue G-250 (CBB) was from Genlantis (San Diego, CA, United States); and Broad-range (200–10 kDa) Unstained Protein Standard was from New England Biolabs (Ipswich, MA, United States). Ammonium persulfate and aprotinin were from Thermo Fisher Scientific (Waltham, MA, United States). Acetonitrile, formic acid, and methanol were from EMD Millipore (Burlington, MA, United States). Acrylamide/bis-acrylamide (37.5:1) solution and all other chemicals utilized were from Alfa Aesar (Haverhill, MA, United States). Double glass-distilled water (ddH_2O) was used throughout.

Study Design, Body Composition and VO_{2max} Measurements

This study was approved by the Brock University ethics committee and was conducted in accordance with the Declaration of Helsinki II. Written informed consent was obtained from each participant prior to beginning the study. Study design is outlined in **Figure 1A**. Briefly, six healthy male participants [age = 24.5 ± 1.3 years; weight = 85.8 ± 10.3 kg; height = 184.1 ± 5.1 cm; body fat% = 12.8 ± 6.8% (mean ± SD)] volunteered to participate, with testing conducted at the Applied Physiology Lab, Brock University. Height was measured with a stadiometer to the nearest millimeter. Body composition was measured via air displacement plethysmography (BodPod;

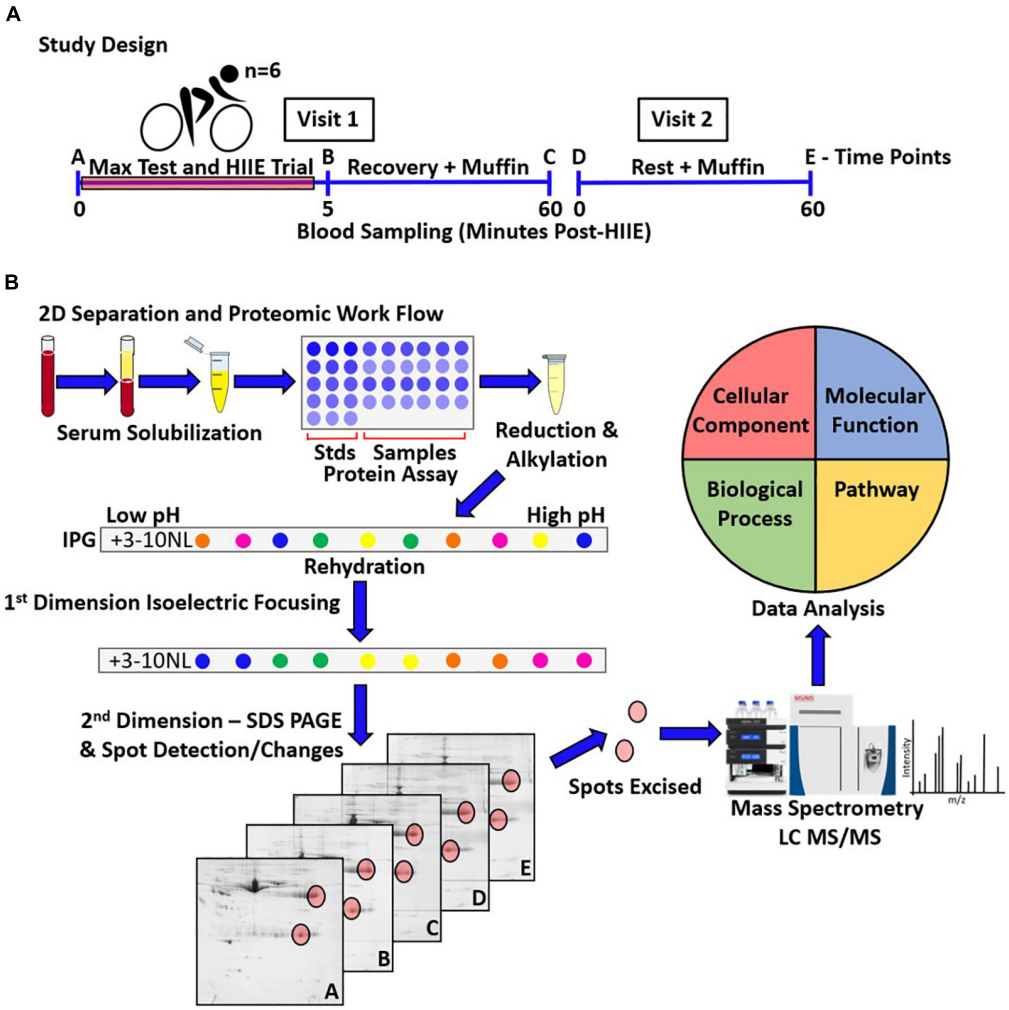

FIGURE 1 | Schematic representation of the study design, and proteomic work flow (i.e., 2DE-LC-MS/MS) for the assessment of the serum proteome following HIIE and during recovery. **(A)** The studied sample consisted of 6 healthy young adult males, who performed one acute bout of HIIE. Blood sampling occurred on an exercise trial visit, at pre- **(A)**, and 5 min **(B)** and 1 h post-HIIE **(C)** (visit 1), as well as on a control trial visit, pre- **(D)** and post-muffin **(E)** (visit 2). **(B)** Triplicates for samples taken at the 5-time points for each participant were resolved by 2DE (90 gels in total) and used to assess spot changes over time. LC-MS/MS was used to analyze and identify the proteoforms present within the protein spots of interest, and gene ontology (Uniprot) and PANTHER were used to analyze and catalog the functions/interactions and biological pathways potentially involved.

Life Measurement, Inc., United States) to obtain measures of body, fat, and fat-free mass (kg), and body fat percentage. Using a continuous, incremental exercise protocol (described in more detailed below), VO_{2max} was measured on a cycle ergometer (Monark, Vansbro, Sweden). Heart rate was recorded continuously during the assessment using a chest band heart monitor (TIMEX Group Inc., Toronto, ON, Canada), and metabolic gasses were analyzed using an AEI metabolic cart (Model S-3A, AIE Technologies, Pittsburgh, PA, United States). A respiratory gas exchange ratio of at least 1.15 and a heart rate >90% of age-predicted maximal heart rate were criteria for achieving peak aerobic capacity.

For the HIIE trials, participants were asked to abstain from exercise for 24 h prior and to arrive in a fasted state (at least 12 h). Testing began between 0700 and 0900 h, with one participant tested at a time. Upon arrival, the participant rested in a seated position for 15 min prior to their first rested blood draw (A). Venous blood was collected from the medial cubital vein into a 5 ml clot activator serum collection tube. The participant then warmed up on a cycle ergometer for 2 min at 50 W before undergoing a maximal exercise (VO_{2max}) test that started at 50 W and increased by 1.5 W every second. The participant was required to remain seated and maintain ≥80 revolutions per minute (RPM). The test was concluded once the participant either stood up from their seat or fell below 60 RPM. The maximum workload (W_{max}) was then recorded as the maximum power achieved at the final stage of the incremental test. A 5–10 min recovery period was permitted prior to commencing HIIE, which consisted of 8 min × 1 min trials at 90% W_{max}, with 1 min non-active recovery between trials. Heart rate was

continuously recorded as above. Following the HIIE trials, exertion was measured by the Borg scale (Borg, 1998).

The participant was permitted to cool-down for 2 min at 50 W before stepping off the cycle ergometer for the 2nd blood draw, taken ∼5 min post-HIIE to assess acute effects of exercise (B). Following this blood draw the participant consumed ∼250 ml of water and one store-bought blueberry muffin (∼115 g). Blood was drawn 1 h post-HIIE to assess recovery from exercise (C).

A second visit parallel to the first except *without* exercise, in which blood was drawn prior to (D) and 1 h following the consumption of a blueberry muffin (E), served to control for potential day-to-day variability as well as food consumption.

Sample Preparation

2D separation and proteomic work flow is outlined in **Figure 1B**. The initial step of this work flow was sample preparation. Blood samples in collection tubes were inverted 5 times and allowed to clot at room temperature for 25 min prior to 4°C centrifugation at 1,500 × *g* for 15 min. Serum was collected and supplemented with kinase, phosphatase, and protease inhibitors [inhibitor cocktail: 4 μM staurosporine; 5 mM sodium fluoride; 1 mM benzamidine; 0.2 mM DTT; 0.3 μM aprotinin; 0.6 μM pepstatin A; 0.85 μM leupeptin; 8 μM AEBSF (Coorssen et al., 1998; Butt and Coorssen, 2005)]. Serum aliquots were snap-frozen in liquid nitrogen and stored at −80°C.

Per participant, one aliquot of serum per time-point was thawed and combined with 2DE lysis buffer [8 M urea; 2 M thiourea; 4% (w/v) CHAPS] at a ratio of 1:7, supplemented with inhibitor cocktail as detailed above. Total protein solubilization was carried out over 2 h at 4°C with intermittent (15–30 min) gentle vortexing and centrifugation at 500 × *g*. Solubilized serum samples were aliquoted and snap frozen, with 10 μl of each reserved for estimation of total protein concentration.

Protein Concentration Estimation

Estimation of total protein concentration was carried out using a solid-phase protein assay as previously described (Noaman and Coorssen, 2018). Briefly, solubilized serum samples were serially diluted to yield concentrations appropriate for measurement against a linear calibration curve (0.5–0.05 mg/ml of BSA in 2DE lysis buffer). 1 μl of each dilution was dot-blotted in triplicate onto Whatman^{TM} 3MM chromatography paper (GE Healthcare, Chicago, IL, United States). Dried blots were washed with methanol for 5 min, dried under ambient conditions, and stained for 10 min with colloidal CBB (cCBB). Destaining was carried out for 5 min × 5 min with ddH_2O, and blots were dried prior to imaging via reflective densitometry using the GS-900 Calibrated Densitometer (Bio-Rad, Hercules, CA, United States) followed by quantitation using ImageLab (Bio-Rad, Hercules, CA, United States) and Microsoft Excel 2013.

Two-Dimensional Gel Electrophoresis (2DE)

Samples were assigned a numerical code and the order of sample analysis was determined with the use of a random number generator. Whole-serum 2DE was carried out as described previously (D'Silva et al., 2018). Briefly, 500 μg total serum protein was combined with rehydration buffer [2DE lysis buffer with 0.75% (v/v) pH 3–10 and 0.25% (v/v) pH 4–6 carrier ampholytes (Bio-Rad, Hercules CA, United States)], supplemented with TBP/DTT in the 1st hour, and acrylamide in the 2nd hour, for protein reduction and alkylation, respectively. 17 cm pH 3–10 non-linear IPGs were passively rehydrated for 16 h prior to isoelectric focussing (IEF), carried out at 17°C and 10,000 V for 75000 VH using a Protean i12 IEF cell (Bio-Rad, St. Louis, MO, United States), with multiple electrode wick changes during voltage ramping to facilitate desalting. Following IEF and prior to SDS-PAGE, IPGs were incubated for 20 min in equilibration solution {6 M urea, 0.375 M tris [pH 8.8), 2% (w/v) SDS, 10% (w/v) glycerol}, supplemented with 2% (w/v) DTT in the first 10 min followed by 350 mM acrylamide in the last, for reduction and alkylation, respectively.

SDS-PAGE was in hand-cast large-format (18 cm × 18 cm × 0.1 cm) 7–20%T gradient gels {0.375 M Tris [pH 8.8]; 0.1% (w/v) SDS; 0.1% (w/v) LDS; 0.05% (w/v) APS; 0.05% (v/v) TEMED (D'Silva et al., 2017)}, poured using a gradient former and multi-caster produced by the Brock University Machine Shop (such equipment may be purchased commercially). Electrophoresis was carried out at 4°C and 300 V for 15 min followed by 120 V until completion, typically for 24–26 h, using the Protean II XL system (Bio-Rad, Hercules, CA, United States). Resolved proteins were fixed in-gel with 10% (v/v) methanol, 7% (v/v) acetic acid for a minimum of 1 h, washed 3 min × 20 min with ddH_2O, and stained for 20 h with cCBB followed by 5 min × 15 min destaining with 0.5 M NaCl (Noaman et al., 2017). Gels were imaged via transmissive densitometry using the GS-900 at highest scanning resolution (36.3 μm). For each of the five serum samples from each of the six participants, three 2DE replicates (technical $n = 3$) were resolved to ensure reproducibility.

Image Analysis

Quantitative 2DE gel image analysis was done using Delta2D (DECODON Gmbh v4.7, Greifswald, Germany). Gel images were grouped into (A) pre-exercise, (B) 5 min post-exercise, and (C) 1 h post-exercise, as well as visit 2 (D) pre-muffin and (E) 1 h post-muffin. Images were meticulously warped, and a consensus gel image was created using 'union fusion' and automatically established detection parameters (average spot size, local background region in pixels, and sensitivity). From this image, a protein spot pattern was generated, manually edited to exclude artifacts, and transferred to all individual gel images for '100% spot matching.'

The resulting quantitation table which displayed average normalized spot volumes for each spot across each condition was used to determine significant changes in protein abundances. Spots which were statistically different (p-value < 0.05) in normalized volume between pre-exercise and 5 min and/or 1 h post-exercise, and 5 min post-exercise to 1 h post-exercise, with a ratio ≥ 1.1 or ≤ 0.9 and a relative standard deviation $\leq 30\%$, were considered genuine changes, and thus candidates for analysis by LC-MS/MS.

Comparisons of visit 1 pre-exercise and visit 2 pre-muffin samples, as well as visit 2 pre- and post-muffin samples, were carried out to ensure significant changes following HIIE were not attributed to day-to-day variability and/or food consumption.

In-Gel Digestion and LC-MS/MS

In-gel digestion was carried out essentially as described previously (Wright et al., 2014). Manually excised protein spots combined from multiple gels were equilibrated briefly in 100 mM ammonium bicarbonate, and destained with 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate prior to dehydration with 100% (v/v) acetonitrile. In-gel tryptic digestion with 3 ng·μl^{-1} trypsin in ammonium bicarbonate was carried out for 30 min at 4°C followed by 12 h at 37°C. Peptide solutions were recovered in microcentrifuge tubes and dried in a speed vacuum. Samples were shipped in microcentrifuge tubes at ambient temperature for LC-MS/MS analysis.

The MS analysis was carried out using a Q-Exactive mass spectrometer (Thermo Scientific), using a top 10 data dependent acquisition method with automatic switching between MS and MS/MS. Full-scan MS mode (375–1600 m/z) was operated at a resolution of 70,000 with automatic gain control and a target of 1×10^6 ions. Ions selected for MS/MS were subjected to the following parameters: resolution 17,500, target of 1×10^5 ions, 1.5 m/z isolation window, normalized collision energy 27.0 V and dynamic exclusion 20.0 s. Source ionization parameters were as follows: spray voltage, 1.9 kV; capillary temperature, 280°C; and s-lens RF level 50.0.

LC-MS/MS results were searched using Proteome Discoverer (version 2.2, Thermo Scientific) against the SwissProt human database (49,070 entries) in which raw files were searched using the Sequest HT algorithm. Peptides produced by trypsin proteolysis with a maximum of two missed cleavages were matched using precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively. Propanamide (C) selected as a fixed modification, and oxidation (M), deamidation (NQ) and acetyl (protein N-term) were chosen as variable modifications. Peptide spectrum matches (PSMs) were verified based on *q*-values set to 1% false discovery rate (FDR). This resulted in the identification of multiple proteoforms (defined as differences in pI and/or MW). In these instances, only those hits with high sequence coverage (i.e., ≥5%) and number of unique peptides (i.e., ≥2) were accepted as identified proteoforms (**Table 1**). All raw mass spectrometry data has been deposited and is publicly available: ftp://massive.ucsd.edu/MSV000083129/raw/.

RESULTS

Each of the six young, healthy, male participants had an initial blood draw before engaging in an HIIE testing phase. Mean VO_{2max} was 50.4 ± 1.0 ml/kg/min, and W_{max} was 391.8 ± 8.3 W, the latter within the 80th percentile for healthy men in the age category assessed (Wang et al., 2010), indicating that the participants had adequate endurance potential. During HIIE trials, mean heart rates were >90% of the participants predicted HR_{max} (94.1 ± 5.3%) and immediately following they all responded ≥19/20 on the Borg scale (Borg, 1998). This confirmed that the HIIE trial was indeed high-intensity and successful in exhausting the participants. Following HIIE trials, blood was collected 5 min and 1 h post-exercise for 2DE analysis.

2DE enabled the resolution and detection of 977 consensus spots (i.e., spots which resolved consistently and were thus analyzed across all gels). Twenty spots were identified to have changed significantly from pre-exercise to 5 min post-exercise and/or 1 h post-exercise ($p < 0.05$; **Figures 2A,B**). Several high-quality/confidence database hits were returned following LC-MS/MS, identifying at least 15 different proteoforms within these spots (**Table 1**). These spot changes were not observed between visit 1 and 2 (pre-exercise, and pre-muffin consumption, respectively) samples, nor visit 2 pre- and post-muffin- consumption samples (not shown), indicating that neither day-to-day variability nor food consumption affected the proteoforms shown here to be associated with acute HIIE.

Table 1 summarizes the most confidently identified proteoforms within each spot analyzed which changed from pre- to 5 min and/or 1 h post-exercise. Additional peptide data from each spot can be found in **Supplementary Data File S1**. Average spot volume ratio changes for each participant are summarized in a heat map in **Figure 2B**, demonstrating the extents of biological variability.

Proteoforms that changed post-HIIE were largely from the protein families including serpins, apolipoproteins, fetuins, immunoglobulins, and albumins. The proteoforms that were found to have increased post-HIIE were fetuin-a, α-1-antitrypsin, vitamin D binding protein, histidine-rich glycoprotein, apolipoprotein J (clusterin), apolipoproteins E and A1, and immunoglobulin J chain; and those that decreased included immunoglobulin heavy constant α 1, immunoglobulin k constant, and β-2-glycoprotein 1. Apolipoprotein E, vitamin D binding protein, immunoglobulin J chain, and clusterin were each found in more than one fixed MW and pI gel region, suggesting resolution of different proteoforms (Jungblut et al., 2008; Thiede et al., 2013; Oliveira et al., 2014; Coorssen and Yergey, 2015; Zhan et al., 2018).

Of the spots changing in abundance, 8 of 20 were found to decrease in volume at 5 min post-HIIE and remained decreased 1 h post-HIIE. The proteoforms in these spots were identified as immunoglobulin heavy constant α 1 and immunoglobulin κ constant, respectively. The other 18 spots either increased only 1 h post-HIIE (α-1-antichymotrypsin, fetuin-a, α-1-antitrypsin, kininogen-1, serotransferrin, histidine-rich glycoprotein, and immunoglobulin κ constant) or at both 5 min and 1 h post-HIIE (clusterin, apolipoprotein E, immunoglobulin J chain, apolipoprotein A-1, and retinol binding protein 4).

Bioinformatic analyses suggested that most of the proteoforms which changed in abundance following HIIE were related to inflammatory responses, protein and lipid binding, antioxidant activity, metabolism and exosome formation (**Figures 3A,B**). Specifically, the apolipoproteins that increased post-HIIE (apolipoproteins A1, E, and clusterin) are associated with LDL/HDL particle receptor binding, amyloid β binding, tau

TABLE 1 | Ratios of spot volumes for 5 min and 1 h post-HIIE when compared to pre-exercise and the proteins identified within those spots by LC-MS/M.

Spot number	Response 5-min PE (ratio/*p*-value)	Response 1-h PE (ratio/*p*-value)	Gene	Protein identified	Accession number	Theoretical MW/pI* (kDa/pI)	Observed MW/pI (kDa/pI)	Score/ PSM	Seq. Cov %	Number of peptides/ unique peptides
1	↔	↑	*SERPINA3*	α-1-antichymotrypsin	P01011	47.6/5.5	63.3/4.6	14/9	13	5/5
	1.1	1.2								
	0.4	0.02								
2	↔	↑	*AHSG*	α-2-HS-glycoprotein	C9JV77	39.4/5.7	61.2/4.5	529/232	13	5/5
	1.1	1.2	*SERPINA1*	(fetuin-a)						
	0.08	0.02		α-1-antitrypsin	A0A024R6I7	46.7/5.6		67/44	21	10/10
			KNG1	Kininogen-1	P01042	71.9/6.8			7	4/4
3	↔	↑	*AHSG*	α-2-HS-glycoprotein	P02765	39.4/5.7	60.0/4.5	674/274	19	11/11
	1.1	1.2		(fetuin-a)						
	0.08	0.02		α-1-antitrypsin	A0A024R6I7					
			SERPINA1			46.7/5.6		117/77	25	13/13
4	↑	↑	*SPERINC1*	Antithrombin-III	P01008	52.6/6.7	59.8/4.5	58/52	29	9/9
	1.2	1.3								
	0.02	0.005								
5	↔	↑	*SERPINA1*	α-1-antitrypsin	A0A024R6I7	46.7/5.6	60.0/4.7	805/524	55	27/27
	1.1	1.2								
	0.2	0.02								
6	↔	↑	*SERPINA1*	α-1-antitrypsin	A0A024R6I7	46.7/5.6	58.8/4.9	1676/940	61	33/33
	1.0	1.1								
	0.4	0.02								
7	↔	↑	*GN*	Vitamin D binding protein	P02774	53.0/5.5	57.4/5.0	1194/650	46	28/4
	1.1	1.3								
	0.3	0.004			D6RF35					
8	↔	↓	*IGHA1*	Immunoglobulin	P01876	37.6/6.5	64.5/5.1	391/184	44	15/5
	0.8	0.7		heavy constant α 1	A0A0G2JMB2					
	0.2	0.008								
			IGHV3OR16-9	Immunoglobulin heavy variable 3/OR16-9 (non-functional)	A0A0B4J2B5	10.7/8.7		9.9/4	31	2/2
9	↔	↑	*TF*	Serotransferrin	P02787	77.0/7.1	72.3/6.4	1868/1087	78	89/89
	1.0	1.2	*IGHM*	Immunoglobulin heavy constant μ	A0A1B0GUU9	51.9/5.8		101/63	35	17/17
	0.4	0.02								
			HRG	Histidine-rich glycoprotein	P04196	59.5/7.5		31/21	18	8/8
10	↑	↑	*CLU*	Clusterin (APOJ)	P10909	52.5/6.3	60.1/58.5	165/105	24	12/12
	1.4	1.7								
	0.002	0.0008								
11	↑	↑	*CLU*	Clusterin (APOJ)	P10909	52.5/6.3	43.5/4.6	338/230	26	11/11
	1.6	1.5								
	0.0000	0.0003								
12	↑	↑	*CLU*	Clusterin (APOJ)	P10909	52.5/6.3	41.8/4.7	14/10	11	2/2
	1.2	1.3								
	0.004	0.01								

(Continued)

TABLE 1 | Continued

Spot number	Response 5-min PE (ratio/*p*-value)	Response 1-h PE (ratio/*p*-value)	Gene	Protein identified	Accession number	Theoretical MW/pI* (kDa/pI)	Observed MW/pI (kDa/pI)	Score/ PSM	Seq. Cov %	Number of peptides/ unique peptides
13	↑ 1.4 0.007	↑ 1.6 0.0002	*APOE*	Apolipoprotein E	P02649	36.1/5.7	37.7/5.0	31/26	29	9/9
14	↔ 1.1 0.4	↑ 1.3 0.02	*APOE*	Apolipoprotein E	P02649	36.1/5.7	36.6/5.1	68/74	34	10/10
15	↔ 1.1 0.1	↑ 1.2 0.02	*APOE*	Apolipoprotein E	P02649	36.1/5.7	35.8/5.2	1511/815	68	26/21
16	↑ 1.2 0.04	↑ 1.3 0.01	*JCHAIN*	Immunoglobulin J chain	P01591	18.1/5.2	27.3/4.4	26/24	11	3/3
17	↑ 1.3 0.02	↑ 1.3 0.007	*JCHAIN*	Immunoglobulin J chain	P01591	18.1/5.2	27.3/4.5	86/83	26	4/4
18	↑ 1.3 0.02	↑ 1.3 0.007	*APOA1*	Apolipoprotein A-1	P02647	30.8/5.8	25.2/5.0	4400/1835	86	52/52
19	↑ 1.3 0.04	↔ 1.0 0.8	*IGKC*	Immunoglobulin κ constant	P01834	11.8/6.5	29.0/6.2	393/178	86	9/9
			IGLC2	Immunoglobulin λ constant 2	P0DOY2	11.3/7.2		240/124	56	4/2
			IGKV3-15	Immunoglobulin κ variable 3-15	P01624	12.5/5.2		58/34	26	2/2
			IGLL5	Immunoglobulin λ-like polypeptide 5	A0A0B4J231	23.1/8.8		52/35	27	4/2
20	↓ 0.8 0.006	↓ 0.8 0.0004	*IGKC*	Immunoglobulin κ constant	P01834	11.8/6.5	27.5/6.2	462/194	53	6/6
			IGKV3-20	Immunoglobulin κ variable 3-20	P01619	12.5/4.9		150/71	41	3/2
			IGLC2	Immunoglobulin λ constant 2	P0DOY2	11.3/7.2		129/67	60	5/2
			IGLL5	Immunoglobulin λ-like polypeptide 5	A0A0B4J231	23.1/8.8		42/29	29	5/2

*Some of the spots contained more than one clearly identifiable protein; presented here are the best hits (i.e., highest coverage and peptide count). PE, post-exercise; MW, molecular weight; kDa, kilodaltons; PSM, peptide spectrum matches; ↔, ↑, and ↓, directional change from pre-exercise levels; ratio, either 5 min or 1 h post-exercise spot volume/pre-exercise spot volume. *Tabulated theoretical molecular masses and isoelectric points predominantly relate to intact precursors. Please refer to databases (e.g., UniProt and ExPASy ProtParam) for MW and pI of potential cleavage products and gene/splice isoforms, as well as common PTMs.*

protein binding, heat-shock protein binding and antioxidant activity; and the pathways they affect include GPCR signaling, mineral absorption, HDL remodeling, and PPAR signaling. Protease inhibitors and binding proteins that increased in abundance following HIIE (α-1-antichymotrypsin and α-1-trypsin and fetuin-a, respectively) were associated with amyloid-β binding (fetuin-a), and their pathways include neutrophil degranulation, platelet degranulation, Nrf2 pathways, complement and coagulation cascades, and mineralization (fetuin-a). Immunoglobulins affected by HIIE are associated with antigen binding and innate immunity (**Figures 3B,C**).

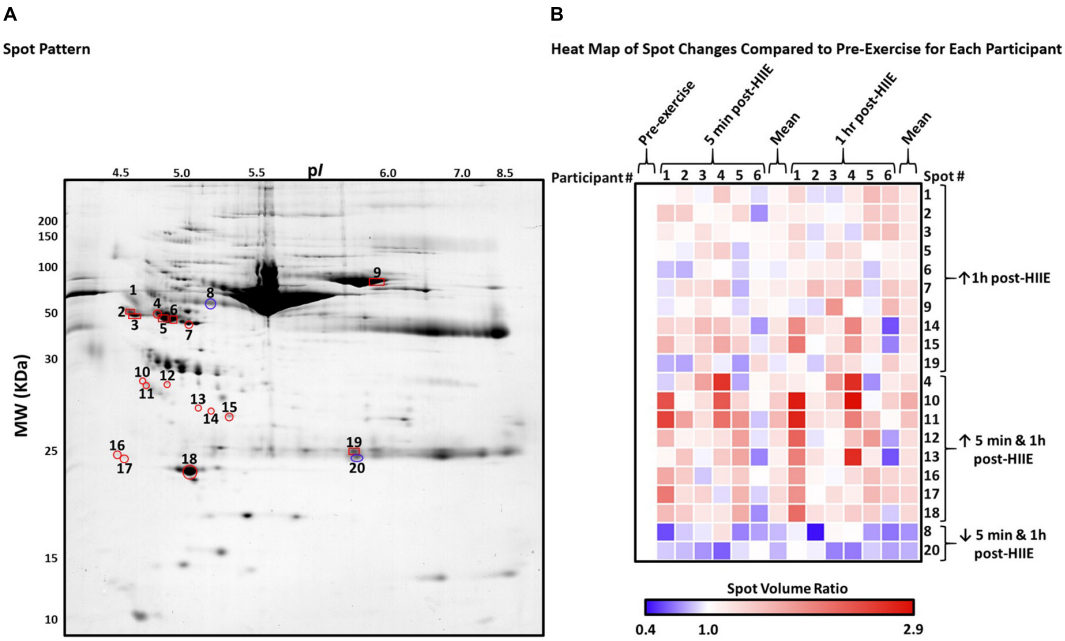

FIGURE 2 | Spot pattern and fold changes for spots that changed significantly post-exercise and/or during recovery when compared to pre-exercise. **(A)** Representative gel image of the serum proteome, indicating the spots that changed significantly 5-min/1 h post-HIIE. Spots in red indicate a significant increase while blue indicates a decrease. Spots with a triangle indicate changes only 5 min post-HIIE, while squares indicate changes only during 1 h post-HIIE, and circles indicate changes both 5 min post-HIIE and 1 h post-HIIE when compared to pre-exercise. **(B)** Heat map of the changes for each participant for each spot; each cell is the average spot volume ratio change from 5 min and 1 h post-HIIE when compared to pre-exercise for each participant from the triplicate gels resolved for each participant. Blue and red indicate a decrease and increase, respectively, while white is no change when compared to pre-exercise levels. Since the ratios presented in the heat map were calculated based on the pre-exercise spot volume for each individual, pre-exercise then indicates no change; the first column (pre-exercise) is thus white to better visually emphasize the changes for each spot for each participant. Averages are also presented, which represent the mean of all participants and their replicates. Statistics (i.e., *p*-values and quantities) are presented in **Table 1**.

DISCUSSION

2DE coupled with LC-MS/MS enabled the resolution and identification of differentially abundant intact proteoforms, unlikely to have been specifically detected by a bottom-up MS-based 'shotgun' proteomic approach nor through targeted proteomic assays (e.g., western blotting, ELISA, and SOMAscan) since both methodologies generally assess only gross changes in abundances, with critical information pertaining to intact proteoforms and their unique physicochemical characteristics (that modulate function and localization) lost or unaccounted for. This is the first study to sequentially assess the human serum proteome following HIIE using a comprehensive top-down approach to identify significant changes in proteoforms (i.e., resolved protein species deviating in pI and/or MW from the theoretical values that are defined only on the basis of amino acid sequence; **Table 1**) (Jungblut et al., 2008; Thiede et al., 2013; Coorssen and Yergey, 2015; Zhan et al., 2018), implicating proteoforms associated with immune function, coagulation cascades, vitamins, protein and lipid metabolism, and proliferative and apoptotic pathways.

Many of these novel exercise-regulated proteoforms (i.e., exerkines) have been previously suggested as potential therapeutic targets in chronic diseases [i.e., Alzheimer's disease (AD), type II diabetes mellitus and cardiovascular disease (CVD)] (Booth et al., 2012), supporting the notion that these proteoforms may mediate the longer-term health benefits associated with exercise training. These findings serve as initial evidence to further investigate the utility of these proteoforms as biomarkers for long-term positive health outcomes, though perhaps also for acute exhaustive exercise. Herein, the discussion will focus on the identified proteoforms that are altered following acute HIIE and their potential impact on immune function and the subsequent health benefits; however, it is important to keep in mind that these protein species have roles in health and disease other than those discussed here.

HIIE Modulates Serum Proteins Known to Regulate Immune Function

It has been suggested that acute bouts of exercise may lead to a state of immune suppression which increases the risk of opportunistic infection (Peters and Bateman, 1983; Nieman et al., 1990; Gleeson, 2007). In contrast, others propose that immunity is improved as immune cells begin to localize in peripheral tissues (e.g., lungs and bone marrow) to increase surveillance and remove damaged/malignant cells (Matthews et al., 2002; Dhabhar, 2014; Campbell and Turner, 2018). Changes in proteoform abundance may be representative of varying post-translational modifications (PTMs) (Jungblut et al., 2008;

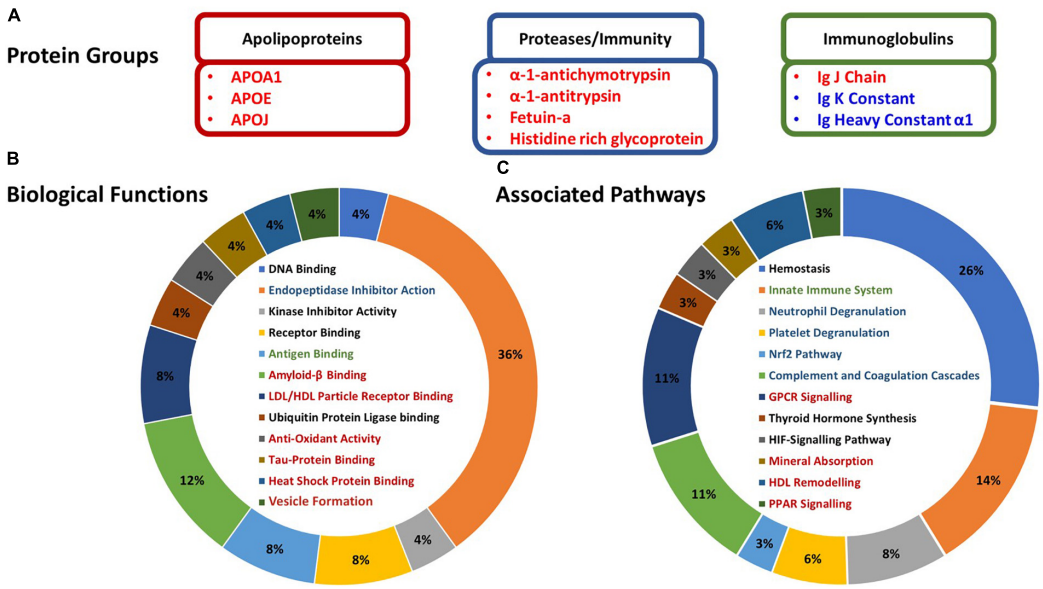

FIGURE 3 | Characterization and associations of the proteoforms altered in response to HIIE. **(A)** Candidate exercise-regulated proteoforms that may be mediating the numerous multi-system health benefits we observe with exercise training. **(B)** Gene Ontology biological functions of the proteoforms altered post-exercise – identified with Uniprot. **(C)** Protein associated pathways of the proteoforms altered post-exercise – identified with PANTHER. Protein names in red indicate proteins that increased post-exercise, and proteins in blue indicate a decrease. Biological functions and associated pathways in red, blue, and green are related to apolipoproteins, proteases/immunity, and immunoglobulins, respectively.

Thiede et al., 2013), changes in expression, secretion or rate of degradation, or indicate an increase in distribution to peripheral tissues. Thus, certain tissues may experience an increase in homing factors resulting in an increase in inflammatory cells/factors being recruited to the periphery when a decrease is measured in serum. This is important to consider when moving forward with a discussion of these initial results, as well as in considering the design of future studies.

These data indicate that proteoforms of immune-regulatory proteins either increase (α-1-antichymotrypsin, fetuin-a, α-1-antitrypsin, apolipoprotein A1, clusterin, and immunoglobulin J chain) or decrease (immunoglobulin heavy constant α 1 and immunoglobulin k constant) in abundance post-HIIE. The immunoglobulin J chain is critical for production of secretory antibodies, which suggests there may be an increase in the secretion and priming of IgA and IgM, which are the initial responders to a specific immunologic defense (i.e., mucosal defense) (Woof and Mestecky, 2015). On the other hand, the decrease in immunoglobulins k and α indicates either that B cells are suppressed during HIIE or that there is a redistribution of immunoglobulins to the periphery to increase surveillance during and immediately following HIIE. Exercise induces the mobilization of B cells into the circulation; however, naïve B cells increase more in number than effector B cells (Turner et al., 2016). Although B cell function was not assessed in this study, given that J chain increases and immunoglobulin α decreases in abundance, we speculate that there is likely a redistribution of immunoglobulin α. These findings are thus consistent with the suggestions that exercise may play an important role in preventing infection, and indicate a potential mechanism as to how acute and chronic exercise may modulate or ameliorate the effects of pro-inflammatory cytokine (e.g., TNF-α and IL-1β) production in response to infection (Kohut et al., 2009).

The increase in circulating protease inhibitors α-1-antichymotrypsin and α-1-antitrypsin and their pro-forms, and the binding protein fetuin-a, suggest a suppression of neutrophil, platelet and mast cell degranulation (Hercz, 1974; Lebreton et al., 1979). This may be a compensatory response to the observed HIIE-induced increase in circulating neutrophils (Neves et al., 2015). α-1-antichymotrypsin also acts as a negative regulator of pro-inflammatory cytokine production (e.g., TNF-α and IL-1β) and innate immunity, thus protecting against systemic inflammation (i.e., lethal endotoxemia and sepsis) (Wang and Sama, 2012). Furthermore, α-1-antitrypsin appears to have other anti-inflammatory properties (Bergin et al., 2012), which include protection against apoptosis induced by pro-inflammatory cytokines (e.g., TNF-α, IL-1B, and IFN-γ) (Kalis et al., 2010), inhibition of toll-like receptor 4 and 2 signaling (Jonigk et al., 2013), and regulation of neutrophil chemotaxis induced by soluble immune complexes (Bergin et al., 2010). Additionally, apolipoprotein A1 appears to be a critical anti-inflammatory mediator during the acute phase, and is known to inhibit pro-inflammatory cytokine production in monocyte-macrophages (e.g., TNF-α and IL-1β) (Hyka et al., 2001). Furthermore, in a model of non-alcoholic steatohepatitis, overexpression of clusterin has been shown to inhibit pro-inflammatory cytokine production (e.g., TNF-α and IL-1β) and hepatic macrophage infiltration, and to abolish hepatic fibrogenesis through the activation of the transcription factor Nrf2 which activates the antioxidant response element (Park et al., 2018), thus

increasing proteins that lead to detoxification and elimination of reactive oxygen species and electrophilic agents (Falgarone and Chiocchia, 2009; Nguyen et al., 2009).

Other identified proteoforms, including histidine-rich glycoprotein (Poon et al., 2011), transferrin (Mancini et al., 2016), antithrombin III (Inthorn et al., 1998), kininogen I (Bryant and Shariat-Madar, 2009), and vitamin D binding protein (Gomme and Bertolini, 2004), have also been shown to have either anti-inflammatory properties or regulate the immune response. Taken together, these post-HIIE molecular responses suggest an overarching anti-inflammatory response rather than immune suppression, which is often said to occur following intense exercise. While these proteoforms are not the classical inflammatory cytokines used to describe systemic inflammation, many have been extensively shown to have anti-inflammatory effects. We have previously shown that pro-inflammatory cytokines, TNF-α and IL-1β, transiently increase post-HIIE and return to baseline 1 h post-HIIE (Mezil et al., 2015). The increase in α-1-antichymotrypsin, α-1-antitrypsin, apolipoprotein A1, and clusterin shown here, may indicate a protective mechanism through anti-inflammatory and antioxidant activity, which is sustained through recovery (i.e., 1 h post-HIIE).

One of the major underpinnings of chronic disease is inflammation caused by obesity or a sedentary lifestyle. There is an overt variation in immune cells as well as platelets, and molecular makeup within the circulation and in peripheral tissues in individuals with chronic disease (i.e., metabolic syndrome). The data here thus indicate that there is a shift toward the regulation of these inflammatory responses/pathways through an increase in anti-inflammatory agents. Thus, the modulation of these immune-regulatory proteins post-HIIE may have a role in the prevention and treatment of many chronic diseases.

HIIE Modulates Proteoforms That May Mediate the Prevention of Several Chronic Diseases

Exercise is the ideal first option therapy for prevention and treatment of numerous chronic diseases (e.g., accelerated aging, metabolic syndrome, Type II diabetes mellitus, and CVD) (Booth et al., 2012). Specifically, fetuin-a has been shown to have cytoprotective activity against oxidative injury in neuronal cells (Kanno et al., 2017) and inhibits calcification of atherosclerotic plaques in patients with type II diabetes mellitus (Emoto et al., 2010). The anti-inflammatory effects of fetuin-a and α-1-antitrypsin, as well as their potential roles in mediating insulin sensitivity in response to exercise training (Kalis et al., 2010; Blumenthal et al., 2017), may thus be an avenue of interest in efforts to find therapeutics that act as mimetics for exercise and thus combat metabolic diseases/insulin resistance. In this regard, however, it is important to note that α-1-antitrypsin was identified in five different resolved spots, and fetuin-a in three, each of which differed markedly in MW and pI from the theoretical values. Therefore, these effects are likely mediated (perhaps selectively) by various proteoforms of the canonical species, emphasizing the importance of clearly identifying active species prior to the development of therapeutics. Focussing on amino acid sequences alone is insufficient in terms of identifying effective biomarkers and designing new therapeutics.

Lipid handling/storage appears to have an important role in the inflammatory response in individuals with atherosclerosis, CVD, and neurodegenerative diseases (Libby et al., 2013). Apolipoproteins are a major class of lipid binding proteins that are thought to be associated with several chronic diseases, given their roles in binding and clearing various lipids (i.e., cholesterol), forming HDL (apolipoprotein A1), and degrading LDL (Horejsi and Ceska, 2000). Cholesterol accumulation contributes to the activation of the immune response and subsequent increase in pro-inflammatory cytokines, which can lead to pathological chronic inflammation (Libby et al., 2013). Thus, the increase in abundance of apolipoproteins following HIIE, which regulate the handling and clearance of cholesterol and promote an increased HDL:LDL phenotype, may be a means of preventing systemic inflammation and adequately regulating lipoprotein abundance/ratios.

Clusterin protects cardiomyocytes from apoptosis through the Akt/GSK-3β signaling pathway (Jun et al., 2011), and protects the heart from damage caused by myocardial infarction (Foglio et al., 2015), transplant (Li et al., 2011), or myocarditis (McLaughlin et al., 2000) (reviewed in: Pereira et al., 2018). It is thought that apoptosis of cardiomyocytes is one of the main age-related contributors to development of heart disease (Kumar et al., 2002; Goldspink et al., 2003), thus the increase in abundances of three clusterin proteoforms following acute-exercise may be responsible for the lower levels of heart disease seen in individuals who are more active (Buchner, 2009). Clusterin appears to be associated with AD risk (Schrijvers et al., 2011); however, it is most likely a compensatory/neuroprotective response (reviewed in: Nuutinen et al., 2009) as it is associated with AD severity, but not with the risk of developing AD at follow up. Interestingly, clusterin has been shown to inhibit amyloid formation through (1) binding amyloid-β or enhancing its clearance across the blood–brain barrier (Yerbury et al., 2007); (2) clearance by endocytosis of amyloid-β aggregates and cell debris by brain phagocytes (Bell et al., 2007); and (3) inhibition of complement activation (Nuutinen et al., 2009). Taken together, these findings suggest clusterin is neuroprotective rather than complicit in AD progression and is most likely a component of a compensatory response in AD.

Exercise also has neuroprotective effects through the stabilization of apolipoprotein E (Soto et al., 2015), and is integral for maintaining blood–brain barrier integrity (reviewed in: Montagne et al., 2017). Multiple sclerosis is a disease that is characterized in-part by inflammation which provokes the progression and pathogenesis of the disease. Exercise training has been shown to moderate symptoms and disease progression, thereby improving the quality of life of individuals with multiple sclerosis (Stuifbergen et al., 2006). Apolipoproteins E and A play critical roles in cholesterol homeostasis and subsequent anti-inflammatory actions, as well as having a potential role in clearing amyloid-β from the brain into the circulation (Robert et al., 2017). These functions are critical for neuronal health and regeneration, indicating potential mechanisms for how exercise attenuates the progression and improves the

quality of life of patients with multiple sclerosis (reviewed in: Gardner and Levin, 2015) and other neurodegenerative diseases. Apolipoproteins E and A1 also appear to promote the regression of atherosclerosis in diet-induced hypercholesterolemia and advanced aortic atherosclerotic lesions (Raffai et al., 2005), and appear to be critical in the regulation of lipid profiles (prevention of hyperlipidemia) and subsequent inflammation-induced atherosclerosis (Centa et al., 2018; Favari et al., 2018). Apolipoprotein E also appears to be a significant component of extracellular vesicles (e.g., exosomes, ectosomes) and may have a role in their formation (Looze et al., 2009; Nikitidou et al., 2017), which could indicate a novel mechanism for increased clearance of amyloid-β. Apolipoprotein A1 has been suggested to be an emerging risk biomarker for CVD (Florvall et al., 2006; Upadhyay, 2015), is a critical component of HDL, and appears to be a key component in the inhibition of atherosclerotic plaque formation (Favari et al., 2018). Taken together, there appear to be several plausible mechanisms and targets that HIIE may work through to explain the role of exercise in the prevention of metabolic syndrome, CVD, and neurodegenerative diseases.

Despite our efforts to control experimental variables there are some limitations to this study, which include (1) a relatively small sample size ($n = 6$); (2) only two time-points post-exercise, which did not allow us to determine if or when the identified proteoforms returned to baseline (or whether there are critical later changes); and (3) relatively limited in-gel protein detection sensitivity [∼2 ng/ml or ∼0.06 ng of total protein in a spot with cCBB staining (Noaman et al., 2017)], which, while rivaling standard shotgun approaches for peptides, is less than claims made for targeted albeit likely less selective proteomic techniques (e.g., ELISA). Future studies will focus on validating the species identified here as post-exercise biomarkers. Importantly, the characterization of the acute response before and after exercise training will aid in understanding the (mal)adaptation events that occur with these proteoforms. Thus, repeating the study with more time-points following acute exercise (recovery) and looking at the effect of exercise training may reveal if, and when these exercise-regulated proteoforms (exerkines) return to baseline. Additionally, assessing other sub-fractions of whole blood in this way will increase the detection sensitivity of lower abundance proteoforms (e.g., microvesicles) (Whitham et al., 2018), allow for the detection of proteoforms that may be lost during clotting (i.e., serum vs. plasma) (Kim et al., 2007), provide further understanding of the anti-inflammatory effects of exercise (e.g., peripheral blood mononuclear cells), and elucidate the tissues/cell types of origin and assess which tissues are affected by these exercise regulated proteoforms (i.e., tissue cross talk).

Overall, it is noteworthy that few of the proteins identified closely matched their theoretical pI and MW (i.e., did not conform to identification based *only* on amino acid sequence; a major shortcoming of relying purely on existing databases) and that ∼50% were found in more than one spot, clearly emphasizing the fundamental importance of proteoforms in biological mechanisms and thus the critical importance of using analytical approaches that can resolve these, as they represent specific targets for the development of future biomarkers and therapeutics.

CONCLUSION

The 2DE-based top-down approach used here to assess the serum proteome response to HIIE revealed significant perturbations in the abundance of proteoforms, with roles in regulating immune function in health and disease during the minutes and hour following exercise, providing new insights into the mechanism(s) underlying how exercise may exert some of its numerous associated health benefits. We propose that these potential serum biomarkers be further validated for their utility in assessing recovery, exertion, and health status as well their importance in the anti-inflammatory mechanisms modulated by exercise.

DATA AVAILABILITY

The datasets generated for this study can be found in Mass Spectrometry Interactive Virtual Environment (MassIVE), ftp://massive.ucsd.edu/MSV000083129/raw/.

AUTHOR CONTRIBUTIONS

NK, NN, JC, and PK conceived and designed the study. SC, JC, and PK obtained funding. NK and NN collected the samples. NK, NN, MP, and SC performed the experiments. All authors contributed to data analysis and interpretation of the results. NK and NN drafted the manuscript and did the subsequent revisions. All authors edited and revised the manuscript and approved the final version submitted for publication.

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SUPPLEMENTARY MATERIAL

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Circulating Irisin and Myostatin as Markers of Muscle Strength and Physical Condition in Elderly Subjects

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Background and objective: Aging is a physiological process known to produce changes in body composition, affecting the musculature and leading to decreased muscle strength. Muscle in response to exercise acts as an endocrine organ, producing and releasing myokines such as irisin and myostatin that modulate muscular growth. Here, we aimed to evaluate the effects of low intensity resistance exercise, with or without protein supplementation, on body composition, anthropometric parameters and circulating irisin and myostatin in elderly subjects.

Methods: This is a prospective and controlled clinical trial in which subjects were randomized into 3 groups: (1) control group (n = 20), (2) low intensity resistance exercise group (RE) (n = 14), and (3) low intensity resistance exercise and nutritional support group (RENS) (n = 9). Participants, aged 60–75 years, were studied at baseline and 16 weeks thereafter. Body composition was evaluated through bioelectric impedance. Serum irisin and myostatin was measured using ELISA.

Results: At follow-up, RENS resulted in a significant increase in fat free mass (47.4 ± 7.4 vs. 46.5 ± 7.4, p = 0.046), the calf muscle circumference (36.4 ± 1.3 vs. 32.3 ± 4.3, p = 0.025), and circulating irisin (3 ± 1.1 vs. 2.6 ± 1.3, p = 0.030) compared to baseline. RE resulted in a significant increase in grip strength (17.2 ± 4.6 vs. 15.3 ± 4.6, p = 0.011) and irisin (3.1 ± 0.8 vs. 2.4 ± 0.3, p = 0.011) and decreased walking speed at different distance (p < 0.02). Opposite findings in these parameters were observed in control intervention. In line with these findings, the percent change of calf muscle circumference (p = 0.003) and fat free mass (p < 0.0001) were significantly increased in RENS compared to control, whereas fat mass (p = 0.033) was decreased. Interestingly, in this group, strength was positively correlated with fat free mass (r = 0.782, p = 0.008), and circulating irisin was significantly decreased in

those participants with strength loss at the end of the study ($p = 0.002$). No significant correlation between circulating irisin and myostatin in any group was observed.

Conclusion: Circulating irisin, but not myostatin, constitutes a marker for improved muscular performance in elderly subjects.

Keywords: exercise, elderly, myokines, protein supplementation, irisin

INTRODUCTION

Aging of population is rapidly accelerating, with dramatic increase of people older than 65 years and a change in the age structure of worldwide population (Christensen et al., 2009). Aging is a physiologic process characterized by a gradual impairment in many body functions and growing risk of disease (Holloszy, 2000), with a decrease in strength, flexibility, aerobic capacity, and force output due to progressive loss of muscle tissue and free fat mass whereas fat mass tend to increase (Vinciguerra et al., 2010).

Evidence from human studies supports the notion that regular, vigorous aerobic exercise is a useful tool, with a dose-response effect, to improve the overall health status and longevity (Teramoto and Bungum, 2010; Ruiz et al., 2011). Exercise stimulates the release of cytokines with autocrine, paracrine and endocrine functions produced in skeletal muscle, termed myokines. Thus, skeletal muscle can be classified as endocrine organ. Irisin and myostatin are myokines involved in adaptations to regular training such as increased muscle mass or muscle hypertrophy. Irisin seems a positive regulator whereas myostatin inhibits muscle growth (Kalinkovichs and Livshi, 2015).

Irisin is the result of proteolytic cleavage product of the fibronectin type III "domain-containing" protein 5 (FDNC5) and acts on skeletal muscle, resulting in increased energy expenditure and oxidative metabolism through the induction of metabolic genes (Li et al., 2017). Previous reports demonstrated anti-diabetic effect of irisin through increased energy expenditure in mice (Boström et al., 2012). Moreover, irisin plays a key role on the skeleton by increasing cortical bone mineral density, modifying its structure and improving bone strength (Colaianni et al., 2015). In humans, contradictory effects of exercise in irisin levels have been reported (Moreno-Navarrete et al., 2013; Lopez-Legarrea et al., 2014; Shi et al., 2016). While higher irisin plasma levels after exercise in humans have been described, Timmons et al. (2012) using gene expression arrays, failed to detect a robust and consistent increase in FNDC5 mRNA in human muscle biopsies after exercise. The threshold of exercise required to produce these effects might be crucial, because acute exercise has been demonstrated to result in increased serum irisin concentrations in humans (Huh et al., 2012; Anastasilakis et al., 2014). Lower circulating irisin is a marker of muscle weakness and atrophy and is associated with total muscle mass (Huh et al., 2012; Kim et al., 2015; Chang et al., 2017). Taken together, these data suggest that resistance exercise may result in increased circulating irisin.

Myostatin, also known as growth differentiation factor 8, is a secreted TGF-β superfamily protein that is expressed in skeletal muscle, controlling myoblast proliferation, but being a potent negative regulator of skeletal muscle growth and development (Lee, 2004). Myostatin is synthesized as a 376 amino acid (aa) preprotein that consists of 24 aa signal peptide, a 243 aa propeptide, and a 109 aa mature protein (McPherron et al., 1997). The secreted proprotein is cleaved by BMP-1 family proteases to separate the propeptide from the bioactive mature protein (Lee and McPherron, 2001; Zimmers et al., 2002; Wolfaman et al., 2003). This cleavage results in a latent complex containing a disulfide-linked dimer of the mature protein and two associated propeptides (Lee and McPherron, 2001; Zimmers et al., 2002), in which the myostatin propeptide inhibits the active form.

In the current study, we aimed to investigate the association between resistance exercise and circulating levels of irisin and myostatin in elderly subjects (more than 60 years).

MATERIALS AND METHODS

Subjects' Recruitment

This is a prospective and controlled clinical trial in which subjects were randomized into 3 groups: (1) control group ($n = 15$), (2) resistance exercise group (RE) ($n = 14$), and (3) resistance exercise nutritional support group (RENS) ($n = 9$) during 16 weeks. To increase the sample size of control group, five additional patients were recruited. Randomization was done with the EPIDAT 4.0 program. The subjects (men and women aged 60–75 years) residing in province of Girona, Spain; users of civic centers and primary health care center were invited to participate. Those who accepted were enrolled after giving written informed consent, being studied at baseline and after 16 weeks. This study excluded subjects who (i) were known to have associated chronic diseases (i.e., type 2 diabetes poorly controlled); (ii) were under treatment that could cause myopathy; and (iii) suffer from dementia, cognitive limitation, respiratory impairment or malnutrition (IMC <17 kg/m2, involuntary weight loss >10% and albumin levels <2.5 g/dl).

The night before the visit, all participants followed their usual diet. The post-intervention visit was performed a week after the last session of exercise. For each patient the following measurements at baseline and at post-intervention visit were carried out: (i) Analysis of the biochemical parameters at fasting state; (ii) Anthropometric measurements: brachial circumference, arm circumference, calf circumference, abdominal circumference, tricipital fold; (iii) Fragility test

(Fried et al., 2001); (iv) World Health Organization Quality of Life – BREF (WHOQOL-BREF); (v) Mini nutrition assessment (MNA); (vi) Evaluation of muscle strength with a dynamometer; (vii) Assessment of the physical condition with the Short Physical Performance Battery (SPPB).

Exercise Program

The exercise program lasted 16 weeks and followed the basic principles of the training: (1) loading principle, (2) progression, (3) specificity and individuality, and (4) recovery [American college of sport medicine position stand (1998)]. The subjects performed two weekly sessions of approximately 45 min, with a minimum day of rest between sessions. The session consisted of 3 parts: (i) Warm up with stretching and joint mobilization exercise; (ii) Main part where the resistance exercises will be worked out: 4 upper body exercises and 3 lower body exercises; (iii) Return to the calm with stretching and relaxation. The first weeks of the program were for the anatomical adaptation and the knowledge for the exercises execution; later and progressively increased the load and the volume. All subjects were directly supervised by the physical educator who was in charge.

Nutritional Intervention

The individuals in the control group (C) and the resistance exercise group (RE) followed their usual diet. Individuals in the resistance exercise and nutritional support group (RENS) followed a balanced and varied diet. A protein intake of 0.8–1 g of protein per kg of weight was guaranteed, distributed in 3 meals per day (breakfast/lunch/dinner). The protein intake was calculated from three 24-h reminders as previously reported (Wu et al., 2017). Dietary advice was given to follow a diet enriched with proteins and also was provided with powdered protein (calcium caseinate, T. Aliment – Espècies Teixidor, Barcelona, Spain).

Nutritional Support

The 10 g of protein (calcium caseinate) powder diluted in water or juice was taken daily at breakfast and twice week additional 10 g of protein administration just after exercise performance.

Analytical Methods

Serum glucose, glycated hemoglobin (HbA1c), serum insulin, total cholesterol, LDL and HDL cholesterol, triglycerides, were determined as described elsewhere (Moreno-Navarrete et al., 2013). C-reactive protein (ultrasensitive assay; 110 Beckman, Fullerton, CA, United States) was determined by a routine laboratory test. Leucocytes, neutrophils and lymphocytes cell count and albumin and creatinine measurement was performed by routine laboratory analysis. Irisin were determined using Irisin ELISA Kit (Catalog Number RAG018R, BIOVENDOR, Brno, Czechia). According to manufacturer information, this ELISA is specific for the measurement of natural and recombinant irisin in human samples, and it does not cross-react with FNDC4, human adiponectin, human Nampt, human RBP4, human clusterin, human leptin, human vaspin, human GPX3, human resistin, human ACE2, human lipocalin-2, human ANGPTL3, human ANGPTL6, human DNER, human DLK1, human calreticulin, and human IL-33. Myostatin concentrations were measured by Quantikine®ELISA GDF-8 / Myostatin Immunoassay (Catalog Number DGDF80, R&D Systems, Inc., Minneapolis, MN, United States). In both irisin and myostatin ELISA, intra- and interassay coefficients of variation were lower than 10%. Irisin was analyzed in all participants from RE and RENS group, but only in first recruited control (n = 15) subjects. Otherwise myostatin, which was measured several months after the end of the study, was analyzed in all participants.

Statistical Analyses

Statistical analyses were performed using SPSS 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables or median and interquartile range. The relation between variables was analyzed by simple correlation (Spearman's test). One factor ANOVA with *post hoc* Bonferroni test and paired t-test were used to compare categorical parameters. Levels of statistical significance were set at $p < 0.05$.

RESULTS

The current study included 20 participants (18 women and 2 men) in C, 14 participants (12 women and 2 men) in RE and 9 participants (only women) in RENS, with an age range of 60–75 years-old (**Table 1**). Mean age was significantly higher in RENS compared to RE group (71.2 ± 3.3 vs. 64.9 ± 5.5, $p = 0.02$) (**Table 1**).

Changes in body composition, anthropometric and physical condition parameters at baseline and at the end of study in each group were shown in **Table 1**. At baseline, no significant differences were found between control and intervention groups (**Table 1**).

Compared to C, RENS resulted in increased percent change of fat free mass kg and calf circumference and in decreased percent change of fat mass kg (**Figure 1A**). RE also resulted in increased percent change of fat free mass kg compared to C ($p = 0.027$) (**Figure 1A**).

In C group, a significant decrease in waist and calf circumference, fat free mass, HbA1c, serum myostatin and speed at 3 m, but in the context of increased fat mass was found (**Tables 1**, **2**). In RE group, a significant increase in strength and circulating irisin, and a decrease in speed at 2.44, 4.5, and 3 m was found (**Tables 1**, **2**). In RENS group, a significant increase in muscle arm circumference, calf circumference, fat free mass and circulating irisin was observed (**Tables 1**, **2**). Interestingly, in this group, strength was positively correlated with fat free mass [($r = 0.782$, $p = 0.008$), **Table 3**].

Since mean age in RENS was higher than in RE, multivariate regression analysis was performed after adjusting for age. This analysis indicated that fat-free mass ($\beta = 0.850$, $p = 0.004$)

TABLE 1 | Evolution of body composition, anthropometric parameters walking speed and strength.

	Control (*n* = 20)			RE (*n* = 14)			RENS (*n* = 9)				
	At baseline	**At follow-up**	**p intra ***	**At baseline**	**At follow-up**	**p intra***	**At baseline**	**At follow-up**	**p intra***	**P inter** At baseline**	**P inter** At follow-up**
Age	66.4 (±4.6)			64.9 (±5.5)			71.2 (±3.3)$^{\#\nabla}$			**0.016**	
Weight (kg)	71 (±14.3)	70.7 (±14.7)	0.530	68.9 (±14.2)	68.7 (±14.1)	0.444	72.3 (±11.6)	72.5 (±12)	0.465	0.813	0.800
BMI (kg/m^2)	28.9 (±4.6)	28.7 (±4.8)	0.231	29.1 (±4.8)	29 (±4.7)	0.505	30 (±2.8)	30.1 (±3.1)	0.525	0.861	0.723
Waist perimeter (cm)	93 (±11.9)	90 (±10.7)	**0.003**	92.8 (±14.4)	92.6 (±14.2)	0.837	96.1 (±9.9*)	96.1 (±10.5)	0.809	0.727	0.670
Arm circumference (cm)	31.6 (±4)	31.8 (±4.2)	0.589	31 (±2.3)	31.4 (±3)	0.243	32.2 (±3.3)	32.4 (±3)	0.635	0.675	0.806
Muscle arm circumference (cm)	23.6 (±2.7)	24 (±2.1)	0.443	22.5 (±2)	23.3 (±2.6)	0.088	23.3 (±3)	24.3 (±2)	**0.038**	0.451	0.621
Abdominal circumference (cm)	105 (±11)	105 (±10)	0.664	92 (±10)	84 (±9)	0.287	97.5 (±12.9)	102 (±7.9)	0.231	0.160	**0.040**
Calf circumference (cm)	33.1 (±3)	32 (±2.8)	**0.008**	33 (±2.4)	32.7 (±2.1)	0.504	32.3 (±4.3)	36.4 (±1.3)	**0.025**	0.735	**0.001**
Basal metabolism (kcal)	1372 (±218)	1347 (222)	**0.004**	1307 (±254)	1306 (±259)	0.824	1390 (±208)	1409 (±208)	**0.038**	0.600	0.554
Fat mass (kg)	25.1 (±8.6)	25.9 (±8.8)	**0.043**	24.8 (±8.5)	24.6 (±7.7)	0.763	25.7 (±6.5)	25.1 (±7.1)	0.294	1	0.911
% Fat mass	34.9 (±5.2)	36.1 (±5.6)	**0.005**	36.3 (±6.6)	36.1 (±5.8)	0.665	35.2 (±6)	34.1 (±6.7)	0.111	0.859	0.560
Fat free mass (kg)	46 (±7.5)	45 (±7.7)	**0.002**	43.8 (±9.5)	43.7 (±9.9)	0.733	46.5 (±7.4)	47.4 (±7.4)	**0.046**	0.511	0.583
% Fat free mass	65.4 (±5.6)	64 (±5.2)	**0.003**	63.1 (±6.6)	63.1 (±5.9)	0.909	65 (±6)	65.7 (±6.7)	0.377	0.692	0.5534
Total water (%)	45.4 (±3.4)	44.7 (±3.4)	0.051	45.3 (±4.6)	45.2 (±4)	0.6781	45.1 (±3.9)	456 (±4.5)	0.102	0.739	0.699
Total water (kg)	31.8 (±4.9)	31.1 (±4.9)	**0.016**	30.5 (±6.6)	30.4 (±4)	0.696	32.5 (±5)	32 (±7.9)	0.457	0.369	0.906
Speed at 2.44 m	3.2 (±0.75)	3 (±0.73)	0.082	3.5 (±0.7)	2.9 (±0.5)	**0.005**	3.5 (±0.7)	3.5 (±0.8)	0.881	0.589	0.107
Speed at 4.5 m	5.6 (±1.2)	5.2 (±0.9)	0.134	5.9 (±0.9)	5.2 (±0.1)	**0.013**	5.9 (±1)	5.9 (±1.1)	0.961	0.460	0.140
Speed at 3 m	3.7 (±0.7)	3.4 (±0.7)	**0.016**	3.89 (±0.7)	3.2 (±0.6)	**0.003**	3.9 (0.9)	3.8 (±1)	0.791	0.773	0.210
Grip strength (kg)	16.7 (±4.9)	17 (±5.6)	0.639	15.3 (±4.6)	17.2 (±4.6)	**0.011**	16.8 (±5.9)	17.8 (±6.5)	0.185	0.638	0.946

**p-value applying T student for matched data. **p-value applying one-way anova. $^{\#}p = 0.03$ compared to C. $^{\nabla}p = 0.02$ compared to RE. Bolded values mean statistical significance ($p < 0.05$).*

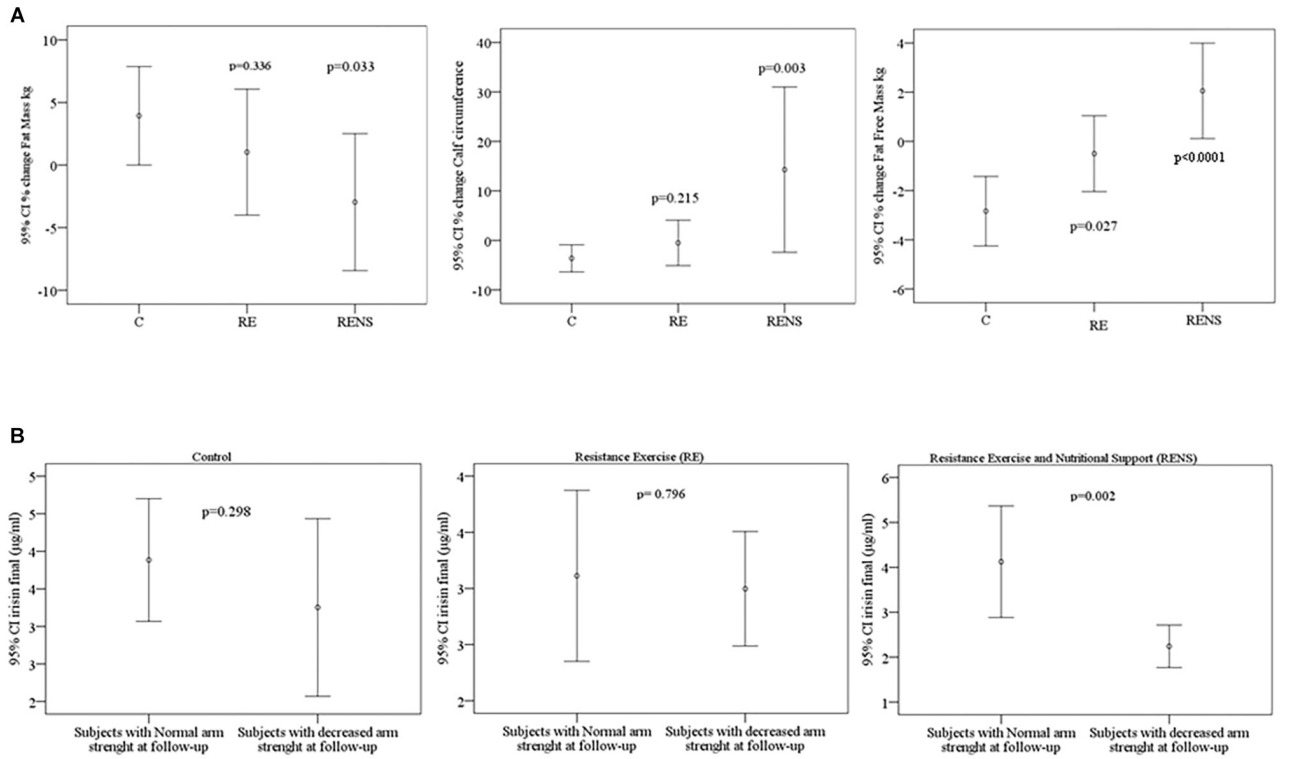

FIGURE 1 | (A) Comparison of percent change of fat free mass, calf circumference and fat mass according to control (C), resistance exercise (RE), and resistance exercise plus nutritional support (RENS). **(B)** Comparison of circulating irisin according to arm strength in C, RE, and RENS groups.

contributed independently to strength variance at follow up after controlling for age in RENS group.

The study showed a percentage of change in force of 2.9% in C group, of 16% in RE and 4% in RENS (p = 0.181). In RE group, the gain of strength expressed in kg significantly increased from an at baseline strength (from 15.3 to 17.2 kg, p = 0.011), and in RENS group also tended to increase (from 16.8 to 17.8 kg, p = 0.185). Moreover, in RENS group, the force was positively correlated with kg of fat-free mass (p = 0.008), kg of muscle mass (p = 0.008), kg of bone (p = 0.008) and kg protein mass (p = 0.009) (**Tables 1**, **3**).

Of note, in RENS group, circulating irisin was significantly decreased in those participants with decreased arm strength at the end of the study (**Figure 1B**).

No significant correlation between circulating irisin and myostatin in any group was observed (**Table 4**).

At baseline, no significant correlation between circulating irisin and anthropometrical and clinical parameters were observed. Circulating myostatin was positively correlated with weight, muscle arm circumference, basal metabolism, fat-free mass, total water and negatively correlated with C-reactive protein (**Table 5**). In control group, circulating myostatin was positively correlated with speed at 2.44 m (**Figure 2**).

At follow up, no significant associations among irisin, anthropometrical and clinical parameters in control (**Table 6**), RE (**Table 7**) or RENS (**Table 8**) were found. In control group, circulating myostatin was positively correlated BMI, abdominal circumference, total water, HOMA, triglycerides, C-reactive protein (**Table 6**), but these associations were not observed in RE (**Table 7**) or RENS groups (**Table 8**).

The walking speed decreased significantly in C and RE group, moving from 3.75 to 3.4 m/s at the end (p = 0.016) in C and from 3.9 m/s to a at follow-up speed of 3.2 m/s (p = 0.003) in RE, but not in RENS, in which walking speed change from 3.9 m/s to a at follow-up speed of 3.8 m/s (p = 0.791) (**Table 2**).

DISCUSSION

In this clinical trial, the impact of low-intensity resistance exercise and nutritional assistance program on body composition, anthropometric parameters, strength, speed of motion and myokines were examined in elderly people.

Body Composition and Anthropometric Parameters

Current data confirmed that low intensity exercising program and nutritional assistance prevented muscle deterioration in people aged 60–75 years old. Exercise improves muscle mass and contributes to the maintenance of daily life activities (Löllgen et al., 2009). Both aerobic and resistance exercise delay the deterioration of the muscles, but resistance exercise seems the most effective (Visvanathan and Chapman, 2010). Here, we

TABLE 2 | Clinical and biochemical parameters in study cohort.

	Control (*n* = 20)			RE (*n* = 14)			RENS (*n* = 9)				
	At baseline	**At follow-up**	**p intra ***	**At baseline**	**At follow-up**	**p intra***	**At baseline**	**At follow-up**	**p intra***	**P inter** At baseline**	**P inter** At follow-up**
Glucose (mg/dl)	110 (±33)	109 (±19)	0.878	104 (±26)	102 (±22)	0.613	97 (±10)	98 (±8)	0.557	0.473	0.279
Insulin (μIU/ml)	7.2±15)	7.1 (±4)	0.954	14.1 (±23)	7.6 (±4.8)	0.295	4.4 (±8.7)	8.4 (±5.8)	0.075	0.316	0.793
HbA1c (%)	5.8 (±0.5)	5.7 (±0.5)	**0.008**	5.8 (±0.8)	6 (±0.5)	0.088	5.5 (±0.3)	5.6 (±0.3)	0.399	0.3475	0.634
HOMA	2.8 (±2.5)	2 (±1.4)	0.243	5.3 (±10.7)	2 (±1.6)	0.297	1 (±2.3)	2.1 (±1.6)	0.076	0.297	0.966
Albumin (g/dl)	4.5 (±0.2)	4.4 (±0.2)	**0.043**	4.5 (±0.2)	4.4 (±0.2)	0.497	4.3 (±0.1)	4.2 (±0.1)	0.282	0.052	0.128
Protein (g/dl)	7.1 (±0.3)	7.1 (±0.3)	0.523	7.1 (±0.3)	7 (±0.3)	0.088	6.9 (±0.1)	6.9 (±0.1)	0.255	0.354	0.302
Total cholesterol (mg/dl)	210 (±30)	203 (±30)	0.073	224 (±56)	227 (±68)	0.728	214 (±37)	217 (±42)	0.0624	0.619	0.336
HDL-cholesterol (mg/dl)	65 (±14)	61 (±14)	0.110	61 (14)	62 (±15)	0.805	64 (±16)	63 (±15)	0.566	0.758	0.972
LDL-cholesterol (mg/dl)	125 (±28)	120 (±128)	0.192	141 (±52)	144 (±60)	0.755	130 (±36)	134 (±41)	0.622	0.467	0.266
Triglycerides (mg/dl)	101 (±36)	105 (±38)	0.570	117 (±76)	122 (±94)	0.453	96 (±41)	100 (±47)	0.544	0.592	0.623
C-reactive protein (mg/dl)	2.2 (8.3)	1.5 (±3)	0.642	0.7 (±1.3)	0.8 (±2.4)	0.858	0.3 (±0.2)	0.4 (±0.3)	0.326	0.614	0.533
Leucocytes	6.8 (±2.3)	6.1 (±1.4)	0.128	5.7 (±1.7)	6 (±2)	0.141	5.6 (±0.9)	5.7 (±1)	0.471	0.120	0.821
Neutrophils	3825 (±1910)	3310 (±1042)	0.134	3100 (±1204)	3240 (±1382)	0.348	3211 (±726)	3211 (±765)	0.405	0.289	0.973
Lymphocytes	2059 (±608)	2047 (±587)	0.482	2000 (±633)	1866 (±588)	0.191	1940 (±765)	1760 (±613)	0.301	0.449	0.709
Irisin (μg/ml)[#]	3.1 (±0.9)	3.5 (±1.1)	0.127	2.4 (±0.3)	3.1 (±0.8)	**0.011**	2.6 (±1.3)	3 (±1.1)	**0.030**	0.116	0.407
Myostatin (ng/dl)	2.5 (1.8–3.1)	1.9 (1.5–2.3)	**0.003**	1.37 (1.2–2.2)	2 (1.4–2.5)	0.203	2.3 (2–2.9)	2.1 (1.8–3.4)	0.736	**0.025**	**0.048**

**p-value applying T student for matched data. **p-value applying one-way anova. [#]In control group was analyzed only in 15 participants (as explained in methods), whereas in RE and RENS was analyzed in all participants. Bolded values mean statistical significance (p < 0.05).*

TABLE 3 | Bivariate correlations between strength with anthropometric parameters.

	Strength (at follow-up)					
	Control (*n* = 20)		RE (*n* = 14)		RENS (*n* = 9)	
	r	p	r	p	r	p
Fat free mass at follow-up (kg)	0.232	0.311	0.413	0.183	0.782	**0.008**
Muscle mass at follow-up (kg)	0.232	0.311	0.413	0.183	0.782	**0.008**
Protein mass at follow-up (kg)	0.122	0.608	0.521	0.101	0.770	**0.009**
Bone at follow-up (kg)	0.141	0.532	0.393	0.165	0.782	**0.008**

Bold values mean statistical significance ($p < 0.05$).

showed that 16-week low intensity resistance program produced significant changes on body composition (fat mass, percent fat mass, fat-free mass, muscle mass kg and the calf circumference). These results were expected and are in agreement with previous studies (Liao et al., 2017), and indicated that low intensity resistance training promotes musculature improvement, is a useful and simple therapeutic approach to prevent sarcopenia, and when was combined with protein supplementation these beneficial effects were enhanced (Liao et al., 2017). However, resistance training presents some limitations (Morgan, 2012; Malafarina et al., 2013; Yu, 2015). For instance, one important limitation was the rapid loss of beneficial effects, such as the gain of muscle mass (in the study the improvement is 0.7% $p < 0.001$) and adherence to exercise. The study's approach of performing low-intensity resistance exercises aims to inform to the subject about exercise strategies that can be performed at home, since equipment is not required to be able to perform them, and the importance of training as long as possible without causing muscle overload injuries (Johnston et al., 2008).

In control group, waist and calf circumference were reduced in parallel to fat free mass and decreased speed at 3 m, indicating aging-associated body weight reduction (Dzien et al., 2013), muscle mass loss and worsening of physical condition. Elderly associated physical inactivity is known to accelerate muscle mass loss (Evans, 2010). In fact, physical activity reduced the progression of muscle aging and decreased the strength of muscle contraction (Hughes et al., 2004; Zampieri et al., 2015), attenuating sarcopenia in pre- and post-menopausal women (Walsh et al., 2006) and in older men (Szulc et al., 2005). On the other hand, 10 days of bed rest resulted in a significant decreased lean mass and skeletal muscle loss in healthy older adults, even greater than young individuals after 28 days (Kortebein et al., 2007).

Otherwise, the reduction of HbA1c from 5.8 to 5.7 has not clinical relevance.

Here, we found the following effects of resistance training on strength, speed and myokines:

(i) *Strength.* Resistance training is the most effective strategy to increase muscle mass and strength (Leenders et al., 2013; Yoshimura et al., 2017). Being the grip strength is a good indicator of low muscle mass (Lauretani et al., 2003), this parameter detects individuals with low muscle mass that will make them more fragile and with greater risk of disability (Ling et al., 2010; Taekema et al., 2010). We observed that there was no significant association between the concentration of myostatin and muscular force, as concluded by Carvalho et al. (2018).

(ii) *Speed.* It is known that exercise interventions that increase muscle strength are very effective in improving walking speed (Gottlob, 2008). In the present study, the walking speed decreased in all groups producing in improvement of 15% in control group, 14% in Resistance Exercise group, and 7% in Resistance Exercise and Nutritional Support group. Different studies have shown improvements between 3 and 10% (Mendieta et al., 2015), being the results obtained in the current study similar to those reported. The walking speed reflects the health and functionality of the person (Abellan van Kan et al.,

TABLE 4 | Bivariate correlations between Irisin and Myostatin.

	Control (*n* = 15)							
	Irisin (at baseline) (μg/ml)		Myostatin (at baseline) (ng/ml)		Irisin (at follow-up) (μg/ml)		Myostatin (at follow-up) (ng/ml)	
	r	p	r	p	r	p	r	p
Irisin (at baseline) (μg/ml)	–	–	−0.027	0.928	0.470	0.077	0.333	0.266
Irisin (at follow-up) (μg/ml)	0.470	0.077	0.099	0.748	–	–	−0.041	0.894
RE (*n* = 14)								
Irisin (at baseline) (μg/ml)	–	–	0.057	0.846	−0.013	0.965	−0.537	**0.048**
Irisin (at follow-up) (μg/ml)	−0.013	0.965	0.011	0.970	–	–	0.132	0.653
RENS (*n* = 9)								
Irisin (at baseline) (μg/ml)	–	–	−0.683	0.042	0.833	**0.005**	−0.483	0.187
Irisin (at follow-up) (μg/ml)	0.833	**0.005**	−0.467	0.205	–	–	−0.483	0.187

Bold values mean statistical significance ($p < 0.05$).

TABLE 5 | Bivariate correlations between anthropometrical and clinical parameters and circulating irisin and myostatin in all participants at baseline.

	Irisin		Myostatin	
	r	p	r	p
Weight (kg)	−0.039	0.809	0.316	**0.039**
BMI (kg/m^2)	0.009	0.955	0.198	0.203
Arm circumference (cm)	−0.201	0.207	0.285	0.064
Muscle arm circumference (cm)	−0.174	0.276	0.396	**0.009**
Waist perimeter (cm)	0.113	0.506	0.040	0.806
Abdominal circumference (cm)	0.033	0.878	0.255	0.191
Calf circumference (cm)	0.094	0.621	0.180	0.316
Basal metabolism (kcal)	−0.019	0.909	0.365	**0.016**
Fat mass (kg)	−0.070	0.622	0.144	0.357
%Fat mass	−0.128	0.430	0.053	0.741
Fat free mass (kg)	0.044	0.785	0.358	**0.018**
% Fat free mass	0.194	0.224	−0.028	0.858
Total water (kg)	0.042	0.794	0.364	**0.017**
%Total water	0.154	0.336	−0.077	0.623
Speed at 2.44 m (seconds)	−0.018	0.912	0.258	0.095
Speed at 4.55 m (seconds)	0.165	0.310	−0.086	0.588
Speed at 3 m (seconds)	−0.144	0.370	0.106	0.498
Grip strength (kg)	0.264	0.096	−0.048	0.761
Glucose (mg/dl)	−0.036	0.829	−0.071	0.665
Insulina (μIU/ml)	−0.164	0.339	−0.192	0.218
HbA1c (%)	0.146	0.368	−0.181	0.249
HOMA	−0.070	0.698	−0.163	0.316
Albumin (g/dl)	0.153	0.358	0.023	0.890
Protein (g/dl)	−0.102	0.535	−0.197	0.212
Total cholesterol (mg/dl)	−0.044	0.784	−0.107	0.494
HDL cholesterol (mg/dl)	−0.058	0.718	0.008	0.958
LDL cholesterol mg/dl)	−0.107	0.513	−0.008	0.580
Tryglicerides (mg/dl)	0.144	0.369	−0.086	0.582
C-reactive protein (mg/dl)	0.249	0.126	−0.388	**0.012**
Leucocytes	−0.187	0.254	−0.039	0.808
Neutrophils	0.079	0.635	−0.066	0.681
Lymphocytes	−0.159	0.335	−0.200	0.209

Bold values mean statistical significance ($p < 0.05$).

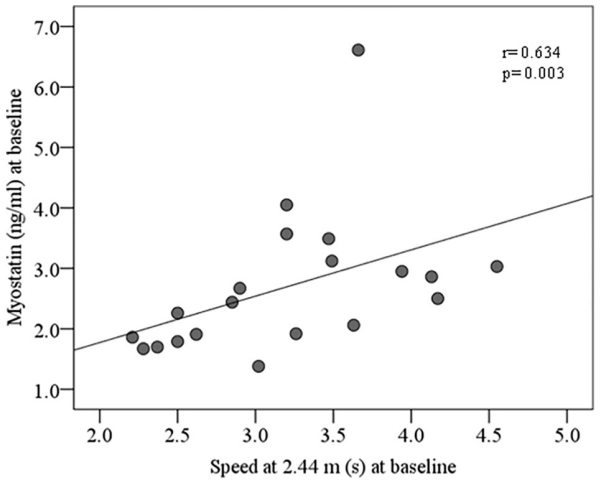

FIGURE 2 | Bivariate correlation between circulating myostatin and speed at 2.44 m at baseline in control group.

2009). This is recommended as a clinical indicator to assess survival. In a meta-analysis published in 2011 in the journal JAMA by Studenski et al. (2011) walking speed within their range of normality behaves as a protective factor against mortality, being the lowest in those individuals with faster walking. A lower driving speed is usually associated with disability, cognitive deterioration, accidental falls, various neurological diseases, cardiopulmonary and orthopedic diseases that contribute to increase mortality rate (Abellan van Kan et al., 2009; Studenski et al., 2011).

(iii) *Myokines.* Myostatin is a hormone that is mainly expressed in the muscle and inhibits the formation of muscle mass. Myostatin is generated as a precursor protein that requires the proteolytic division to release the N-terminal and the C-terminal propeptide. The active form of myostatin is a disulfide dimer attached to the C-terminal fragment (White and LeBrasseur, 2014).

Myostatin increases with age, although differences in total vs. active form have not been well investigated (Yarasheski et al., 2002). The current study, that analyzed serum myostatin concentrations before and after the resistance exercise program, found results that are contradictory with those reported in the literature. In the control group, circulating myostatin concentration decreased significantly after 16 weeks, in parallel to decreased fat free mass, whereas, in RE and RENS group, myostatin remained unchanged after the exercise, and fat free mass increased. These findings were in hard contrast to previous studies that demonstrated negative effects of myostatin in muscle mass development (McPherron et al., 1997; Lee and McPherron, 2001; Zimmers et al., 2002; Wolfaman et al., 2003; Lee, 2004). Possibly, in control group, a reduction of myostatin causes significant decrease in primary fibers of skeletal muscle, which is associated with increased fatigue susceptibility (Hennebry et al., 2009), indicating physical condition decline. In line with current data, in a recent study low serum myostatin levels have been associated with low skeletal muscle (Peng et al., 2018). Muscular force exercise is known to inhibit local myostatin (Brotto and Abreu, 2012). However, Laksmi et al. (2017) observed increased secretion of myostatin in blood in response to exercise. As, the antibodies used to detect myostatin (current ELISA) did not distinguish between the active or latent form, further research is needed.

Irisin is a myokine produced by the proteolytic cleavage of the membrane-protein, fibronectin type III "domain-containing" protein-5 (FNDC5) (Boström et al., 2012). It is regulated by peroxisome proliferator activated receptor (PPAR) and peroxisome proliferator activated receptor γ co-activator-1-α (PGC1α). It is suggested that irisin may act in some of the beneficial effects of exercise

TABLE 6 | Bivariate correlations between anthropometrical and clinical parameters and circulating irisin and myostatin in control group at follow-up.

	Irisin		Myostatin	
	r	p	r	p
Weight (kg)	−0.021	0.940	0.370	0.109
BMI (kg/m^2)	0.002	0.995	0.464	**0.039**
Arm circumference (cm)	−0.013	0.964	0.196	0.422
Muscle arm circumference (cm)	0.143	0.626	0.345	0.148
Waist perimeter (cm)	−0.040	0.893	0.423	0.071
Abdominal circumference (cm)	0.014	0.966	0.487	**0.041**
Calf circumference (cm)	0.353	0.215	−0.107	0.662
Basal metabolism (kcal)	0.011	0.970	0.388	0.091
Fat mass (kg)	−0.103	0.725	0.366	0.123
%Fat mass	−0.263	0.344	0.065	0.784
Fat free mass (kg)	−0.046	0.876	0.337	0.158
% Fat free mass	0.169	0.563	−0.131	0.593
Total water (kg)	0.058	0.851	0.547	**0.019**
%Total water	0.236	0.437	−0.024	0.925
Speed at 2.44 m (seconds)	−0.109	0.699	0.387	0.092
Speed at 4.55 m (seconds)	0.091	0.747	0.239	0.310
Speed at 3 m (seconds)	−0.247	0.375	0.268	0.253
Grip strength (kg)	0.071	0.800	−0.078	0.743
Glucose (mg/dl)	−0.446	0.110	0.297	0.217
Insulina (μIU/ml)	−0.258	0.394	0.308	0.187
HbA1c (%)	−0.201	0.472	0.051	0.829
HOMA	−0.371	0.236	0.508	**0.026**
Albumin (g/dl)	−0.013	0.964	−0.130	0.585
Protein (g/dl)	−0.069	0.816	0.131	0.594
Total cholesterol (mg/dl)	−0.339	0.216	−0.333	0.151
HDL cholesterol (mg/dl)	−0.232	0.405	−0.348	0.133
LDL cholesterol mg/dl)	−0.338	0.218	−0.348	0.133
Tryglicerides (mg/dl)	0.289	0.297	0.462	**0.040**
C-reactive protein (mg/dl)	−0.068	0.810	0.528	**0.017**
Leucocytes	−0.147	0.615	0.120	0.624
Neutrophils	−0.101	0.730	0.227	0.349
Lymphocytes	0.056	0.844	−0.320	0.169

Bolded values mean statistical significance ($p < 0.05$).

TABLE 7 | Bivariate correlations between anthropometrical and clinical parameters and circulating irisin and myostatin in resistance exercise group at follow-up.

	Irisin		Myostatin	
	r	p	r	p
Weight (kg)	0.059	0.835	−0.147	0.615
BMI (kg/m^2)	−0.157	0.576	0.055	0.852
Arm circumference (cm)	0.122	0.664	−0.283	0.327
Muscle arm circumference (cm)	0.018	0.950	−0.218	0.455
Waist perimeter (cm)	0.030	0.914	−0.081	0.782
Abdominal circumference (cm)	0.500	0.667	−0.500	0.667
Calf circumference (cm)	−0.109	0.711	−0.320	0.287
Basal metabolism (kcal)	0.113	0.689	−0.385	0.175
Fat mass (kg)	−0.027	0.937	0.115	0.751
%Fat mass	−0.122	0.666	0.415	0.140
Fat free mass (kg)	0.172	0.594	−0.391	0.235
% Fat free mass	0.193	0.490	−0.431	0.124
Total water (kg)	0.077	0.821	−0.745	**0.013**
%Total water	0.296	0.377	−0.091	0.803
Speed at 2.44 m (seconds)	0.161	0.566	−0.167	0.568
Speed at 4.55 m (seconds)	0.165	0.558	−0.123	0.675
Speed at 3 m (seconds)	−0.113	0.689	−0.279	0.334
Grip strength (kg)	0.227	0.416	−0.266	0.358
Glucose (mg/dl)	0.065	0.819	−0.651	**0.012**
Insulina (μIU/ml)	−0.134	0.647	0.385	0.175
HbA1c (%)	0.174	0.534	−0.527	0.053
HOMA	−0.147	0.615	0.345	0.227
Albumin (g/dl)	0.045	0.874	−0.070	0.812
Protein (g/dl)	−0.274	0.322	0.372	0.191
Total cholesterol (mg/dl)	−0.441	0.099	0.402	0.154
HDL cholesterol (mg/dl)	−0.213	0.446	0.284	0.325
LDL cholesterol mg/dl)	−0.396	0.161	0.560	**0.046**
Tryglicerides (mg/dl)	−0.133	0.636	−0.009	0.976
C-reactive protein (mg/dl)	−0.184	0.529	0.033	0.915
Leucocytes	−0.214	0.443	0.051	0.864
Neutrophils	0.014	0.960	0.221	0.449
Lymphocytes	−0.432	0.108	−0.150	0.609

Bold values mean statistical significance ($p < 0.05$).

by inducing the uncoupling protein one (UCP-1), that afterward increases the energy expenditure of white adipocytes, process called "browning." This process is characterized by a switch of white to beige adipose tissue in response to β-adrenergic stimuli (exposure to cold and physical activity) (Novelle et al., 2013). In humans, the effect of exercise on irisin production has provided contradictory findings (Huh et al., 2012; Lecker et al., 2012; Timmons et al., 2012). Huh et al. (2012) correlated the decrease in the circulating irisin concentration with the loss of muscle mass associated to aging. A recent study by Chang et al. (2017) concluded that a low blood concentration of irisin was a sensitive molecular marker for muscle weakness and atrophy. In post-menopausal women, decreased serum irisin concentration is an independent predictor of sarcopenia (Park et al., 2018). In fact, irisin has been proposed as a molecule that combines beneficial effects for treating osteoporosis and muscular atrophy through its effects restoring bone and preventing muscle wasting (Kim et al., 2015; Colaianni et al., 2017).

In agreement with these studies, current findings indicated that RE and RENS resulted in increased circulating irisin in parallel to strength and walking speed in elderly subjects. In fact, decreased circulating irisin was significantly associated with strength loss at the end of the study (**Figure 1B**). Thus, irisin could be a potential biomarker of muscle dysfunction (Chang et al., 2017).

In relation to protein supplementation, several studies indicated that protein supplementation improved the adaptations of resistance exercise (Burke et al., 2001; Candow et al., 2006; Cribb et al., 2006; Hulmi et al., 2009; Volek et al., 2013;

TABLE 8 | Bivariate correlations between anthropometrical and clinical parameters and circulating irisin and myostatin in resistance exercise and nutritional support group at follow-up.

	Irisin		Myostatin	
	r	p	r	p
Weight (kg)	−0.233	0.546	0.517	0.154
BMI (kg/m^2)	−0.267	0.488	0.150	0.700
Arm circumference (cm)	−0.400	0.286	0.033	0.932
Muscle arm circumference (cm)	−0.517	0.154	0.717	**0.030**
Waist perimeter (cm)	0.133	0.732	0.467	0.205
Abdominal circumference (cm)	−0.143	0.736	0.238	0.570
Calf circumference (cm)	0.554	0.154	−0.289	0.487
Basal metabolism (kcal)	0.183	0.637	0.350	0.356
Fat mass (kg)	−0.250	0.516	0.133	0.732
%Fat mass	−0.350	0.356	−0.150	0.700
Fat free mass (kg)	0.183	0.637	0.350	0.356
% Fat free mass	0.350	0.356	0.150	0.700
Total water (kg)	0.083	0.831	−0.117	0.765
%Total water	0.343	0.366	0.142	0.715
Speed at 2.44 m (seconds)	−0.550	0.125	0.267	0.488
Speed at 4.55 m (seconds)	−0.483	0.187	0.300	0.433
Speed at 3 m (seconds)	−0.583	0.099	0.183	0.637
Grip strength (kg)	0.467	0.205	0.250	0.516
Glucose (mg/dl)	0.633	0.067	−0.133	0.732
Insulina (μIU/ml)	0.450	0.224	−0.150	0.700
HbA1c (%)	0.611	0.081	−0.569	0.110
HOMA	0.450	0.224	−0.150	0.700
Albumin (g/dl)	0.061	0.877	0.087	0.325
Protein (g/dl)	−0.042	0.915	0.244	0.527
Total cholesterol (mg/dl)	0.276	0.472	−0.377	0.318
HDL cholesterol (mg/dl)	−0.159	0.683	−0.460	0.213
LDL cholesterol mg/dl)	0.200	0.606	−0.150	0.700
Tryglicerides (mg/dl)	0.483	0.187	−0.233	0.546
C-reactive protein (mg/dl)	0.310	0.417	−0.870	**0.002**
Leucocytes	0.500	0.207	−0.381	0.352
Neutrophils	0.790	**0.020**	−0.323	0.435
Lymphocytes	−0.211	0.586	−0.270	0.482

Bold values mean statistical significance ($p < 0.05$).

Morton et al., 2017). Cribb et al. (2006) reported a 5 kg increase of fat-free mass after 10 weeks of resistance training combined with 45 g/day ingest of milk protein. Burke et al. (2001), Candow et al. (2006) demonstrated that milk protein supplementation during 6 weeks of resistance training promoted an increase of 2.3–2.5 kg of fat-free mass. Hulmi et al. (2009) also reported that supplementation with milk protein and 10 weeks of resistance exercise resulted in an increase of 2.5 kg fat free mass. Volek et al. (2013) reported that supplementation with milk protein from cow or soy in addition with resistance training for 9 months increased by 3.6 and 2.6 kg of fat-free mass, respectively. Morton et al. (2017) suggest that one of the mechanisms responsible for the enhancement of skeletal mass could be explained for the improvement of the anabolism of skeletal muscle. These positive effects of protein supplementation do not have the same effects as isolated supplementation with amino acids (Bird et al., 2006; Vieillevoye et al., 2010; Aguiar et al., 2017). In line with all these studies, current findings confirmed an improvement in fat-free mass in the exercise group with cow milk protein supplementation. Even though, this improvement was not so marked as that described in the literature, probably due to relative low protein supplementation (10 g of protein every day and 20 g during exercise days) did not reach the threshold to promote anabolism of muscle protein in older people (Traylor et al., 2018). An important consideration is that the attachment to nutritional support was not homogeneous in all participants.

Resistance exercise and nutritional support resulted in improved anthropometric parameters (muscle arm circumference, calf circumference, basal metabolism and fat free mass) compared with RE intervention. The improvement in walking speed and grip strength was not so marked in the RENS group compared to RE intervention. These differences could be due to significant increased mean age in RENS compared to RE subjects.

CONCLUSION

A program of physical exercise of low intensity resistance, together with a nutritional support, improved the deterioration of the musculature in people aged 60–75. Circulating irisin, but not myostatin, constituted a marker for improved muscle strength after resistance exercise in elderly subjects.

ETHICS STATEMENT

The development of the study was carried out in accordance with the Helsinki Declaration of the World Medical Association on the ethical principles for research in human subjects. The current study followed the ethical principles according to the Organic Law 15/99 (LOPD) and the guideline document of good clinical practices. The protocol was approved by the Ethics Committee of the Dr. Josep Trueta University Hospital of Girona on July 30, 2012 under the code 2012099.

AUTHOR CONTRIBUTIONS

CP-F, WR, and JF-R participated in the study design and analysis of data. FC, MS-M, MM, JMM-N, and OR participated in acquisition of data. CP-F, WR, JMM-N, and JF-R participated in interpretation of data. CP-F, FC, and JF-R wrote and edited the manuscript. JMM-N and WR revised the manuscript critically for important intellectual content. All authors participated in final approval of the version to be published.

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REVIEW
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Contribution of Extracellular Vesicles in Rebuilding Injured Muscles

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Skeletal myofibers are injured due to mechanical stresses experienced during physical activity, or due to myofiber fragility caused by genetic diseases. The injured myofiber needs to be repaired or regenerated to restore the loss in muscle tissue function. Myofiber repair and regeneration requires coordinated action of various intercellular signaling factors—including proteins, inflammatory cytokines, miRNAs, and membrane lipids. It is increasingly being recognized release and transmission of these signaling factors involves extracellular vesicle (EV) released by myofibers and other cells in the injured muscle. Intercellular signaling by these EVs alters the phenotype of their target cells either by directly delivering the functional proteins and lipids or by modifying longer-term gene expression. These changes in the target cells activate downstream pathways involved in tissue homeostasis and repair. The EVs are heterogeneous with regards to their size, composition, cargo, location, as well as time-course of genesis and release. These differences impact on the subsequent repair and regeneration of injured skeletal muscles. This review focuses on how intracellular vesicle production, cargo packaging, and secretion by injured muscle, modulates specific reparative, and regenerative processes. Insights into the formation of these vesicles and their signaling properties offer new understandings of the orchestrated response necessary for optimal muscle repair and regeneration.

Keywords: injury, exosomes, ectosomes, skeletal muscle, myogenesis, miRNA, endocytosis, ESCRT

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INTRODUCTION

Skeletal muscle comprises over a third of the total human body mass, making it one of our largest organ systems. The contractile activity of the skeletal myofiber generates the force that enables and controls physical movement. This mechanical activity constantly subjects the skeletal muscle to stresses and strains that results in myofiber injury. Minor lesions inflicted upon skeletal myofibers, cause sarcolemmal disruption. The process of "myofiber repair" rectifies such membrane disruptions, preventing death of the injured myofiber. However, severe muscle injuries incurred via heavy overload, resistance training stresses, or genetic defects (e.g., muscular dystrophy), cause myofiber death. Such injuries are repaired by a highly orchestrated process involving inflammation and satellite cell activation and fusion that replaces the dead myofiber through the process of "myofiber regeneration." Together, repair and regeneration of skeletal myofibers are essential for skeletal muscle maintenance in patients with chronic muscle disorders as well as restoration of functional performance in individuals that suffer sport

injuries (Counsel and Breidahl, 2010; Tidball, 2017). Understanding the complexity of intercellular and intracellular interactions involved in myofiber repair and regeneration is essential for treating muscle diseases, and for the rehabilitation of individuals following muscle injury. Cellular and molecular events involved in repairing injured myofibers, and in regenerating damaged muscles, are extensively reviewed (Bentzinger et al., 2013; Yin et al., 2013; Andrews et al., 2014; Cooper and Mcneil, 2015; Horn and Jaiswal, 2018; Wosczyna and Rando, 2018). Here we will discuss how extracellular vesicle (EV)—membrane bound compartment released by cells, enable intracellular, and intercellular communication to coordinate repair and regeneration of the injured myofiber.

EVENTS THAT FACILITATE MYOFIBER REPAIR AND REGENERATION

While myofiber repair relies on coordinated intracellular events, the process of myofiber regeneration involves coordinated intercellular interactions. The discussion below focuses on the role of vesicles and membrane trafficking processes that are involved in inter-and intra-cellular events that enable repair and regeneration of the injured skeletal myofibers.

Myofiber Repair

Plasma membrane damage results in a rapid influx of extracellular calcium, which triggers vesicular activity—internalization (endocytosis) and externalization (exocytosis, ectocytosis), and non-vesicular activity—local actin cytoskeleton reorganization to help repair the injury (**Figures 1B,C**). These activities help the myofiber repair, by sequestering the damaged part of the plasma membrane into endosomal vesicles or shedding it via EVs (Babiychuk et al., 2011; Keyel et al., 2011; Corrotte et al., 2013; Jimenez et al., 2014; Scheffer et al., 2014). Vesicle shedding can occur passively (Keyel et al., 2011; Boye et al., 2018) or through Endosomal Sorting Complexes Required for Transport (ESCRT)-mediated scission of the damaged membrane (Andrews et al., 2014; Jimenez et al., 2014; Scheffer et al., 2014; Demonbreun and Mcnally, 2017; Romero et al., 2017). Increase in intracellular calcium triggers exocytosis of vesicles such as lysosomes and late endosomes/multivesicular bodies (MVBs), by activating calcium binding proteins such as dysferlin and synaptotagmin. This allows the released vesicles to then accumulate locally and or distribute systemically (**Figure 2**).

Lysosome exocytosis contributes endomembrane to help close the wound, and also enables release of the lysosomal enzyme acid sphingomyelinase (ASM) (Chakrabarti et al., 2003; Jaiswal et al., 2004; Defour et al., 2014; Sreetama et al., 2016; **Figure 1A**). Upon its release ASM gains access to the plasma membrane lipids including sphingomyelin, and hydrolyzes it to generate ceramide—a lipid that is enriched in injured cells (Babiychuk and Draeger, 2000; Tam et al., 2010; Corrotte et al., 2013; Romancino et al., 2013; **Figure 1A**). This ceramide facilitates plasma membrane repair by removing the damaged membrane through internalization (via endosomes) and shedding (via ectosomes) (Draeger and Babiychuk, 2013; **Figures 1A–C**). ASM inhibitors and ASM deficiency blocks membrane removal (Bianco et al., 2009) and membrane repair (Corrotte et al., 2013; Deng et al., 2017; Michailowsky et al., 2019). The endocytosed vesicles can progress through the endosomal pathway, whereby inward budding of the injured membrane forms ∼300–500 nm-sized intracellular endocytic vesicles that fuse together to form late endosomes and MVBs in the injured cells (Murphy et al., 2018; **Figures 1A**, **3A**). These endosomes may degrade the internalized damaged proteins and lipids, or undergo inward budding to create intraluminal vesicles (ILV) (Murphy et al., 2018). The MVBs can then traffic and fuse to the membrane to exocytose their contents. These ILVs are subsequently released from the cell upon MVB exocytosis, and these released vesicles are called "exosomes" (**Figures 1C**, **3B**). Through these vesicle trafficking events, the injured plasma membrane is not only repaired within minutes of injury, but the EVs generated and released in the process of repair can subsequently signal extracellularly to affect a tissue-level repair response that can continue beyond the brief myofiber repair phase (see **Figures 1**, **2**).

For the above vesicular events to remodel damaged membranes, cells rely on cytoskeletal proteins to stabilize the damaged membrane and assist in ferrying vesicles to and from the damaged membrane (Mcdade et al., 2014; Vaughan et al., 2014; Horn and Jaiswal, 2018; **Figure 1D**). Calcium influx due to cell membrane disruption activates kinases and lipid modifying enzymes, creating a signaling platform that assists with vesicle fusion, lipid, and actin reorganization, and constriction of membrane wound during repair (Floyd et al., 2001; Mandato and Bement, 2001; Benink and Bement, 2005; Vaughan et al., 2014; Horn and Jaiswal, 2018). Injury-induced calcium entry also activates Calpains, which help disassemble the cortical actin cytoskeleton allowing intracellular vesicles to access the cell membrane and repair the membrane by fusion. This calpain-mediated actin loss also relieves membrane tension, preventing cytoskeletal contraction from pulling the lipids in the damaged free-edges of the wounded membrane outward, which would cause the wound to expand (Gitler and Spira, 1998; Togo et al., 2000; Mcneil et al., 2001; Hendricks and Shi, 2014; Redpath et al., 2014). Moreover, lipids in the wounded membrane facilitate binding of specific proteins such as annexins. Annexin family proteins subsequently assist in closing the wound by curving the membrane wound edges via their construction of 2-dimensional proteins arrays to stabilize the membrane, and by stimulating actin reorganization and membrane scission (Bouter et al., 2011; Boye et al., 2017; **Figures 1B,D**). Another signal that regulates reorganization of actin and cytoskeletal proteins is reactive oxygen species (ROS) produced by mitochondria. However ROS is also produced during-, and has an additional role in-, membrane repair (Cai et al., 2009; Spaeth et al., 2012; Duan et al., 2015; Horn et al., 2017; **Figure 1B**). Restoration of membrane structure and function is a key early feature in successful repair and regeneration of skeletal muscle, failure of which leads to myofiber degeneration, requiring myofiber regeneration through intracellular signaling (Tidball, 2011; Demonbreun et al., 2016). However, even myofibers that successfully repair release signals in the form of secreted molecules and vesicles that informs the tissues of the muscle damage. Thus, a continuum of extracellular

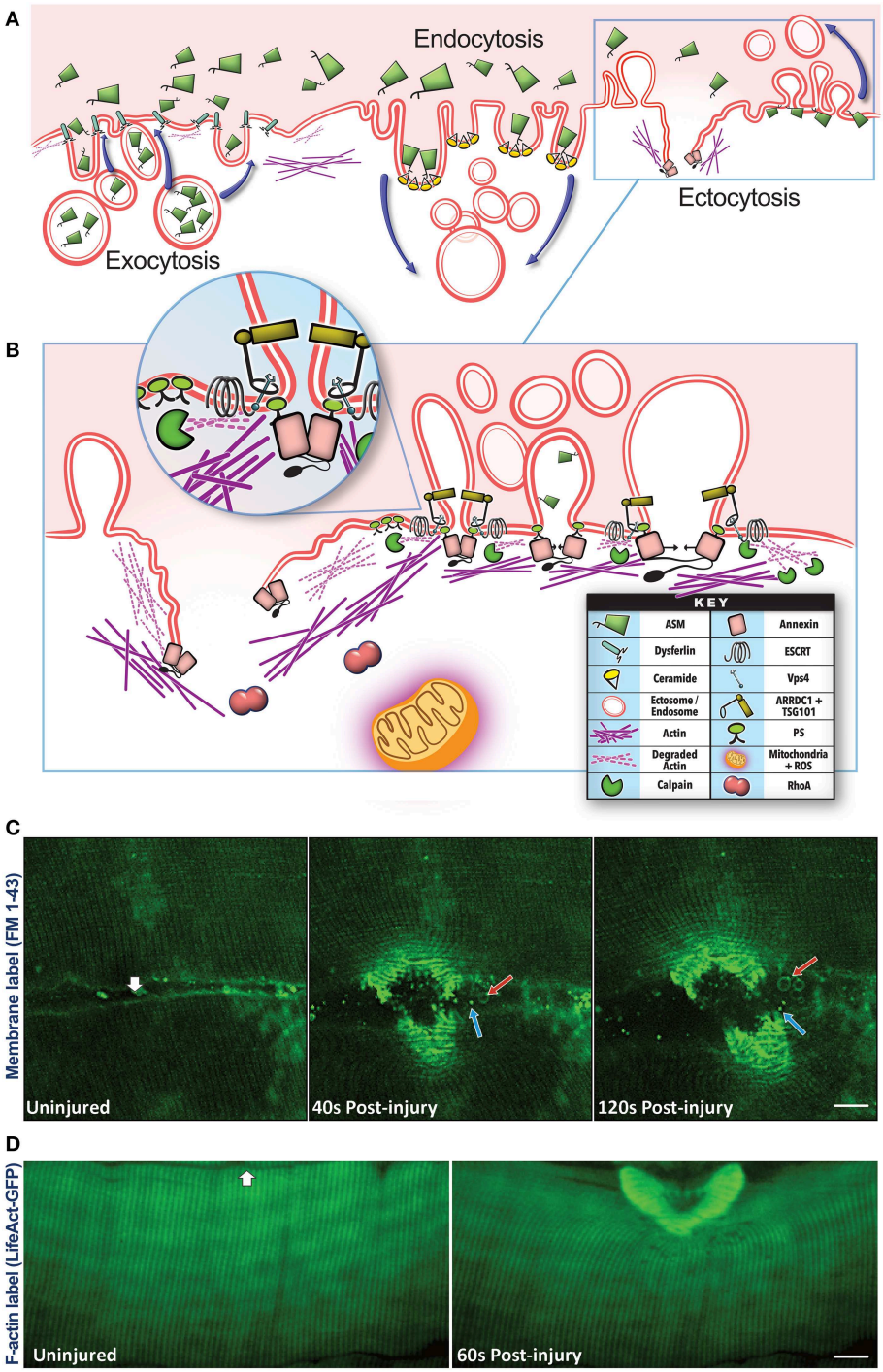

FIGURE 1 | Vesicular pathways involved in plasma membrane repair. **(A)** Plasma membrane injury triggers membrane trafficking by Exocytosis—fusion of the intracellular vesicles such as lysosomes with the injured plasma membrane, Endocytosis—internalization of the plasma membrane, and Ectocytosis—shedding of the plasma membrane by way of microvesicles/ectosomes. Exocytosis is aided by calcium-binding membrane proteins such as dysferlin resulting in the release of lysosomal luminal proteins such as acid sphingomyelinase (ASM). The secreted ASM can access the outer and inner leaflets of the injured plasma membrane and hydrolyze the sphingomyelin lipids in these membranes to ceramide. Presence of ceramide in the outer leaflet will facilitate inward curvature and endocytosis, ceramide in the inner leaflet will cause outward curvature and ectocytosis. Both these processes enable removal of damaged membrane lipids from the site of injury by internalizing or shedding these lipids. **(B)** Ectocytosis is also facilitated by the interaction of proteins such as TSG101, ARRDC1, ESCRT III, and VPS4 as well as rearrangement of cortical actin beneath the membrane which help with vesicle budding and scission (*see inset*). Membrane shedding is also facilitated by the interaction of membrane lipids (phosphatidylserine) with the Annexin proteins and disassembly/reassembly of the cortical actin cytoskeleton with the help of calpain,

(Continued)

FIGURE 1 | Rho A, and Annexin proteins as well as mitochondrial ROS signaling. These latter processes also play a role in facilitating exocytosis and endocytosis indicating a complex set of membrane trafficking events that occur in concert, and failure, or delay in the any of these processes results in the failure of the injured myofiber to repair the plasma membrane injury. **(C)** Confocal images of live myofibers injured focally (white arrow) in the presence of membrane-impermeable FM 1–43 dye (green). Upon membrane injury, FM dye labels the intracellular membrane as well as all the vesicles secreted by the injured myofibers. Images of the same myofiber taken prior to and 40- or 120-s post injury show the formation of large (500–2,000 nm, red arrow) and small (<500 nm, blue arrow) extracellular vesicles. The released vesicles subsequently traffic away from the site of injury, but remain within the interfiber space (see **Video 1**). **(D)** Confocal images of a myofiber in an intact biceps muscle of Lifeact-GFP transgenic mouse showing F-actin response to focal injury (white arrow) by a 10 ms laser pulse. Images of the same fiber were taken just before and 60 s after injury, and show F-actin reorganization and buildup at the injury site.

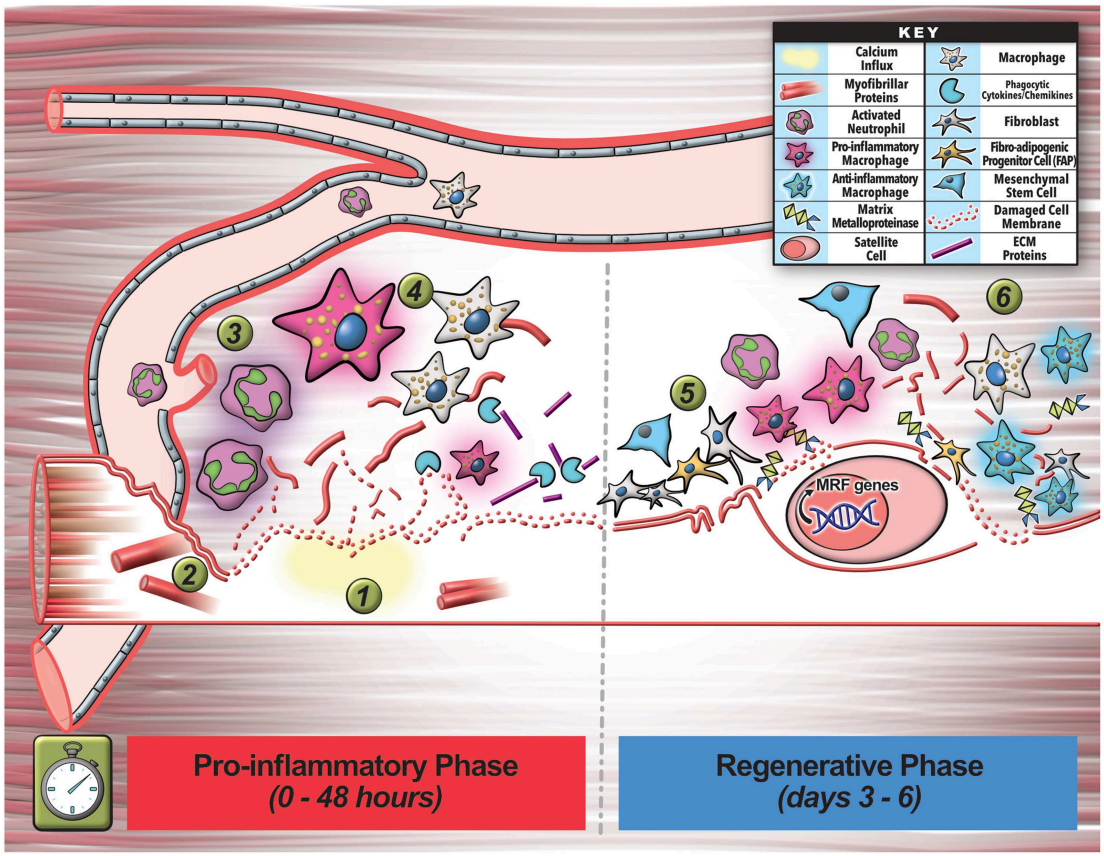

FIGURE 2 | Multicellular interactions involved in early stages of myofiber regeneration. **(1)** Myofiber regeneration starts with the initial damage that causes calcium influx into the myofiber leading to release of damaged membrane, as well as breakdown and release of myofibrils and other cellular contents. **(2)** These and other factors released by the injured myofiber activates and recruits circulating immune cells (neutrophils) to the injury site to commence inflammation and initial phagocytosis of cellular debris. **(3)** Invading neutrophils secrete pro-inflammatory cytokines that promote inflammatory macrophage enrichment. **(4)** These cells help clear debris during initial inflammation and also secrete additional cytokines and chemokines that further assist in clearance of cell debris. **(5)** The inflammatory cells also signal proliferation of cells including the fibroblasts (FAPs) that assist in secretion of growth factors, cytokines, and extracellular matrix-remodeling enzymes (MMPs) that facilitate SC escape from the basal lamina and matrix remodeling required for regeneration. **(6)** In the final phase of the inflammatory response to injury, macrophages turn pro-regenerative, and together with the other additional cell types activate satellite cell (SC) myogenic program by activating regenerative transcription factors. This tightly orchestrated cellular choreography relies on intercellular communication via secretory factors including EVs that ultimately facilitates regeneration of the lost myofiber.

responses triggered by damaged, repaired, regenerating, and degenerating myofibers, facilitate the intracellular signaling following membrane injury that supports healthy regeneration and growth of the injured muscle.

Myofiber Regeneration

Similar to the complexity of myofiber repair, myofiber regeneration also depends on a series of highly coordinated events, orchestrated between multiple cell types that engage in intercellular communication (Chazaud, 2016; Wosczyna and Rando, 2018). Inadequate membrane repair initiates myofiber necrosis, triggering an inflammatory response, and subsequently culminating in regeneration of the lost myofiber by activation of muscle-resident stem cells satellite cells (SCs). Dysfunction in this process results in myofiber loss and replacement by fibrosis, adipogenesis, or calcification, preventing structural, and functional restoration of the injured muscle (Wosczyna and Rando, 2018). Unlike myofiber repair, where local/mild

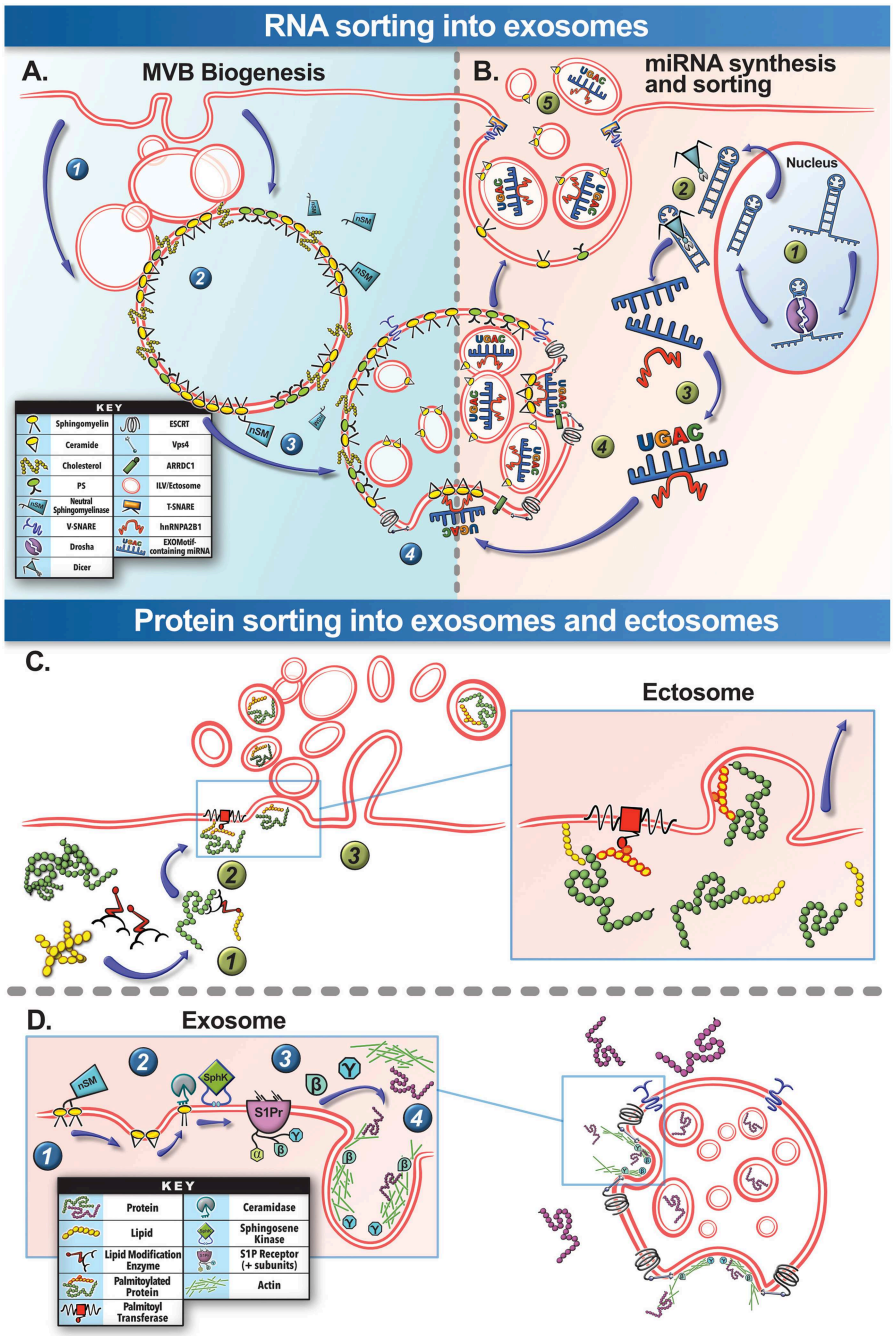

FIGURE 3 | Biogenesis and selective cargo packaging into EVs. **(A)** MVB Biogenesis: Spontaneous or injury-triggered endocytosis of the plasma membrane forms **(1)** endosomes that **(2)** progress through the endosomal pathway to create the late endosome and the "multivesicular body" (MVB). The MVB membrane contains domains rich in sphingomyelin, cholesterol ceramide, and other specific lipids (lipid raft). **(3)** Cytosolic neutral sphingomyelinase (nSM), can hydrolyze sphingomyelin on the MVB membrane to generate ceramide that enables spontaneous inward budding of this region of the MVB membrane. **(4)** This inward budding is also assisted by the assembly of the ESCRT family proteins and is finally cleaved by Vps4, allowing the newly formed vesicles to be released into the MVB lumen as "intraluminal vesicles" (ILVs). **(B)** RNA Cargo Packaging: **(1)** Specific cellular stresses, such as cell injury, can trigger miRNA duplex transcription in the cell nucleus and then processed by Drosha. **(2)** This is followed by cleavage of the miRNA passenger strand by Dicer protein within the cytosol. **(3)** Based on the sequence of the miRNA, it may preferentially bind the cytosolic protein hnRNPA2B1. **(4)** These complexes can then associate the miRNA to ceramide at the nascent ILV and packaged into the ILV. **(5)** Subsequently these miRNA-containing ILVs are secreted as exosomes through the fusion of MVB via membrane-embedded v-SNARE proteins that interact with sarcolemmal t-SNARE proteins. This process helps exosomes functionally traffic miRNA to other cells and tissues. **(C)** Ectosome Protein Sorting: **(1)** Post-translational covalent lipid modification of the cysteine residues in the proteins is one of the mechanisms for protein sorting into ectosomes. This process is

(Continued)

FIGURE 3 | assisted by a family of lipid modification enzymes collectively known as acyltransferases. **(2)** Protein-membrane anchoring for stable ectosome packaging is achieved through palmitoylation by the membrane-bound palmitoyltransferase, anchoring the protein to the budding ectosome (*see inset for details*). **(3)** The protein containing ectosome can then be released by the cell. **(D)** Exosome Protein Sorting: For protein sorting into exosomes lipid signaling pathways on the MVB membrane play an important role. **(1)** Neutral sphingomyelinase hydrolyze sphingomyelin in the MVB membrane to ceramide. **(2)** Ceramide is subsequently catabolized to sphingosine and sphingosine-1-phosphate (S1P) by cytosolic ceramidase and sphingosine kinase (SphK), respectively. **(3)** S1P continuously activates S1P receptors on the MVB membrane, stimulating the release of their β and γ subunits. **(4)** This catalyzes GTPases and actin-mediated sorting pathways for loading proteins into the budding ILVs/exosomes. A parallel pathway not depicted here involves ESCRT complex-mediated sorting and packaging of ubiquitinated proteins on the MVB membrane into ILVs for subsequent degradation or secretion.

activation of calpain helps restore myofiber integrity, excessive calcium influx activates calpain-mediated loss of myofibrils and extracellular exposure of phosphatidylserine (PS) membrane lipids (**Figure 2**). While myofibril damage causes myofibers to degenerate, PS exposure is one of many signals that activate the recruitment of circulating immune cells to initiate the inflammatory response and initial phagocytosis of myofibrillar debris (Demonbreun and Mcnally, 2017; Lemke, 2019). Amongst the first responders to muscle injury are neutrophils that rapidly invade the damaged tissue within hours, reaching their peak at 24–48 h after muscle injury (Bentzinger et al., 2013; Demonbreun and Mcnally, 2017; Tidball, 2017; **Figure 2**). These neutrophils signal to enrich the muscle environment with pro-inflammatory cytokines, activating a pro-inflammatory response by macrophages (Tidball, 2017). These pro-inflammatory macrophages help with phagocytic clearance of debris and stimulate SCs to proliferate and proceed down the myogenic program (**Figure 2**). This initial inflammatory response also involves other cell types including regulatory T-cells, fibroblasts, and fibro-adipogenic-progenitors (FAPs) that assist in removal and cleanup of damaged cells and proteins over the course of 3–4 days post-injury—processes needed for proper extracellular matrix (ECM) turnover and subsequent regeneration of functional muscle tissue (Tidball, 2017; Wosczyna and Rando, 2018; **Figure 2**). These immune cells secrete a wealth of diffusible factors, such as growth factors, inflammatory cytokines (IL-6), globular adiponectin, ECM components, and ECM-remodeling matrix-metalloproteinases (MMPs) that not only generate ECM chemoattractive fragments, but help SCs escape from the basal lamina to engage in regeneration (Bentzinger et al., 2013; Chazaud, 2016; **Figure 2**).

In the second phase of the inflammatory response to muscle injury—around 4 days post-injury, macrophages shift to a pro-regenerative phenotype and secrete anti-inflammatory cytokines and growth factors to facilitate SC proliferation and differentiation leading to their fusion and formation of nascent myofibers (Bentzinger et al., 2013; Chazaud, 2016; **Figure 2**). This regenerative and remodeling phase also hinges upon intercellular coordination and communication involving immune cells, SCs, FAPs, pericytes, fibroblasts, and endothelial cells that secrete proteins and molecules influencing neighboring cells, notably culminating in SC differentiation (Bentzinger et al., 2013; Wosczyna and Rando, 2018; **Figure 2**). This stimulated myogenic differentiation program is an irreversible process driven by the sequential expression of transcription factors myogenic regulatory factors (MRFs) that induce gene expression signals to target genes within the SCs. A large proportion of these MRF target genes largely encode muscle-specific structural and contractile proteins (actins, myosins, and troponins) essential for formation of functional skeletal muscle (Bentzinger et al., 2013). However, other cofactors such as miRNAs (e.g., miR-1, miR-133, miR-206) also influence myogenic differentiation through modulation of MRF levels as well (Bentzinger et al., 2013).

It has become evident that muscle repair and regeneration requires coordinate expression of various factors including secreted proteins, inflammatory cytokines, miRNAs, and membrane lipids that transfer intercellular signals (Murphy et al., 2018). However, an increasingly recognized means of intercellular signaling in muscle repair and regeneration involves release and uptake of vesicles (Demonbreun and Mcnally, 2017). A recent study in a murine model, examining the effects of acute muscle injury due to a 20 min downhill running exercise, demonstrated increased circulating vesicles, both during the initial hours after myofiber injury and during subsequent regeneration 5–7 days post-exercise damage (Coenen-Stass et al., 2016). This suggests that the vesicles that carry signals during regeneration are produced by the injured myofiber and regenerative cells alike, and that these may play distinct but complimentary roles in the repair response to injury (Coenen-Stass et al., 2016). Insights into the formation of these vesicles and their signaling properties may reveal new understandings of the orchestrated response necessary for proper muscle repair and regeneration.

EXTRACELLULAR VESICLES—EXOSOMES AND ECTOSOMES

Similar to other cells, skeletal myofibers broadly produce 3 types of extracellular vesicles—apoptotic bodies, exosomes, and ectosomes. These vesicles are distinguished by their size, composition, and cellular origin. The largest of these vesicles—apoptotic bodies (1,000–8,000 nm diameter), derive from apoptotic tissues in which cell repair and regeneration has failed (Crescitelli et al., 2013; Silva et al., 2017; Caruso and Poon, 2018). Hence, we will focus on the other two groups of EVs—exosomes and ectosomes (microvesicles). Exosomes are smaller in size, with diameter ranging from ∼30–150 nm, while ectosomes range from ∼150–1,000 nm (Crescitelli et al., 2013; Villarroya-Beltri et al., 2013; Janas et al., 2015; Leoni et al., 2015; Choi et al., 2016; Wang and Wang, 2016; Demonbreun and Mcnally, 2017; Meldolesi, 2018; Murphy et al., 2018). Aside from their size however, these vesicles

TABLE 1 | Properties of muscle derived exosomes and ectosomes.

Exosomes	Ectosomes
Size: Cup-shaped Smaller (~30-150 nm diameter)	**Size:** Larger (~150-1000 nm diameter)
Biogenesis: Derive from Endocytosis Originate as ILVs in MVBs – once secreted, called exosomes	**Biogenesis:** Derive directly from membrane budding
Lipids: - cholesterol, sphingomyelin, ceramide - more enriched in lipid raft lipids	**Lipids:** - cholesterol, sphingomyelin, ceramide - more heterogeneous sarcolemma-like lipid composition
Proteins: - more luminal proteins - Transmembrane proteins (tetraspanins, ESCRTs, Rabs, integrins) - Contain proteins related to their biogenesis (Alix, TSG101, CD63, CD81, AnnexinA5, HSP90, etc.) - Myogenic growth factors (VEGF, IGFBP, HGF, IGF-1, bFGF, PDGF-A) - Contractile proteins - Actin, Vimentin, Talin, GTPases, RNA-binding proteins - More enriched in membrane integrins, and testraspanins - GO Term – "cell-cell-signaling"	**Proteins:** - more membrane-embedded protein - Transmembrane proteins (tetraspanins, ESCRTs, Rabs, integrins) - ER proteins (CALU, CALR, HSP90B1, HSPA5) - Mitochondrial proteins and DNA - Ribosomal subunits (RPL4, RPL10, RPS5, RPS17) - Translation initiation factors (EIF3A, EIF5) - MMPs - cytoskeletal proteins (ACTB, EIF5) - Glycoprotein receptors (GP1b, and GPIIb/GPIIa) - golgi proteins - cytosolic proteins
RNAs: - miRNAs, tRNA, mRNAs, lncRNAs, DNA fragments - more enriched in miRNAs than ectosomes	**RNAs:** - miRNAs, tRNA, mRNAs, lncRNAs, DNA fragments

Exosomes and Ectosomes share many common features (red text), but also present a number of unique features and cargoes (blue text).

fundamentally differ in their composition and the cellular compartments from which they are manufactured (**Table 1**).

Exosomes, also known as "intraluminal vesicles" (ILVs), develop from a sequential process of MVB membrane remodeling (**Figure 3A**). The MVB membrane contains lipid domains not unlike the muscle sarcolemma from which they are derived (Murphy et al., 2018; **Figure 3A**). Lipid-protein interactions and lipid reorganization at MVB membrane domains enables spontaneous inward budding of these regions, that is further aided by ESCRT-family proteins and released into the MVB lumen by Vps4 ATPase-mediated cleavage as ILVs (Trajkovic et al., 2008; Colombo et al., 2014; Cocucci and Meldolesi, 2015; Janas et al., 2015). The ILVs are thus enriched in the structural lipids of the MVB membrane that help their formation (Janas et al., 2015). The MVBs subsequently traffic to the plasma membrane where SNARE proteins and tethering factors such as SNAP23, Syntaxin1a, VAMP7 and 8, Rabs 11, 27, and 35 aid in MVB exocytosis, releasing ILVs as exosomes (Colombo et al., 2014; Cocucci and Meldolesi, 2015; Janas et al., 2015; Meldolesi, 2018; **Figures 3A,B**).

Ectosomes, are produced at the plasma membrane by a different cellular process that involves small GTPases Arf6, Rab22, RhoA, CDC42, and Rac1 and the contractile activity of cortical actin beneath the cell membrane (Muralidharan-Chari et al., 2009; Wang et al., 2014; Meldolesi, 2018). However, ectosomes can also be formed using some of the same cellular machinery (e.g., ESCRTs) used by exosomes (Muralidharan-Chari et al., 2009; Scheffer et al., 2014; Cocucci and Meldolesi, 2015; Meldolesi, 2018; **Figure 1**). A proteomic screen in muscle cells identified several ESCRT III proteins (Chmps 1, 4, and 6) and only one ESCRT I protein (Tsg101) accumulates at the site of muscle cell membrane injury (Scheffer et al., 2014).TSG101 assists in ectosome formation by interacting at the ectosome neck with the arrestin domain-containing protein-1 (ARRDC1) and with ESCRT III/ Vps4 complex to enable pinching and release of the ectosome (Nabhan et al., 2012; Dobro et al., 2013; Scheffer et al., 2014; **Figure 1B**).

FUNCTIONALIZING THE EXTRACELLULAR VESICLES BY ACTIVE AND SELECTIVE CARGO LOADING

The differences in cellular origin of exosomes and ectosomes lead to differences in their structural components and the cargoes they carry (Cocucci and Meldolesi, 2015; Mcguinness et al., 2016; Silva et al., 2017; Taverna et al., 2017). Lipid, protein and RNA cargo composition in these vesicles are distinct, not only from that of the cytoplasm but also between exosomes and ectosomes (**Table 1**). This indicates that cargo packaging in the vesicles is an active and selective process that may be responsive to the cell's physiology (Roberts et al., 2012; Matsuzaka et al., 2016; Panagiotou et al., 2016; Fry et al., 2017; D'souza et al., 2018). However, despite these differences, these vesicle subtypes share a range of common lipids, transmembrane proteins, surface ligands, and cargoes (Mcguinness et al., 2016; Silva et al., 2017; Taverna et al., 2017; **Table 1**, red text).

Protein and Lipid Cargo

Due to their generation from the raft-like region of the MVB membrane, exosomes contain lipids that are enriched in the MVB lipid raft domains, as discussed above (Janas et al., 2015). Conversely, being derived from the plasma membrane, ectosomes contain a more heterogeneous plasma membrane-like lipid composition (Meldolesi, 2018; **Table 1**). Moreover, vesicle differences extend beyond lipid composition, with as much as 65% of protein cargoes reported to differ between muscle exosomes and ectosomes (Le Bihan et al., 2012). This highlights the divergent protein sorting pathways employed by the vesicle generating cell to control vesicle cargoes and consequentially, their signaling properties after muscle damage (Laterza et al., 2009; Roberts et al., 2012).

Proteomic analysis of muscle-derived exosomes shows that in addition to proteins involved in their biogenesis, the exosomes also contain functionally important proteins such as myogenic growth factors and contractile proteins (Choi et al., 2016; Demonbreun and Mcnally, 2017; **Table 1**). Compared to ectosomes, which are enriched for membrane-embedded proteins, exosomes are enriched in proteins including integrins, MHC molecules, tetraspanins, ESCRTs, endosome proteins, and even zinc-finger transcription factors (Le Bihan et al., 2012; **Tables 1**, **2**). Gene-ontology analysis of muscle exosome proteins show that they largely belong to the protein families involved in "cell-cell signaling" (Le Bihan et al., 2012). Unlike their exosome counterparts, due to their immediate generation at the muscle sarcolemma, ectosomes, contain more transmembrane proteins, receptors, glycoproteins and metalloproteinases (MMPs) (Meldolesi, 2018; **Table 1**). They are also enriched in proteins normally found in the endoplasmic reticulum, mitochondria (dehydrogenase proteins, respiratory electron transport chain), golgi, cytoskeleton, and cytosol (Le Bihan et al., 2012; Kowal et al., 2014; Phinney et al., 2015; Willms et al., 2016; Meldolesi, 2018; **Tables 1**, **2**). Gene ontology analysis of muscle ectosome proteins shows predominance of proteins involved in RNA post-translational modification, amino acid metabolism, protein synthesis, molecular transport, and protein degradation (Le Bihan et al., 2012). Thus, there are notable differences in the protein and membrane lipid composition of exosomes and ectosomes, which in-turn influences uptake and function of these EVs in the target cells (see the section Extracellular Vesicle Uptake by the Target Cells for details). However, aside from cellular compartment location, cell sorting pathways may influence the differential cargo packaging in these vesicles as well.

Aside from the cellular location where the EVs are produced, active protein sorting pathways also influence the cargo packaging in these vesicles (Yang and Gould, 2013). One example of this is "curvature-based sorting" (Hanson et al., 2009; Yáñez-Mó et al., 2009, 2015; Nazarenko et al., 2010; Perez-Hernandez et al., 2013).Protein intrinsic molecular shape can drive its diffusion and membrane routing to flat membrane spaces or curved, vesicle-generating areas, as this limits membrane free energy, while proteins with curvature-favoring structural elements (i.e., BAR domains), can actually drive the formation of vesicles themselves (Arkhipov et al., 2008). For instance, in ectosome formation, ESCRT complex proteins energetically favor the neck region of the budding ectosome, while tetraspanins tend to form tetraspanin-enriched microdomains that help drive ILV budding on the MVB membrane (Hanson et al., 2009; Yáñez-Mó et al., 2009). Such protein sorting mechanisms may partially account for the frequent findings of ESCRT proteins within ectosome vesicles and tetraspanins within exosomes (Nazarenko et al., 2010; Perez-Hernandez et al., 2013). Of particular relevance, BAR domain proteins, such as BIN-3, may be required for muscle regeneration due to its ability to promote satellite cell fusion and migration via lamellipodia formation (Simionescu-Bankston et al., 2013). However, further studies are warranted to investigate if BAR-domain proteins in EVs play a role in muscle regeneration.

Assembly of cytosolic cargo proteins into the lumen of ectosomes requires binding of these proteins to the plasma membrane—a process reliant on post-translational protein lipid-modification (Yang and Gould, 2013; **Figure 3C**). These modifications include covalent lipid attachment on the cysteine side chain of myristoyl (myristoylation), isoprenoid (prenylation), or palmitoyl (palmitoylation) moieties (Aicart-Ramos et al., 2011). These modifications can attract and sustain/anchor the tagged cytosolic proteins from their N- or C-terminal ends to the ectosome budding site on the membrane (Yang and Gould, 2013; Cocucci and Meldolesi, 2015; **Figure 3C**). This process is dynamic, highly regulated, reversible, and governed by a family of enzymes e.g., DHHC palmitoyl transferases, deacylases, and prenyltransferases (Aicart-Ramos et al., 2011; Cocucci and Meldolesi, 2015). Interestingly, these protein anchors are not efficient in protein targeting for sorting into exosomes (Yang and Gould, 2013). Examination of skeletal muscle cell ectosome proteome reveals an abundance of proteins with predicted palmitoylated cysteine residues, while exosome-enriched proteins do not possess such anchoring/modification sites (Blanc et al., 2015; **Table 2**). In this respect, protein lipid

TABLE 2 | Proteins enriched in skeletal muscle exosome and ectosome fractions.

Proteins enriched in Ectosomes	SwissPalm palmitoylation site	Cellular role (skeletal muscle)
Endoplasmic reticulum proteins		
CALU, HSP90	CALU—Cysteine 9, 14 HSP90—Cysteine 420	ER calcium-binding protein (protein folding/sorting) HSP90-induces inflammation, activates TLRs on macrophages and muscle cells to stimulate muscle catabolism (Zhang et al., 2017)
TCP-1 chaperonins TCP-1	Cysteine 147	Key protein in actin biogenesis at z-disc during sarcomere assembly (Berger et al., 2018)
Actins and tubulins ACTB, TUBA1B	ACTB—Cysteine 17 TUBA1B—Cysteine 376	TUBA1B-trafficking of dysferlin to the sarcolemma in membrane repair (Azakir et al., 2010)
Ribosomal subunits RPL4, RPL10, RPS5, RPS17	RPL4—Cysteine 3 RPL10—Cysteine 80 RPS5—Cysteine 172, 66 RPS17—Cysteine 35	All involved in ribosomal structure and protein translation in the cytosol for cell growth (Bennet et al., 2018)
Translational initiation Factors EIF5	Cysteine–22, 38	Crucial for satellite cell differentiation – delivers tRNA to ribosome to initiate protein translation (Luchessi et al., 2009; Jennings and Pavitt, 2010)
Matrix metalloproteinases MT1-MMP	Cysteine–574	Required for fibronectin degradation and basement membrane laminin cleavage for proper elongation and myoblast fusion (Ohtake et al., 2006)
Glycoprotein receptors GPIb	Cysteine–352	Involved in gluconeogenesis (Lamanna et al., 2015)
Adhesion proteins P-selectin	Cysteine–807	Required for leukocyte recruitment to injured skeletal muscle (Frenette et al., 2003)
Integrin Mac-1	Cysteine 14	Required for leukocyte recruitment after skeletal muscle injury (Lagrota-Candido et al., 2010)
Proteins enriched in Exosomes	**SwissPalm palmitoylation site**	**Cellular role (skeletal muscle)**
Intermediate Filament (z-disc) Vimentin	Cysteine–328	Required for structural remodeling of skeletal muscle, especially at myotendinous junction (Vaittinen et al., 2001)
Cytoskeletal protein Talin	NA	Required for myoblast fusion, sarcomere assembly, and myotendinous junction maintenance (Conti et al., 2009)
Annexins ANX-A1, ANX-A4, ANX-A6	ANXA1—Cysteine 343, 263 ANXA6—Cysteine 114	Anti-inflammatory signaling, signal transduction, cytoskeleton and ECM integrity, muscle growth, satellite cell differentiation, migration, and fusion, and muscle membrane repair (Bizzarro et al., 2012a,b)
GTPases ARF-6	NA	Regulator of myoblast fusion via phospholipase D activation (triggers phospholipid production and actin reorganization at myoblast fusion sites) (Bach et al., 2010)
RNA-binding proteins Argonaute 2, Y-box1	NA	AGO2—facilitates packaging of miRNAs into exosomes, protects miRNA from lysosomal degradation, required for miRNA silencing of mRNA translation (Lv et al., 2014)
		Y-box1—nucleic acid chaperone involved in DNA replication and repair, transcription, pre-mRNA splicing and mRNA translation, particularly of genes involved in cell division, apoptosis, immune response (Eliseeva et al., 2011)
Integrins ITGA4, ITGA6, ITGA7	ITG4—Cysteine 25 ITGA6—Cysteine 8, 26	Integrins—serve as mechanotransducers connecting ECM to cell cytoskeleton to influence signaling cascades involved in myogenesis, hypertrophy (Carson and Wei, 2000). Also play role in myoblast migration and fusion (Mayer, 2003)
MHC molecules HLA-A, HLA-B	NA	HLA-A and B—MHC class 1 cell surface receptors that present peptides recognized by inflammatory T-cells leading to inflammation and apoptosis (Appleyard et al., 1985)
Endosomes TSG101	NA	TSG101—Governs ILV and exosome formation in the late endosome/MVB (Edgar, 2016)
Lysosome LAMP2	NA	LAMP2—governs protein sorting into degradative lysosomes (autophagasomes) (Zhang et al., 2017)

The palmitoylated cysteines are as reported in SwissPalm [weblink]. All of the validated ectosome-enriched proteins appear to contain the palmitoylation sites, while only a few exosome proteins possess these cysteine residues. The roles reported for these cargoes include roles in the health and maintenance of skeletal muscle, as well as repair and regeneration of muscle following injury.

modifications within the cell may serve as a key mechanism of selective cargo loading to influence intercellular signals produced by skeletal muscle in the repair and regeneration process. It is worth noting that following burn injury, skeletal muscle increases expression of prenyltransferase and of protein prenylation, inhibition of which reduces inflammatory gene expression in skeletal muscle, implicating protein prenylation in inflammation following skeletal muscle injury (Nakazawa et al., 2015). Moreover, tissue regeneration-specific protein palmitoylation has been shown to be critical for neural scaffolding and dendritic spine maturation in neuronal regeneration (Zhang and Hang, 2017). Therefore, given the connection between protein acylation and ectosome packaging and its role in tissue inflammation and regeneration, probing the role of protein lipid modification

in vesicle-mediated intercellular signaling after skeletal muscle injury is an interesting area for future investigation.

Aside from protein-lipid modification and membrane anchoring in cargo sorting into ectosomes, other intracellular lipid signaling processes have also emerged as means of protein cargo sorting into exosomes (Kajimoto et al., 2013, 2018; Janas et al., 2015). For example a chain of sequential lipid intracellular lipid modifications at the MVB membrane, concluding with S1P receptor activation and Rho-family GTPAse stimulation, acts as a signaling axis for protein sorting into exosomes (Kajimoto et al., 2013, 2018; **Figure 3D**). Aberrant lipid hydrolysis, SphK activity/phosphorylation status, and S1P subunit release, alters exosome protein cargo packaging and muscle repair (Saba and De La Garza-Rodea, 2013; Guo et al., 2015; Janas et al., 2015; Badawy et al., 2018; Kajimoto et al., 2018). In skeletal muscles, eccentric-contraction-induced injury activates SphK1 and increases endogenous S1P synthesis, while selective inhibition of SphK1 during eccentric injury promotes muscle fibrosis, attenuates extracellular matrix remodeling, and exacerbates myofiber damage (Sassoli et al., 2011; Loh et al., 2012). Further, increasing skeletal muscle S1P levels improves muscle regeneration in a mouse model of Duchenne muscular dystrophy (DMD), while also increasing myofiber size, force, and SC abundance, and diminishing fibrosis and adipose tissue accrual (Ieronimakis et al., 2013). Given the impact of altered S1P signaling in exosome protein sorting and in skeletal muscle regeneration, there is a need to determine if altered exosome packaging due to aberrant S1P activity contributes to poor reparative response to muscle injury (Donati et al., 2013).

RNA Cargo

EVs differ from their parent cell in RNA composition—unlike the abundance of rRNA in the parent cell, EVs are comparatively enriched in small RNAs such as miRNAs (Crescitelli et al., 2013; Jeppesen et al., 2019; **Table 1**). Further, similar to their differential protein contents, exosomes and ectosomes have different RNA cargoes (Jeppesen et al., 2019; **Table 1**). RNA-seq analysis of EV subpopulations identified that of the RNA transcripts unique to EVs, ectosomes contain only ∼9–14% of the vesicle-enriched RNA species (Chen et al., 2016). This is attributed to differential RNA sorting mechanism—miRNAs are preferentially sorted into exosomes based upon their unique nucleotide sequences (Roberts et al., 2012; Matsuzaka et al., 2016; Fry et al., 2017; D'souza et al., 2018; **Table 3**). In muscle cells damaged by eccentric exercise or due to muscular dystrophy, tissue-enriched miRNAs (myoMirs) are seen to be elevated in the circulation, specifically during the regenerative phase *in-vivo* and during myoblast differentiation *in-vitro* (Coenen-Stass et al., 2016). However, after muscle damage, the level of many of these miRNAs declines within muscle and are detected only in vesicles, suggesting their selective packaging and release, as opposed to passive leak, in response to muscle damage (Siracusa et al., 2016). Moreover, vesicle-associated myomiRs differ between dystrophy-associated damage and eccentric muscle damage, further suggesting a context-specificity of miRNA loading into the exosomes (Roberts et al., 2012; Matsuzaka et al., 2016; Fry et al., 2017; D'souza et al., 2018).

Simultaneous with miRNA transcription and cleavage within the cell cytosol, these cytosolic miRNAs bind the outer MVB membrane to be packaged into nascent ILVs as they bud inwards (Janas et al., 2015; **Figure 3B**). This sorting of these RNAs into the ILVs is based on affinity of the RNAs to the MVB raft regions, which is dependent upon the RNAs nucleotide-sequence termed EXOmotif (Villarroya-Beltri et al., 2013). Thus, the presence of these motif(s) in miRNA preferentially sorts them into exosomes, and mutating these EXOmotifs, prevents their import into exosomes, retaining them in the cytoplasm (Villarroya-Beltri et al., 2013). There are currently 10 annotated EXOmotifs responsible for sorting of the ∼75% of known miRNA found in exosomes, and then there are established CLmotifs that predisposes miRNA retention within the cytosol (∼67% of the time) (Janas and Janas, 2011; Janas et al., 2012; Villarroya-Beltri et al., 2013; **Table 3**). EXOmotifs can dictate RNA binding to- and attraction of- cytoskeleton-associated cytosolic proteins (hnRNPA2B1, Y-box protein 1, Major Vault Protein) that assist in miRNA shuttling to the MVB (Villarroya-Beltri et al., 2013; Santangelo et al., 2016), a process that confers specificity to exosome miRNA packaging (Villarroya-Beltri et al., 2013; **Figure 3B**). Lack of these proteins reduces miRNA sorting into exosomes (Shurtleff et al., 2016; Teng et al., 2017; Statello et al., 2018). The evidence for specific sorting of miRNAs into exosomes following muscle damaging stimuli, comes from studies examining exosome cargoes. Following a bout of muscle damaging cycling, exosomal release of EXOmotif containing miRNAs—miR-208a, miR-126, and miR-16 increases (D'souza et al., 2018). Similarly, overload-induced muscle damage causes release of the CCCG-EXOmotif containing miR-206 within SC-derived exosomes (Fry et al., 2017). Conversely, DMD-associated muscle damage promotes exosomal release of GGAC-EXOmotif-containing miR-1, CCCG-containing miR-206 (Roberts et al., 2012), and CCCU containing miR-133a (Matsuzaka et al., 2016). With the importance of these miRNAs in intercellular signaling for regeneration, their selective packaging and sorting after muscle damage may be a purposeful regulatory mechanism (**Table 3**).

EXTRACELLULAR VESICLE UPTAKE BY THE TARGET CELLS

In order to transmit their signals, both the EVs find and interact similarly with their target cell to deliver their cargoes into the host cell cytosol, this includes direct fusion with the target cell membrane, or internalization via endocytosis (Rejman et al., 2004; Cocucci and Meldolesi, 2015; Silva et al., 2017). Upon arriving at the target cells, EVs roll or "surf" on the cell surface until reaching a "hotspot" for internalization, a process driven by electrostatics and aided by membrane proteins like tetraspanins, integrins, proteoglycans, and lectins (Demonbreun and Mcnally, 2017; Meldolesi, 2018). Both, exosomes and ectosomes exhibit cell-specific signaling via different surface receptors and their ligands (Sahoo and Losordo, 2014). This is supported by the fact that presence of specific peptides in the EV membrane allows EV cargoes

TABLE 3 | miRNA EXOmotif: commonly reported muscle-damage induced miRNAs.

	NA	CLmotif—sorts the miRNA into the cell (not packaged in exosomes). Not demonstrated within commonly reported muscle-damage induced miRNAs.
	miR-133B miR-486	Involved in SC proliferation, differentiation, fiber-type specification, exercise adaptation (Horak et al., 2016; Siracusa et al., 2016; Jin et al., 2017) represses Pten and FoxO-1a to reinforce Akt signaling and enhance muscle growth (Small et al., 2010)
	miR-206	Promotes myoblast differentiation and promotes terminal maturation of muscle fibers (Kim et al., 2006; Windbanks et al., 2013; Siracusa et al., 2016)
	miR-27B miR-14	Increases proliferation of myogenic cells (Ling et al., 2018) positively regulates myogenin and MyHC by targeting the epigenetic regulator Ezh-2 (member of the polycomb repressive complex) (Juan et al., 2009)
	miR-149	Released and peak in circulation 1 h and 1 day after resistance exercise. Its role is unclear (Sawada et al., 2013)
	miR-1	miR-1 promotes differentiation of myoblasts via repression of Hdac-4 that represses Mef-2-depednent expression of myogenic factors (Chen et al., 2010; Di Filippo et al., 2016; Siracusa et al., 2016)
		

With the exception of the CLMotif UGCA, each EXOMotif is found in miRNA's reported to be elevated in skeletal muscle exosomes following injury. The reported roles of these miRNAs in repair and regeneration of skeletal muscle include pro-myogenic effects on muscle satellite cells and activation of myogenic transcription factors.

to be targeted to skeletal muscles or to neurons to alter the cell type (Alvarez-Erviti et al., 2011; Gao et al., 2018). Differential targeting by the EVs occurs during skeletal muscle injury and repair—EVs produced by exercise-induced muscle damage largely hone to the liver, while EVs produced from resting muscle do not do so (Whitham et al., 2018). This may be due to context-specific loading of membrane proteins within the secreted EVs that not only differ between exosomes and ectosomes, but promote different interactions with target cell membranes to ultimately cause their differential uptake (Whitham et al., 2018).

Exosomes are more enriched in tetraspanins—a protein that associates with a wide range of other cell surface proteins including proteoglycans, complement-regulatory proteins, growth factor receptors and ligands (**Table 1**). This is important in the cellular uptake of exosomes as their membrane-embedded tetraspanins may bind more readily with a host of membrane proteins and receptors from potential target cells to facilitate their efficient uptake (Hemler, 2003; Meldolesi, 2018). In the context of muscle injury, tetraspanin-laden exosomes from injured muscle cells facilitate myoblast fusion—tetraspanin uptake by target cells induces cell spreading, and CD9 and CD81 tetraspanins specifically facilitate myoblast spreading and fusion in regeneration (Hemler, 2003). Similarly, exosomes are enriched in syncytin-1—a protein involved in receptor targeting to ILVs of the MVB that also facilitates efficient exosome uptake by target cells via binding to surface fusogens. Syncytin-1-loaded exosomes bind to their neutral amino acid transporters ASCT2 found in skeletal muscle, to induce rapid exosome internalization (Cocucci and Meldolesi, 2015; Kowal et al., 2016). In principle, these unique surface protein properties of exosomes support potentially faster uptake of exosomes (as compared to ectosomes) in muscle repair (**Figure 4**). Indeed, exosomes from muscle cells (myotubes and myoblasts) are taken up twice as fast by their target cells than ectosomes (Le Bihan et al., 2012). Faster uptake of exosomes also occurs in humans where damage-induced spike in exosomes was found to return to baseline within 4 h (Frühbeis et al., 2015; Callegari et al., 2017; D'souza et al., 2018). Conversely, uphill treadmill running to exhaustion under conditions that cause muscle damage (Jung et al., 2011), increases ectosome production, and they are cleared relatively slowly—their level remain elevated even after 6 h (Frühbeis et al., 2015).

The internalization pathway taken by the EV is relevant for vesicle-mediated intercellular signaling, as different endocytic pathways taken by the EV leads to different intracellular sorting

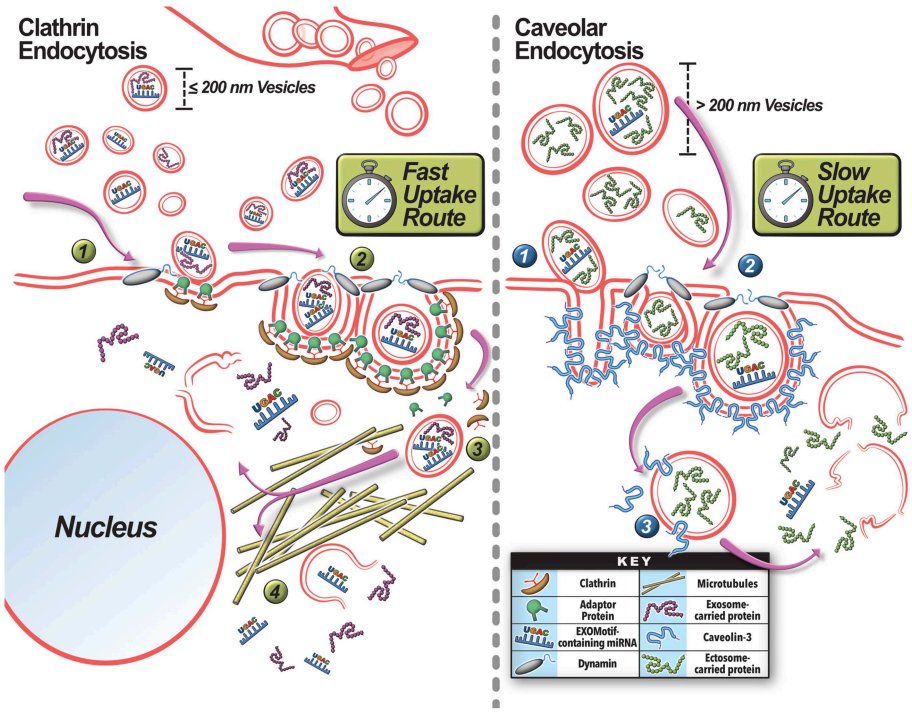

FIGURE 4 | Mechanisms for extracellular vesicle uptake by the target cells. Exosomes and ectosomes produced locally or brought in via blood vessels can be taken up by **(A)** Clathrin-dependent or **(B)** Caveolin-dependent pathways. **(A)** Smaller vesicles are preferentially taken up via clathrin-mediated endocytosis. **(1)** Internalization occurs at a site for EV engagement with the plasma membrane where clathrin-coated vesicle assembly can occur by the deformation of the membrane with the help of the EV and clathrin associated adaptor proteins. **(2)** Dynamin-2 is recruited to the clathrin-pit where it forms a collar-like structure to pinch off the endosome neck. **(3)** The internalized endosome subsequently uncoats and the vesicle is shuttled via microtubules to the perinuclear region. **(4)** There the EV cargo is released. **(B)** Larger vesicles tend to favor the caveolar endocytosis pathway. Caveolar invaginations in the membrane are created by the oligomerization of Caveolin proteins. **(1)** Once the EV encounters a caveolae conducive for internalization, it engages with the caveolae. **(2)** Proteins such as Dynamin-2 and EHD2 are recruited at this site to pinch off the caveolar endosome. **(3)** Subsequently, the caveolar endosome traffics in a microtubule-independent manner and releases the EV cargo in the cytoplasm. The rate of clathrin-mediated internalization compares to rate of receptor-mediated endocytosis, and is faster than caveolae-mediated endocytosis.

and signaling fate of vesicle cargoes as well (Rejman et al., 2004; Svensson et al., 2013; Mulcahy et al., 2014; Costa Verdera et al., 2017; Schneider et al., 2017; **Figure 4**). This topic is discussed in detail elsewhere (Rejman et al., 2004; Le Roy and Wrana, 2005; Svensson et al., 2013; Mulcahy et al., 2014; Tian et al., 2014; Costa Verdera et al., 2017; Schneider et al., 2017). Interestingly, altering the intensity and damaging-potential of exercise can shift EV production to favor exosome secretion (Oliveira et al., 2018). This may be a mechanism to confer a more rapid repair and regenerative response from surrounding target cells after a more damaging stimulus—as exosomes may be more efficiently taken up (Dittrich et al., 2013). Different pathways for exosome and ectosome uptake also impact on their intracellular shuttling and thus potential intracellular site of action by their cargoes. Clathrin-mediated uptake of smaller vesicles uses microtubules (Svensson et al., 2013) to shuttle these cargoes to the perinuclear region of the cell, while the caveolar endocytosis of larger vesicles shuttles them to the cell periphery in a microtubule-independent manner (**Figure 4**; Rejman et al., 2004). Thus, the differences in size and membrane composition of EVs could influence how, when, and where these EVs are taken up and delivered to in the cell. This could then alter the nature of intercellular signaling each of them can engage in during muscle repair and regeneration. Thus, in addition to selective cargo packaging, use of specific EVs allows the injured myofibers control over modulating the timing, site of action, and intracellular location of the signals they communicate to other cells in the injured tissue.

EXTRACELLULAR VESICLE-MEDIATED COMMUNICATION DURING MUSCLE REPAIR AND REGENERATION

Co-ordinated extracellular interactions between inflammatory cells, endothelial cells, mesenchymal stem cells (MSCs), and myogenic stem cells are critical for regeneration of injured skeletal myofibers (Wosczyna and Rando, 2018). EV-mediated transfer of cargoes facilitates repair and regeneration across tissues including cardiac, intestinal, neural, renal, respiratory, and skeletal muscles (Cocucci et al., 2009; Leoni et al., 2013, 2015; Lopez-Verrilli et al., 2013; Demonbreun and Mcnally, 2017; Taverna et al., 2017). These diverse EV cargoes help alter the phenotype of the recipient cells by affecting the recipient cell's mRNA composition, translation, or by directly transferring membrane components such as receptors, transmembrane

proteins, and lipids to activate downstream pathways involved in maintenance of tissue homeostasis and repair (Cocucci et al., 2009; Taverna et al., 2017). Below, we discuss how immediately following muscle injury EVs facilitate "myofiber repair" and later on facilitate "myofiber regeneration" to restore the lost muscle tissue.

Extracellular Vesicles in Myofiber Repair and Early Stages of Muscle Regeneration

While much of the work on EVs has focused on intercellular communication during tissue regeneration, recent studies have started to address their role in facilitating repair of injured cells. As discussed above, cell membrane damage leads to acute actin reorganization and membrane remodeling through the action of mitochondrial redox signaling, annexin binding, and ESCRT activity (Jaiswal et al., 2004, 2014; Bouter et al., 2011; Scheffer et al., 2014; Boye et al., 2017; Horn et al., 2017). Together, these help close the membrane wounds within minutes of being injured. These early stages of repair can be directly influenced by EVs. For example, intestinal epithelial cell injury causes secretion of Annexin-A1 containing vesicles that can induce redox signaling via Nox1, which act on Rac, and p120 proteins to enable faster closure of the epithelial wound (Leoni et al., 2013, 2015). The N-terminal Annexin A1 peptide Ac2-26 enables epithelial wound repair by binding formyl peptide receptor (FPR1) that activate downstream focal adhesion protein Paxillin and focal adhesion kinase (FAK) to stimulate cell migration, and wound closure (Bizzarro et al., 2012a; Leoni et al., 2015). Skeletal muscles also express these FPRs, which are crucial during regenerative stages in stimulating myoblast proliferation by exosome-derived Annexin-A1 (Bizzarro et al., 2012b). Additionally, myofiber sarcolemmal injury causes several Annexins—A1, A2, A5, and A6, to accumulate at or outside of the injured sarcolemma and some of these are also shown to be required for myofiber repair (Roostalu and Strähle, 2012; Demonbreun et al., 2016; D'souza et al., 2018; Hogarth et al., 2019). Annexin A1 is the first to accumulate at the injury site and is shed by the damaged plasma membrane by action of Annexin A2-mediated actin polymerization (Jaiswal et al., 2014). Mice lacking Annexin A2 and those expressing truncated dominant negative form of Annexin A6 poorly repair myofiber injury, implicating Annexins in vesicle shedding and other intracellular vesicle trafficking processes important for repair (Swaggart et al., 2014; D'souza et al., 2018). Interestingly, mice lacking Annexin A1 do not show defects in membrane repair, but are affected in their ability to efficiently regenerate damaged myofibers (Leikina et al., 2015). Thus, while annexins are amongst the earliest responders to myofiber injury, their role extends beyond myofiber repair to myofiber regeneration. Such a role of annexins is supported by the recent finding that annexin A2, an ectosome-enriched protein, which is required for myofiber repair, also influences the muscle resident immune and other cells that leads to adipogenic loss of muscles in Limb Girdle Muscular Dystrophy (LGMD) 2B—a disease associated with poor myofiber repair due to the lack of dysferlin protein (D'souza et al., 2018; Hogarth et al., 2019).

Shedding of vesicles by injured myofibers, muscle and other cells, is enabled by ESCRT-mediated ectosome formation at the plasma membrane. Failure of this process prevents repair of the injured plasma membrane (Jimenez et al., 2014; Scheffer et al., 2014). Unlike ILV formation at the MVB, where the ESCRTs I and II are required for the assembly of ESCRT III to mediate ILV budding and scission, ectosome budding, and scission at the site of injury is initiated by the calcium binding protein ALG-2 (Apoptosis linked gene-2) (Scheffer et al., 2014). ALG-2 accumulates at this injury site in response to the local influx of calcium and it in turn recruits its binding partner—ALG-2 interacting protein X (ALIX), to localize ESCRT III and Vps4 ATPase that cleaves the nascent ectosome (Scheffer et al., 2014). Formation of outwardly budding vesicle at the site of sarcolemmal injury has been observed in intact muscles and also in isolated skeletal muscle fibers, where the vesicle formation involves a coordinated action of multiple proteins including annexins as well as membrane proteins and actin cytoskeleton (Scheffer et al., 2014; Demonbreun et al., 2016; **Figure 1**; **Video S1**). Shedding of damaged membrane at the site of sarcolemmal injury is also observed in Zebrafish, where the membrane repair protein dysferlin is reported to accumulate sarcolemmal phosphatidylserine to the repair site to be recognized and removed through phagocytosis (Middel et al., 2016). Failure of sarcolemmal vesiculation due to the lack of ESCRTs or actin, dysferlin, and other accessory proteins that help with the proper membrane dynamics and outward budding of the injured sarcolemma prevents repair of the injured myofibers (Scheffer et al., 2014).

In addition to facilitating myofiber repair the extracellular vesicles shed by the repairing myofiber and other cells also initiate extracellular interactions for muscle tissue regeneration (**Figure 2**). For instance, EVs trigger pro-inflammatory cascades through transport of antigens loaded onto MHC class 1 and 2 complexes to T cells, as part of the initial inflammatory events required for regeneration following skeletal muscle injury (Taverna et al., 2017). Similarly, hypoxia-injured myotubes produce vesicles that increase macrophage expression of inflammatory IL-6 that in-turn alters myogenesis (Guescini et al., 2017). Initial invading neutrophils with skeletal muscle injury, migrate to the site of damage and release ectosome vesicles that contain F-actin, and membrane-bound Annexin-5, selectins, integrins, complement regulator HLA-1, and matrix metalloproteinases (MMPs) that degrade the ECM, and preferentially bind to monocytes to propagate the inflammatory response (Gasser et al., 2003; Taverna et al., 2017). Additionally, neutrophil-derived ectosomes stimulate the release of anti-inflammatory factors (TGF-β1, and IL-10) from macrophages to fine-tune inflammatory pre-conditioning for eventual pro-inflammatory macrophage induction (Gasser et al., 2003; Tidball, 2017). At later timepoints after injury, ectosomes transfer chemokine receptors (CCR4, CCR5), and stimulate release of IL-6 and monocyte chemotactic protein 1 (MCP1) that promote inflammation (Gasser et al., 2003). However, macrophages are not merely recipients of these EVs, as seen during vascular endothelial cell injury where macrophages secrete ectosome vesicles that bind platelets to initiate the coagulation cascade and wound healing (Del Conde et al., 2005). With muscle membrane injury, the acute mitochondrial ROS production near the site of injury coincides with actin buildup and ectosome release that

peaks within 2–4 min post-injury (Scheffer et al., 2014; Horn et al., 2017; **Figures 1C,D**). With their production near the injury site where mitochondrial ROS levels are the highest, ectosomes are likely to contain oxidized plasma membrane lipids which can stimulate neighboring TLR-expressing macrophages to promote inflammation and cell repair (Zhou et al., 2018). Interestingly, miRNAs delivered via exosomes, reduce macrophage expression of TLRs, and cause these cells to take-up other vesicles and their cargoes without immune activation, a vesicle-signaling effect demonstrated in muscular dystrophy (Mancek-Keber et al., 2015; Phinney et al., 2015; Hindi and Kumar, 2016). Such findings raise the intriguing possibility that exosomes and ectosomes may act in coordinated ways to fine-tune their intercellular signals and cellular stimulation in muscle repair and regeneration.

Extracellular Vesicles in Myofiber Regeneration and Restoration of Muscle Damage

As discussed in section Events that Facilitate Myofiber Repair and Regeneration, injuries that cause myofiber death require regeneration of these myofibers. This occurs through a program that first clears the cellular debris and then guides the regenerative response (Chazaud, 2016). This program involves release of neutrophil-attractants CXCL1 and CCL2, which enrich the muscle environment in cytokines that activate macrophages to a pro-inflammatory phenotype (Tidball, 2017). These pro-inflammatory cytokines cause myotubes to increase production of exosomes that are loaded with the myostatin protein, while concomitantly decreasing the level of packaged myostatin antagonist protein decorin (Kim et al., 2018). These exosomes inhibit myogenic regulatory factors (MRFs)—myoD and myogenin expression and Akt and mTOR-mediated myogenesis, while increasing myoblast COX-2 expression (Kim et al., 2018). Thus, at this early pro-inflammatory stage of muscle repair, exosomes participate in limiting myogenesis and allowing the inflammatory cells to clear the damaged tissue. This is followed by a change in macrophage polarization to pro-regenerative state with a concomitant rise in MSCs (Tidball, 2017; Wosczyna and Rando, 2018). These MSCs secrete vesicles that stimulate MYOD, and Myogenin, which facilitate myofiber regeneration in target cells (Phinney et al., 2015). Moreover, MSC exosomes attenuate fibrosis, improve capillary density, and accelerate regeneration of the injured muscle (Nakamura et al., 2015). This is largely accomplished via cellular transfer of vesicle cargo VEGF, and IL-6, in addition to exosome-enriched miR-1, miR-133, miR-206 (containing EXOmotif's GGAC, CCCU, CCCG, respectively), and miR-125b, miR-494, and miR-601, that promote a variety of pro-regenerative cellular processes (**Table 3**). However, these MSCs not only transfer pro-regenerative vesicles to injured skeletal muscle cells and SCs, they can also package and transfer mitochondria and mitochondrial proteins within ectosomes, to the invading macrophages. This is suggested to enhance macrophage energetics and inflammatory activity vital for regeneration (Phinney et al., 2015; Sansone et al., 2017).

Exosomes also facilitate myogenesis in other muscle injury contexts. Skeletal muscle denervation injury shifts the muscle-derived EV enrichment from miR-133a and miR-720 to the EXOmotif containing miRNA—miR-206. miR-206 stimulates satellite cell differentiation, myofiber maturation, and brain-derived neurotrophic factor (BDNF), and nerve-growth factor (NGF) production, which together enhance myofiber re-innervation (De Gasperi et al., 2017). These pro-myogenic and neuro-regenerative effects of miRNA-206 offer further evidence for the benefits of context-specific EV cargo loading to facilitate distinct regenerative effects (Mccarthy, 2008; Yuasa et al., 2008). SC differentiation during regeneration, also actively increases their secretion of vesicles that contain growth factors (IGF-1, HGF, TGF-B1, FGF2, VEGF, and PDGF) that act in SC chemotaxis, lineage commitment, and neovascularization, while simultaneously transferring miRNA cargoes such as miR-206, and miR-1 that promote SC differentiation by altering myogenic gene expression in neighboring SCs (Braun and Gautel, 2011; Forterre et al., 2014; Murphy et al., 2018). Additionally, exosomes are also released by differentiating satellite cells to help attenuate fibrosis and enhance myofiber regeneration via cargo transfer (Braun and Gautel, 2011; Forterre et al., 2014; Choi et al., 2016; Murphy et al., 2018). Indeed, skeletal muscles injured by laceration, secrete exosomes enriched in myogenic growth factors that subsequently stimulate differentiation of adipose-derived stem cells toward a myogenic lineage to assist in muscle regeneration (Choi et al., 2016). Similarly, oxidatively injured myotubes promote satellite cell proliferation by vesicle-mediated repression of myogenin expression in target satellite cells, resulting in faster wound closure in an *in-vitro* wound assay (Guescini et al., 2017).

Similar to the integral role of vesicles in the regenerative mobilization of tissue resident stem cells, they are also involved in remodeling of damaged tissue by facilitating angiogenesis, fibroblast activation, deposition, and degradation of new ECM, and tissue cell replenishment. Skeletal muscle remodeling involves dynamic changes in extracellular matrix structure and composition. Indeed, exosomes isolated from SCs after overload-induced muscle damage downregulate collagen and fibronectin production by neighboring fibroblasts through the transfer of their miR-206 cargo that inhibits fibroblast Rrbp1—the master regulator of collagen and fibrogenic expression (Fry et al., 2017). This attenuated ECM deposition can then help damaged muscle regenerate and remodel for hypertrophic adaptation. Similarly, high intensity cycling-induced muscle damage induces enrichment in 12 miRNAs within the exosome pool which did not concomitantly increase in the plasma or within the cells themselves, nor was there a global elevation in exosomal miRNAs—suggesting that these miRNAs were neither passively leaked, nor part of a general increase in exosomal miRNA abundance (D'souza et al., 2018). Many of these 12 miRNAs are known to fine-tune muscle regeneration. During muscle regeneration and remodeling, miR-208a regulates fiber type determination, while miR-126 and miR-16 regulate

blood vessel formation (D'souza et al., 2018). These findings support the aforementioned evidence that specific miRNAs may be transcribed by the cell under certain cellular stresses to accomplish highly specific and targeted intercellular signaling goals in myofiber regeneration and remodeling. Both miR-126 and miR-16 contain the UGAC and GGCG EXOmotifs, respectively, and stimulate neovascularization in response to an aerobically taxing and damaging stimulus- implicating these motifs in context-specific and selective packaging in exosomes based on the nature of the stimulus/damage. This specific miRNA production and packaging in exosomes shows that under certain cellular stresses, EVs accomplish specific intercellular signaling goals to enable myofiber regeneration and remodeling. This is not limited to skeletal muscles alone however, as in the early stages of cardiac muscle hypertrophy, the mechanical stress and damage to cardiomyocytes promote secretion of miRNA-378-containing EVs that impair fibroblast hyperplasia and attenuate their production of collagen, to limit cardiac fibrosis (Yuan et al., 2018). These findings implicate specific miRNA transcription by the cell under certain cellular stresses to accomplish highly specific intercellular signaling goals in muscle regeneration and remodeling.

In addition to cargo packaging, purposeful and context-specific release of EVs also regulates contextual signaling by EVs. Unlike damage to healthy muscles, damage associated with Duchenne muscular dystrophy causes muscle-resident fibroblasts to secrete vesicles with increased levels of miR-199a-5p that promotes increased fibrosis in skeletal muscle and surrounding matrix (Zanotti et al., 2018). Similarly, vesicles from the serum of *mdx* mice, when administered to myoblasts, promote survival, and reduce cell death (Murphy et al., 2018). These *mdx* exosomes protect against cell death in muscle cells stressed by excessive reactive oxygen species—in a manner that is linearly associated with the concentration of these vesicles (Matsuzaka et al., 2016). This pro-survival effect of EVs was achieved by the exosome transfer of miR-133a—a miRNA that decreases caspase-mediated proteolysis and decreases expression of apoptosis-associated genes (Matsuzaka et al., 2016). Conversely, inhibiting exosome formation in the MVB (by neutral sphingomyelinase inhibition), or blocking vesicle uptake by target cells via reduction of membrane cholesterol with MBCD treatment, each abolished the protective effect of *mdx* muscle exosomes in hypoxia-stressed muscle cells (Matsuzaka et al., 2016). Interestingly, pro-myogenic miRNA cargoes (miRNAs−1, 133a, and 206) in exosomes produced by *mdx* muscles, are absent from exosomes from muscles where dystrophin has been restored (Roberts et al., 2012). This suggests that muscle instability-induced damage results in selective production and packaging of exosomes to promote regenerative effects, which can be attenuated by improved muscle stability. This exemplifies cell-stress specific vesicle cargo production and packaging. Supporting this feature of *mdx* EVs, dystrophin-deficient cardiomyocytes also produce not only smaller EVs than healthy cardiomyocytes, but these vesicles have the unique capacity to protect neighboring cardiomyocytes from cell death following their uptake (Gartz et al., 2018).

CONCLUSION

It has become increasingly clear from the mounting body of evidence in skeletal muscle injury, that EV secretion and extracellular signaling occurs along the continuum of muscle repair and regeneration. The studies discussed above suggest the existence of an injury-responsive production of EVs packaged with proteins, RNAs, and lipids to facilitate repair and regeneration. This cargo packaging appears to be selective based upon the context and type of injury, required intercellular signaling, and intended cellular targets, thus adding a previously unrecognized layer of complexity to this process beyond the initially postulated cell debris hypothesis. While exosomes and ectosomes are similar in their basic structure and range of cargoes, these vesicles differ in nearly all critical elements—specific cargo, route of cellular uptake, rate of uptake, intracellular handling by the target cell, and time course of secretion by the parent cell. While EVs are produced actively, and their signaling effects help orchestrate the continuum of response from myofiber repair to regeneration, this complex diversity of EVs in the context of healthy and diseased muscle repair needs to be acknowledged in muscle physiology research. These features of EVs raise additional mechanistic questions—how does alteration in total ectosome and exosome production, rate of secretion, and cargoes improve or impair health of injured muscles? Does the improved repair capacity of skeletal muscle with repeated insult (i.e., repeated bout effect of eccentric exercise) derive, in-part, from alterations in vesicle production, secretion, and or cargo loading? Answers to such questions may build upon the current knowledge discussed here, and may highlight the roles of EVs being provided by other tissues and physiological processes. Such findings are bound to offer exciting new insights into the coordination of the complex process of muscle repair, and how this can be optimized for therapeutic purposes in disease as well as in sport.

AUTHOR CONTRIBUTIONS

DB and JJ contributed to the conception and writing of this paper.

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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.2019.00828/full#supplementary-material

Video S1 | Real-time visualization of the release of small and large vesicles by injured skeletal myofibers. Confocal time lapse video shows a pair of live myofibers in an intact mouse biceps muscle injured focally via pulsed laser. Myofiber sarcolemma and released vesicles are labeled with green membrane binding dye FM 1-43 present in the buffer. Laser injury occurs at 20 s into the video—note the secretion of vesicles from the site of injury, which then travel/accumulate in the inter-fiber space. The time is shown in min:ss.msec format and the scale bar represent 10 μm.

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Insights on the Role of Putative Muscle-Derived Factors on Pancreatic Beta Cell Function

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Skeletal muscle is a main target of insulin action that plays a pivotal role in postprandial glucose disposal. Importantly, skeletal muscle insulin sensitivity relates inversely with pancreatic insulin secretion, which prompted the hypothesis of the existence of a skeletal muscle-pancreas crosstalk mediated through an endocrine factor. The observation that changes in skeletal muscle glucose metabolism are accompanied by altered insulin secretion supports this hypothesis. Meanwhile, a muscle-derived circulating factor affecting *in vivo* insulin secretion remains elusive. This factor may correspond to peptides/proteins (so called myokines), exosomes and their cargo, and metabolites. We hereby review the most remarkable evidence encouraging the possibility of such inter-organ communication, with special focus on muscle-derived factors that may potentially mediate such skeletal muscle-pancreas crosstalk.

Keywords: myokines, miRNA, exosomes, insulin secretion, insulin sensitivity, muscle, beta cell, crosstalk

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INTRODUCTION

Skeletal muscle is the largest organ in lean humans that plays a main physiological role in insulin-stimulated glucose disposal (Baron et al., 1988). To accomplish this, muscle cells adapt their metabolism to changes in circulating metabolites and hormones. Muscle cell adaptation also involves autocrine and paracrine interaction among cell types (Pillon et al., 2013). Thus, several muscle cell-derived factors have been detected, including myokines (Bouzakri et al., 2011; Raschke et al., 2013; Scheler et al., 2013; Hartwig et al., 2014; Mizgier et al., 2017), exosomes and their cargo (Forterre et al., 2014), and metabolites (Daskalaki et al., 2018). Whether or not muscle-derived factors increase in blood at a sufficient magnitude to influence distant organs has not yet been elucidated.

Among muscle-targeted organs, a crosstalk between skeletal muscle and the pancreas has been proposed (Bouzakri et al., 2011; Mizgier et al., 2017; Rutti et al., 2018). Pancreatic beta cells secrete insulin to facilitate glucose disposal in insulin-sensitive tissues such as skeletal muscle, so hyper- and hypo-glycemia are prevented. The extent at which insulin is secreted depends of the amount of glucose taken up by beta cells (Rorsman and Braun, 2013). Furthermore, beta cells receive information from other organs to adjust insulin secretion. For instance, when glycemia is low, the brain increases sympathetic activity to suppress insulin secretion, which raises glycemia back to normal levels (Porte, 1969).

Whether a similar mechanism is activated under conditions of high glycemia (e.g., after meals) is unknown. Since skeletal muscle is the main tissue responsible for insulin-mediated glucose disposal (Baron et al., 1988), it is intuitive to propose that skeletal muscle can signal back toward the pancreas. Such organ crosstalk may allow adjusting insulin secretion to insulin demands to maintain glycemia within the physiological range. Here, we will discuss the most remarkable evidence supporting such a muscle-pancreas crosstalk, and the progress made in identifying the endocrine mediator.

PHYSIOLOGICAL RELEVANCE OF A PUTATIVE SKELETAL MUSCLE-PANCREAS CROSSTALK

Pancreatic insulin secretion must tightly match insulin demands for proper glycemic control. Upon glucose ingestion, the increase in glycemia and glucose transport into pancreatic beta cells may suffice to adapt insulin secretion to insulin demand. In turn, higher glycemia often observed in insulin resistance, especially in postprandial conditions (Petersen et al., 2004; Galgani and Ravussin, 2012), may also be sufficient to elevate insulin secretion. Thus, blood glucose concentration might itself be the factor contributing to adjust insulin secretion.

However, convincing evidence shows that blood glucose concentration cannot entirely accommodate insulin secretion to insulin demand (Kahn et al., 1989, 1990). Another physiological mechanism must therefore exist to maintain glycemic control. The skeletal muscle-pancreas crosstalk appears as an attractive hypothesis. The fact that insulin is secreted as an inverse function of insulin sensitivity under fasting and postprandial conditions (Galgani et al., 2014) reinforces the notion of such organ crosstalk (Mizgier et al., 2014). The mechanism underlying this association remains elusive. But, it may be mediated by a factor coming from skeletal muscle (and eventually from other insulin-sensitive tissues). Thus, skeletal muscle may play a pivotal physiological role in the adjustment of insulin secretion to maintain glycemic control throughout the day.

EVIDENCE SUGGESTING A SKELETAL MUSCLE-PANCREAS CROSSTALK

A large-scale, multi-center study investigated which factors determine the change in insulin secretion (by plasma C-peptide concentration) during an isoglycemic-hyperinsulinemic clamp (Mari et al., 2011). The analysis highlighted insulin-stimulated glucose disposal rate as a direct determinant of insulin secretion. That finding was independent of sex, age, family history of diabetes and body mass index. Noteworthy, that association was attenuated in individuals with altered glycemic control. One can suggest that the extent to which glucose is taken up in skeletal muscle, as a main insulin-sensitive glucose disposal site (Baron et al., 1988), somehow determines pancreatic beta cell function. Alternatively, skeletal muscle insulin sensitivity may just reflect beta cell insulin sensitivity. The observation that insulinemia during the isoglycemic clamp related positively with insulin secretion suggests a direct action of insulin on its secretion (Mari et al., 2011). Pursuing that latter hypothesis, i.e., that insulin enhances its secretion through a direct effect on beta cell function, a sophisticated study was conducted in humans (Halperin et al., 2012).

The study compared the effect of saline vs. insulin infusion on glucose-stimulated insulin secretion (GSIS) in individuals with normal and abnormal glycemic control (Halperin et al., 2012). Blood glucose concentration was maintained at similar values in both sessions by co-infusing glucose throughout the insulin infusion session. When compared with saline, the insulin infusion increased GSIS in healthy individuals. Such effect was attenuated in volunteers with altered glycemic control. These results were interpreted as proof that insulin stimulates its own secretion in pancreatic beta cells. Furthermore, lower GSIS in subjects with altered glycemic control may fit with a state of beta cell insulin resistance. Taken together, these findings were considered in agreement with some *in vitro* data showing that insulin stimulates its secretion (Aspinwall et al., 1999; Srivastava and Goren, 2003; Jimenez-Feltstrom et al., 2004; Caporarello et al., 2017; **Supplementary Table 1**).

However, other studies have not confirmed that insulin (*in vitro*) stimulates its secretion (reviewed in Leibiger et al., 2008 and updated in **Supplementary Table 1**). For instance, a neutral effect was reported in isolated rat islets (Zawalich and Zawalich, 2002) and a rat beta cell line (INS-1) (Collier et al., 2004). Even more, an inhibitory action was concluded from experiments in perfused canine pancreases (Iversen and Miles, 1971); human islets (Johnson and Misler, 2002; Persaud et al., 2002); rat islets (Araujo et al., 2002); mouse beta cells; and INS-1 cells (Collier et al., 2004). Unpublished findings from our group also support an inhibitory action. Briefly, isolated mouse islets in which insulin signaling was inhibited (with wortmannin) displayed higher GSIS. This suggests that insulin secreted upon glucose stimulation decreased further insulin secretion.

Aforementioned findings *in vitro* are controversial (**Supplementary Table 1**), although it predominates an inhibitory action of insulin on its secretion. A study that assessed C-peptide release at different insulin concentrations in mouse islets might enlighten an answer. This study showed that insulin stimulated its secretion when tested at concentrations between 0.05 to 0.1 nM. In turn, insulin inhibited its secretion at a higher concentration (1 μM) (Jimenez-Feltstrom et al., 2004). Whether this dose-dependent effect of insulin on its secretion explains such controversial findings is elusive.

Now, if insulin indeed displays an inhibitory action on its own secretion *in vivo*, how can the increase in GSIS after insulin infusion be explained? (Halperin et al., 2012). From studies conducted by the same research group (Bouche et al., 2010; Lopez et al., 2011; Halperin et al., 2012), it is acknowledged that glucagon, cortisol, free-fatty acids or potassium are likely not responsible. We then propose that an endocrine factor signaling from skeletal muscle toward the pancreas may enhance insulin secretion. If insulin plays an inhibitory action on its secretion *in vivo*, this endocrine factor must be potent enough to overcome that inhibition. We speculate that the release into circulation of

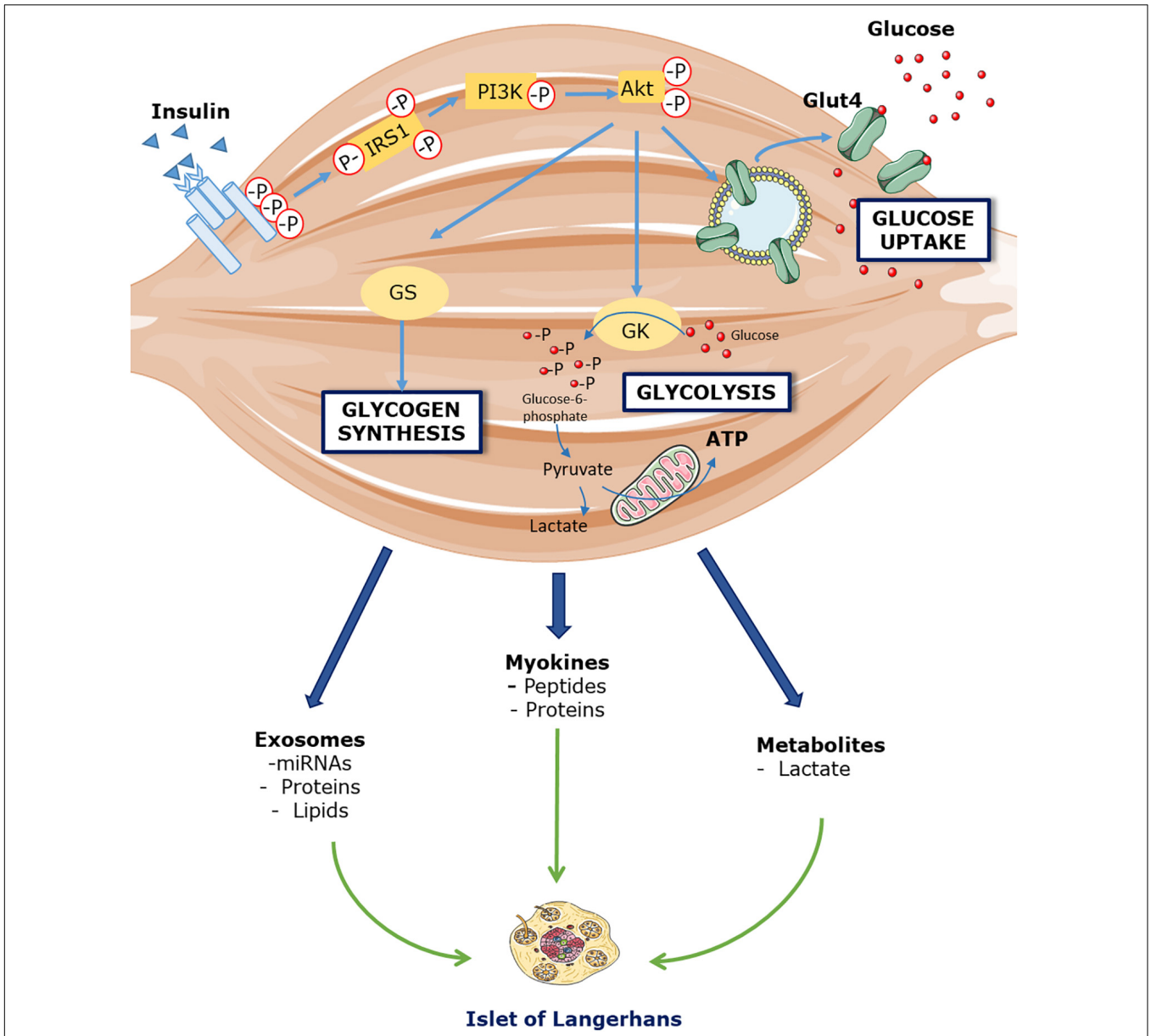

FIGURE 1 | Hypothetical model linking skeletal muscle insulin sensitivity and pancreatic insulin secretion. Insulin binds to its receptor located in skeletal muscle cells that triggers phosphorylation of Akt, a pivotal protein involved in insulin signaling. As a consequence, GLUT4 are translocated from cytosol to cell membrane, which increases facilitated transport of glucose into muscle cells. Increased cellular glucose flux will enhance its disposal by increasing glycogen synthesis and glucose utilization for ATP synthesis. Changes in glucose disposal at the level of uptake, storage or oxidation may be the signal to trigger the release of a putative factor into circulation, which can then influence insulin secretion directly through its action on beta cells, or indirectly through extra-pancreatic cell types.

that factor is triggered by insulin itself or the increase in glucose disposal (**Figure 1**).

Two mouse models with conditioned expression of specific proteins in skeletal muscle support this muscle-pancreas crosstalk. A loss-of-function of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) (Handschin et al., 2007) and a gain-of-function of muscle-specific RING-finger 1 protein (MuRF1) (Hirner et al., 2008) are accompanied by altered skeletal muscle glucose metabolism as well as abnormal insulin secretion *in vivo*. In the former study, the loss of PGC1α expression at the muscle level induced glucose intolerance in mice due to hypoinsulinemia. Such defect in *in vivo* insulin secretion was not detected in isolated islets, suggesting that a circulating muscle-derived factor present *in vivo* plays a role. In this case, IL-6 was suggested as a candidate. The second study showed that overexpression of MuRF1 was accompanied of lower hepatic glycogen content and hyperinsulinemia. The authors concluded that an alteration of MuRF1 expression in skeletal muscle stimulates insulin secretion, providing a regulatory feedback loop between muscle

and pancreas. Besides, we found that conditioned medium from human myotubes increases insulin secretion in isolated pancreatic islets (Bouzakri et al., 2011; Mizgier et al., 2017). Moreover, the contribution of skeletal muscle on GSIS seems to be fiber specific (Rutti et al., 2018), adding further complexity to the muscle-pancreas axis. These data suggest that insulin secretion is influenced through muscle-derived factors released in response to modifications in skeletal muscle glucose metabolism. A muscle-to-pancreas communication therefore seems to exist.

MUSCLE-RELEASED FACTORS TARGETING INSULIN SECRETION

Myokines

Myokines having influence on insulin secretion should express their receptors in pancreatic islets. Because dozens and even hundreds of proteins have been detected in conditioned media from human muscle cells (Hartwig et al., 2014; Weigert et al., 2014), we first compared the presence of selected proteins in different studies (see **Supplementary Table 2** for further details). Nineteen out of 73 (26%) proteins were consistently detected regardless of the detection method (Raschke et al., 2013; Scheler et al., 2013; Hartwig et al., 2014). Among secreted proteins, interleukins (IL) 1-beta, 2, 6, 7, 8, 10, and 12 stand out. Also, chemokines such as monocyte chemoattractant protein-1 (MCP1/CCL2); regulated on activation, normal T cell expressed and secreted (Rantes); and growth-regulated oncogene (GRO) alpha, beta and gamma. The proteins detected by at least two analytical approaches were selected for a gene expression search of their receptors in human, mouse and rat pancreatic islets and beta cells in the Beta Cell Gene Atlas (Smink et al., 2005; **Table 1**). Many of the selected proteins do express their receptors in islets or beta cells, which opens the number of potential endocrine candidates. Most of these proteins have often more than one receptor belonging to CC or CXC receptor families, which adds further complexity to the quest for endocrine muscle-pancreas mediators. As an example of the complexity, CXCL10 appears to interact with the toll-like receptor 4 but not with its known receptor (CXCR3) in beta cells (Schulthess et al., 2009). Additionally, myokine receptors may also be located in extra-pancreatic tissues. For instance, IL-6 binds to receptors located in intestinal L cells to stimulate glucagon-like peptide 1 secretion, which then can enhance GSIS (Ellingsgaard et al., 2011).

The effect of specific myokines on beta cell function has been assessed by using their recombinant forms. IL-6 showed a stimulatory action on GSIS in mice after an acute injection (400 ng) that emulated its circulating concentration in response to exercise or high-fat diet (Ellingsgaard et al., 2011). By contrast, neutral (Ellingsgaard et al., 2008) or inhibitory (Handschin et al., 2007) actions are also reported. These controversial outcomes might be due to different IL-6 levels achieved. Physiological concentrations of IL-6 (<100 pg/mL) had anti-inflammatory effect and show a stimulatory action on GSIS (Andreozzi et al., 2006; Ellingsgaard et al., 2011). In turn, higher IL-6 concentrations (500–25000 pg/mL) show inhibitory action on GSIS (Sandler et al., 1990; Southern et al., 1990; Eizirik et al., 1994).

Additional cytokine assessment includes IL-12, CXCL10 and fractalkine (CX3CL1). Both IL-12 and CXCL10 have shown to impair GSIS in INS-1 cells (Taylor-Fishwick et al., 2013) and in human islets (Schulthess et al., 2009; Taylor-Fishwick et al., 2013). In turn, fractalkine at 100 ng/mL increased GSIS in human islets and in a mouse beta cell line (MIN6) (Lee et al., 2013). However, fractalkine did not influence GSIS when added at a lower concentration in sorted rat beta cells or human islets (Rutti et al., 2014), although it prevented tumor necrosis factor alpha-induced GSIS reduction in sorted rat beta cells (Rutti et al., 2014). The type of receptor also plays a role in determining the action of its ligand. For instance, Rantes appears to have a stimulatory effect when bound to GPR75 receptor (Liu et al., 2013), while an inhibitory action when bound to CCR1 receptor (Pais et al., 2014).

An additional aspect to take into account when searching for potential endocrine mediators is to distinguish among proteins released from cell lysis vs. an active, regulated secretion process. In fact, many detected proteins do not seem to be secreted as they have no signal peptide, known receptor and their described function is at the intracellular level (**Supplementary Table 3**). Thus, the myokinome may include a much lower number of proteins than previously anticipated.

Exosomes and Their Cargo

Extracellular vesicles are a heterogeneous population of cell-derived membranous structures including microvesicles and exosomes. Exosomes are the smallest vesicles (ranging from 30 to 100 nm of diameter) formed by the fusion of multivesicular endosomes with the plasma membrane (van Niel et al., 2018). Exosomes carry proteins, lipids and nucleic acids [e.g., micro (mi)RNAs] (van Niel et al., 2018). Released exosomes can dock to the plasma membrane of a target cell, where they can fuse with the plasma membrane or be endocytosed before delivering its cargo (Raposo and Stoorvogel, 2013). Some evidence suggests a role in cell-to-cell and organ-to-organ communication, particularly through miRNAs (Camussi et al., 2010). However, the role of exosomes in whole-body homeostasis remains unclear.

Skeletal muscle cells can release exosomes (Forterre et al., 2014) and miRNAs (contained or not in exosomes) (Aoi and Sakuma, 2014). Furthermore, exercise and metabolic diseases such as type 2 diabetes (T2D) affect the expression of several muscle-derived miRNAs (myomiRs). For instance, miR-1 and miR-133a expressions are increased by acute endurance exercise in untrained subjects while miR-133b and miR-206 are not affected by acute exercise (Nielsen et al., 2010). In turn, all of these myomiRs were downregulated after a 12 week training period (Nielsen et al., 2010). Other myomiRs such as miR-23b/27b are down-regulated during muscle cell differentiation in individuals with T2D vs. healthy subjects (Henriksen et al., 2017), while the miR-29 family is up-regulated in muscle from T2D vs. non-diabetic donors (Massart et al., 2017).

TABLE 1 | Receptor expression in human, mouse and rat pancreatic islets and beta cells of cytokines and chemokines detected in conditioned media from human myotubes.

Protein	Gene	Uniprot identifier	Receptor	mRNA expression					
				Beta cells			Islets		
				Human	Mouse	Rat	Human	Mouse	Rat
GRO α/β/γ	CXCL1/2/3	P09341/P19875/P19876	CCR-1	–	–	–	–	++	–
			CCR-5	–	–	+	+	++	–
			CXCR-1	++	–	No data	No data	–	+
			CXCR-2	++	–	+	–	–	–
			CXCR-3	+++	–	++	++	+	+
IFN-γ	IFN-G	P01579	INFGR-1	++	+++	+++	++	+++	++
			INFGR-2	++	+++	+	++	+++	++
IL-1β	IL-1B	P01584	IL1R-1	+++	++	+++	+++	+++	+
			IL1R-2	+	+++	++	++	++	++
IL-2	IL-2	P60568	IL2R-α	–	–	+	+	+	–
			IL2R-β	–	+	–	++	++	+
IL-6	IL-6	P05231	IL6R	+	++	+++	+++	–	++
IL-7	IL-7	P13232	IL2R-γ	++	–	+++	–	++	+
			IL7R	++	No data	++	+	+	No data
IL-8	CXCL8	P10145	CXCR-1	++	–	No data	No data	–	+
			CXCR-2	++	–	+	–	–	–
			DARC	++	No data	++	++	–	No data
IL-10	IL-10	P02778	IL10R-α	++	+	–	–	++	–
			IL10R-β	+++	++	+++	++	+++	No data
IL-12	IL-12A/B	P29459/P29460	IL12R-β1	–	No data	+	–	+	No data
			IL12R-β2	+	+	++	++	+	No data
LIF	LIF	P15018	LIFR	–	+	+	++	++	+
M-CSF	CSF1	P09603	CSF1R	+	+	+++	+	++	+
MCP-1	CCL2	P13500	CCR-1	–	–	–	–	++	–
			CCR-2	No data	–	++	+	No data	–
			CCR-5	–	–	+	+	++	–
MIF	MIF	P14174	CXCR-2	++	–	+	–	–	–
			EGFR	+	+++	+++	++	+++	+++
RANTES	CCL5	P13501	CCR-1	–	–	–	–	++	–
			CCR-2	No data	–	++	+	No data	–
			CCR-3	+	+	–	–	–	+
			CCR-4	–	+	+	–	–	+
			CCR-5	–	–	+	+	++	–
			GPR75	No data	+	–	+	+	No data
VEGF-A	VEGFA	P15692	VEGFR-1	++	No data	+++	+	+++	No data
			VEGFR-2	+	++	+++	+	++	++
G-CSF		P09919	CSF3R	+	+	No data	+	++	No data
IL-13		P35225	IL13RA1	+	++	++	+++	++	+++
TNF-α		P01375	TNFRSF1A/TNFR1	+++	+++	+++	+++	+++	+++
			TNFRSF1B/TNFBR	+++	+	–	+	++	–
IL-4		P05112	IL4R	++	+	–	+++	+	–
			IL13RA1	+	++	++	+++	++	+++

*Specific receptor name for each cytokine and chemokine detected in human myotube conditioned media in Hartwig et al. (2014) and Mizgier et al. (2017) studies (see **Supplementary Table 2**) were identified browsing the UniProt and STRING protein databases. Receptor mRNA expression were obtained from the Beta Cell Gene Atlas (Smink et al., 2005). Low (+), moderate (++), enriched (+++) expression level; not expressed (–).*

Besides myomiRs expression being affected by exercise or diabetic status, exosome-associated miRNAs can also be transferred to other cell types. In this regard, Jalabert et al. (2016) found that skeletal muscle-derived exosomes injected into mice specifically targeted pancreatic islet cells and affected gene expression and proliferation of beta cells. Whether or not skeletal muscle-derived exosomes can influence insulin secretion deserves attention.

Metabolites

The interaction between muscle and pancreas may not be restricted to myokines and exosomes. Metabolites such as lactate may also participate, considering that skeletal muscle plays a predominant role in lactate metabolism. In addition, we (Galgani et al., 2013) and others (Lovejoy et al., 1992) have found a direct correlation between plasma lactate concentration and insulin resistance. Thus, lactate might represent a candidate for linking skeletal muscle insulin sensitivity with insulin secretion. However, its actual relevance in driving insulin secretion is controversial. On the one hand, lactate showed to stimulate *in vitro* insulin secretion (Meats et al., 1989; Akiyoshi et al., 1999). On the other hand, lactate was reported to have a null effect on insulin secretion (Ishihara et al., 1999). The latter explained by lacking expression of the monocarboxylate transporter 1 in pancreatic beta cells (Ishihara et al., 1999; Schuit et al., 2012).

CONCLUSION

For many years, the inverse relationship between insulin sensitivity and its secretion is known. However, an underlying mechanism relating these processes remains elusive. Strikingly, *in vivo* animal (Handschin et al., 2007; Hirner et al., 2008) and human (Mari et al., 2011; Halperin et al., 2012) studies show that changes in skeletal muscle glucose metabolism are accompanied by differential insulin secretion. Some of the evidence has been mostly interpreted as a direct role of insulin on its own secretion (Mari et al., 2011; Halperin et al., 2012). Because insulin drastically increases skeletal muscle glucose metabolism, we propose that a putative interaction between skeletal muscle and the pancreas may proceed. Assessing a causal relationship between these processes is challenging. It requires altering muscle glucose metabolism while maintaining similar blood glucose and insulin concentration. Under that setting, it will be essential to combine blood sampling from arterio-venous balance with proteomic analysis. Such assessment will contribute to identifying such an endocrine factor (or refute its existence) in conditions of physiological (e.g., transition from fasting to postprandial) and pathophysiological (e.g., normal vs. abnormal glycemic control) relevance.

The definition of myokine also needs to be revisited. At present, many muscle cell-derived proteins are named myokines. However, there is no evidence that those factors can indeed affect the function of any organ *in vivo*. Perhaps, the strongest evidence for supporting an endocrine role of a myokine on a distant organ is attributed to IL-6. Thus, in individuals undergoing a 12 week exercise training, blocking IL-6 action (by infusing an IL-6 receptor antibody) prevented the decrease in visceral fat mass as detected in participants receiving vehicle infusion (Wedell-Neergaard et al., 2018). Authors interpreted that finding as proof of the lipolytic action of IL-6 shown in earlier studies (van Hall et al., 2003). Certainly, Wedell-Neergaard's study cannot claim that muscle-derived IL-6 was responsible of decreasing visceral fat mass. In any case, it is the strongest current evidence in humans linking skeletal muscle contraction with changes in a distant tissue throughout a circulating factor. Whether or not blocking IL-6 action had any effect on insulin secretion after exercise training deserves analysis.

Finally, one should bear in mind that besides skeletal muscle, organs such as the liver and adipose tissue, may also secrete a number of factors into circulation. Those factors may play a role in mediating the interaction between insulin sensitivity and its secretion. Identifying the mechanism linking insulin sensitivity with insulin secretion, in particular in dynamic states such as the transition from fasting to postprandial conditions, may have a strong impact on designing therapies for improving insulin secretion in pre-diabetic and diabetic individuals.

AUTHOR CONTRIBUTIONS

MM and JG conceived the present idea and drafted the manuscript. All authors revised 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.2019.01024/full#supplementary-material

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REVIEW
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Skeletal Muscle-Released Extracellular Vesicles: State of the Art

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All cells export part of their intracellular content into the extracellular space through the release of various types of extracellular vesicles (EVs). They are synthetized either from the budding of the plasma membrane [i.e., microparticles (MPs, 150–300 nm size)] or from the late endosomes in which intraluminal vesicles progressively (ILVs) accumulate during their maturation into multivesicular bodies (MVBs). ILVs are then released into the extracellular space through MVB fusion with the plasma membrane [i.e., exosomes (50–100 nm size)]. In the context of metabolic diseases, recent data have highlighted the role of EVs in inflammation associated with pancreas dysfunction, adipose tissue homeostasis, liver steatosis, inflammation, and skeletal muscle (SkM) insulin resistance (IR). Among these insulin-sensitive tissues, SkM is the largest organ in human and is responsible for whole-body glucose disposal and locomotion. Therefore, understanding the contribution of SkM-EVs in the development of diabetes/obesity/dystrophy/,-related diseases is a hot topic. In this review, we have summarized the role of SkM-EVs in muscle physiology and in the development of metabolic diseases and identify important gaps that have to be filled in order to have more precise information on SkM-EVs biological actions and to understand the functions of the different subpopulations of SkM-EVs on the whole-body homeostasis.

Keywords: exosomes, microparticles, skeletal muscle (myotubes), extracellular vesicles, organ cross-talks

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INTRODUCTION

Skeletal muscle (SkM) is the largest organ in the human body. It is responsible for whole-body glucose and energy homeostasis, locomotion, and serves as body protein pool. It is a highly adaptable tissue responding to numerous environmental conditions (e.g., physical activity/sedentarity) and physiological challenges (e.g., nutrition, chronic inflammation) by changing fiber size and composition. These modifications are associated with the secretion of myokines into the extracellular milieu capable of modulating homeostatic adaptations in other peripheral organs (e.g., pancreas, adipose tissue, and bone) (Plomgaard et al., 2012; Guo et al., 2017; Leal et al., 2018; Lee and Jun, 2019) or involved in the process of myogenesis (Henriksen et al., 2012). During the last decade, it has been shown that muscle cells also release extracellular vesicles (EVs) into the extracellular milieu, which represent new paracrine and endocrine signals that have modified our conceptual basis to explain

how muscles communicate to other organs (Guescini et al., 2010; Le Bihan et al., 2012; Romancino et al., 2013; Forterre et al., 2014a). In this review, we summarize the knowledge about SkM-EV contents (protein/nucleic acids/lipids) and on the role of SkM-EVs on muscle physiology and on the development of metabolic diseases. We also identify important questions that have to be elucidated in order to have more precise information on SkM-EVs biological actions and to understand the functions of the different subpopulations of SkM-EVs on the whole-body homeostasis.

Skeletal Muscle Cells Release Various Types of Extracellular Vesicles

All cells export part of their content into various types of extracellular vesicles (EVs) (**Figure 1A**). They are synthetized either from the budding of the plasma membrane [i.e., microparticles (MPs), 150–300 nm size)] or from the maturation of late endosomes in which intraluminal vesicles (ILVs) progressively accumulate to form the multivesicular bodies (MVBs). ILVs are then released into the extracellular space through exocytosis of MVB [i.e., exosomes (EXO), 50–110 nm size] (Johnstone et al., 1991; Colombo et al., 2014). Larger vesicles are also formed during cell apoptosis when the cell cytoskeleton breaks up and induces the membrane to bulge outward (i.e., apoptotic bodies of 300–2,000 nm size) (**Figure 1B**; Caruso and Poon, 2018). It has been shown that apoptotic bodies are involved in tissue repair and angiogenesis during atherosclerosis (Zernecke et al., 2009). MPs are involved in inflammation, coagulation, vascular function, and sepsis (Boulanger, 2010; Reich et al., 2011; Das, 2019). They also release proteases for extracellular matrix degradation (Lozito and Tuan, 2012) and promote oncogene propagation among subsets of cancer cells (Al-Nedawi et al., 2008). Shedding of membrane MP is also observed in normal conditions, e.g., MPs released from normal endothelial cells are implicated in angiogenesis. EXO are involved in cell homeostasis to eliminate unwanted and toxic cellular compounds (proteins, lipids, and nucleic acids) (Johnstone et al., 1991; Desdin-Mico and Mittelbrunn, 2017). As these types of vesicles do not originate from the same cellular compartment their composition (lipid/nucleic acids and proteins) are different (Crescitelli et al., 2013; Durcin et al., 2017; Wei et al., 2017; Tucher et al., 2018).

To isolate these different types of EVs from muscle cell conditioned medium (**Figures 1C,D**) differential centrifugations are performed as described in Thery et al. (2006) (**Figure 2**). After MP removal, and in order to enrich the preparation in small EVs, the supernatant is passed through a 0.22-μm filter and then is ultracentrifugated to collect small EVs enriched in exosomes. Sucrose or iodixanol gradients, or size exclusion chromatography with open columns are recommended to remove protein aggregates that may precipitate with the small EVs (Thery et al., 2018; Jeppesen et al., 2019). It has to be noticed, however, that these different methods have not been compared for muscle-released EVs and may result in the selection or enrichment of specific EV subpopulations.

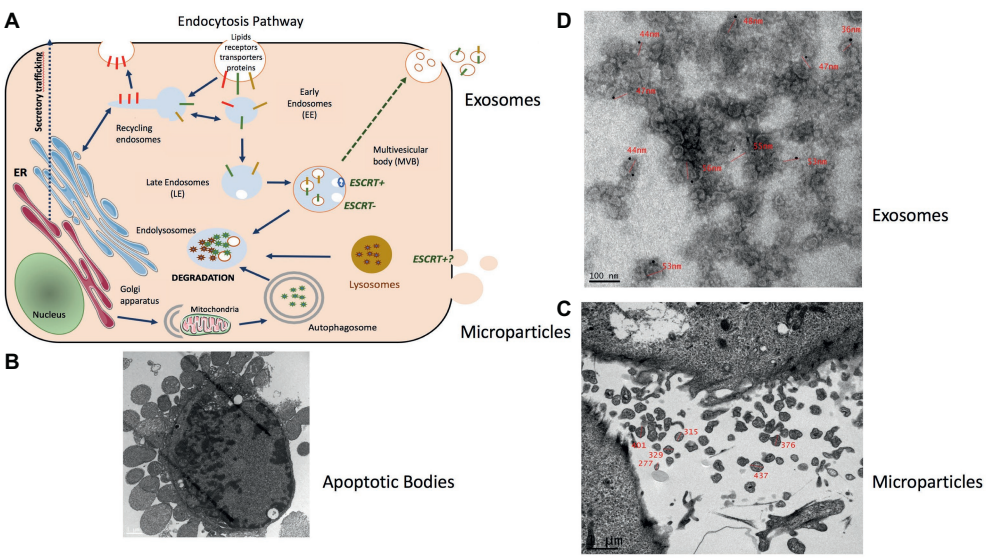

FIGURE 1 | Representation of muscle-release extracellular vesicles (EVs). **(A)** Exosomes are formed in late endosomes called multivesicular endosomes (MVBs), containing internal vesicles (ILVs) that pack and store molecules in membrane-bound structures. Endosomes are an intermediate compartment between the plasma membrane where endocytosis takes place and lysosomes where these molecules are degraded. MVB biogenesis can occur with the ESCRT machinery (ESCRT$^+$) or without (ESCRT$^-$) (Hessvik and Llorente, 2018). MVBs rich in lysobisphosphatidic acid (LBPA) but low in cholesterol migrate toward lysosomes and fuse with them. Those rich in cholesterol but low in LBPA migrate to the plasma membrane to fuse and release their ILVs as exosomes (Laulagnier et al., 2005; Colombo et al., 2014). **(B)** Electron microscopy showing the release of apoptotic bodies from an apoptotic C2C12 myoblast (personal data from S. Rome) (scale = 1 μm). **(C)** Microparticles (MPs) represent a heterogeneous population of small plasma membrane vesicles (Morel et al., 2011). Electron microscopy of human myotubes showing the release of MPs from the plasma membrane (scale = 1 μm) (personal data from S. Rome). **(D)** Due to their small size exosome-like vesicles (ELVs) can be visualized only through electron microscopy. ELVs released from *quadriceps* explants labeled with anti-CD81 gold particles (personal data from S. Rome).

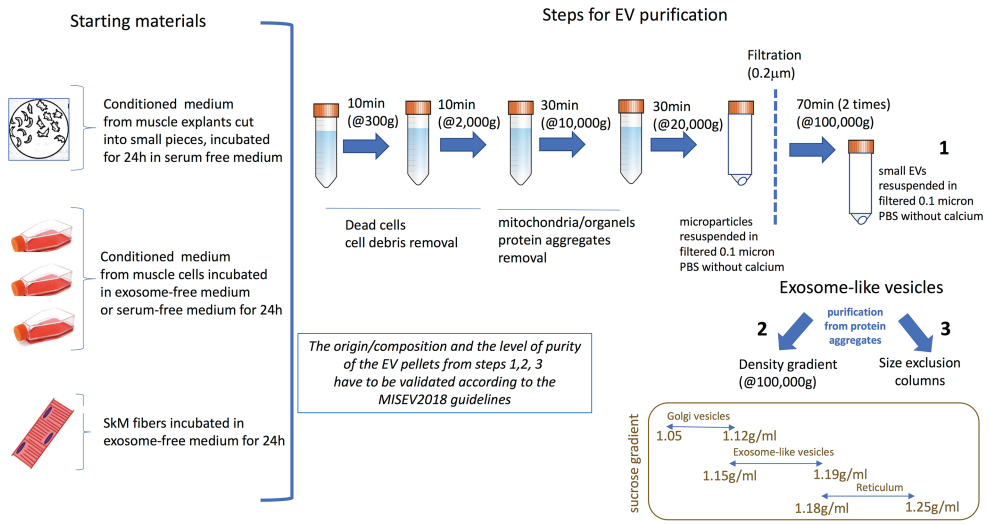

FIGURE 2 | Currently used protocols to purify ELVs from skeletal muscle (SkM) cells conditioned medium, SkM explants, or myofibers. The origin/composition and the level of purity of the EV pellets from steps 1–3 have to be validated according the MISEV2018 guidelines (Thery et al., 2018).

As it is difficult to avoid contaminations of exosomes with other small vesicles, (i.e., small MP or small apoptotic bodies) and because no highly extensive vesicle purification was performed in published studies working on isolated EVs from SkM cells, SkM explants, or myofibers, we will use the term of exosome-like vesicles (ELV) in this review to consider the small EV subpopulation's heterogeneity.

The process of myogenesis involves a first step in which myoblasts proliferate until they reach confluence, then fuse to produce multinucleated myotubes which do not proliferate anymore. Nuclei are located at the periphery of the myofiber and the other organelles are squeezed in between myofibrils or between myofibrils and the plasma membrane. How myotubes and myoblasts, which are very different type of cells, generate these small ELVs has not been yet investigate. It has been described that myoblasts produce small ELVs (ELV-MB) (Guescini et al., 2010; Forterre et al., 2014a,b; Sork et al., 2018) likely reflecting the intense macromolecule/RNA/lipids turn-over that occurs during cell proliferation (Johnstone et al., 1991; Desdin-Mico and Mittelbrunn, 2017). Myotubes also release small ELVs (ELV-MT) (**Figure 1D**; Le Bihan et al., 2012; Romancino et al., 2013; Forterre et al., 2014a,b) and the presence of ELVs was also demonstrated in dispersed mouse myofibers from the mouse hindlimb muscles (De Gasperi et al., 2017). Romancino et al. (2013) have suggested that ELV-MB and ELV-MT may have different origins and that ELVs from MT would be produced mainly through the budding of the plasma membrane. In their study, loss of Alix protein involved in ILV biogenesis in MVBs (Sun et al., 2015) resulted in alteration of membrane budding, a decrease in the number of ELV released and a modifications of ELV-MT composition (of note, in this study the EV pellet was not filtered at 0.22 µm; thus, the authors may have worked with a pool of MPs and ELVs) (Romancino et al., 2013). Interestingly, the involvement of Alix for EV release seems to occur only in muscle cells as the knockdown of Alix did not affect the number of ELVs released from human liver stem-like cells (Iavello et al., 2016).

MPs released from human MT were enriched in tetraspanin CD81 and in CLIC1 and galectin-1 (LGALS1) proteins and did not contain tetraspanins CD63, CD82, and CD9 which were found associated with ELVs from human MT (**Figures 1D, 3**; Le Bihan et al., 2012). Proteomic analyses indicated that ELV-MT contain few of the endosomal/lysosomal CD63 tetraspanin compared with CD81 or CD9 tetraspanin (Forterre et al., 2014a), known to be also expressed at the plasma membrane[1]. Western blot quantifications also showed that CD81 was more enriched in ELV-MT than in ELV-MB (Forterre et al., 2014a) and that Alix protein level was significantly enriched in ELVs released from myotubes than from myoblasts (Romancino et al., 2013). Taken together, these data highlight two pathways for ELV release from muscle cells (i.e., for ELVs and MPs, respectively) and suggest that the ratio between ELVs and MPs may vary between proliferation and differentiation. The contribution of these two types of vesicles in muscle physiology has never been fully characterized, and their specific biological functions on recipient cells are not known.

Beside EVs biogenesis' variability resulting in the release of different vesicle subpopulations, recent data from other cell types indicated that when sucrose gradient purifications were performed, small ELVs might be composed of different subpopulations. For instance, ELVs released from colon carcinoma cell line LIM1863 (Tauro et al., 2013), human dendritic cells (Kowal et al., 2016), human mast cell HMC-1 (Crescitelli et al., 2013), and RBL-2H3 (Laulagnier et al., 2005) are a mixture of two subpopulations of small EVs with distinct densities when separated on sucrose or iodixanol gradients. In addition, they are distinguished by their total RNAs, proteins, and lipids content too. For mesenchymal stem cells, at least three populations

[1]www.proteinatlas.org/ENSG00000110651-CD81/cell

with specific protein and lipid contents were identified (Lai et al., 2016). Based on the recent study of Jeppesen et al. (2019), it is likely that these two populations represent exosomes and small MPs. For human muscle cells, iodixanol gradients have been performed with ELV-MT and resulted in the identification of five potential ELV-MT subpopulations with specific tetraspanin enrichments (**Figure 3**; Le Bihan et al., 2012). Interestingly, mitochondrial DNA (Guescini et al., 2010), histones, and nuclear proteins were also found in ELV-MB (Guescini et al., 2010; Forterre et al., 2014a; Sork et al., 2018) and ELV-MT (Le Bihan et al., 2012; Forterre et al., 2014a). Although we cannot exclude the presence of contaminations with small apoptotic bodies which are formed during myoblast apoptosis, it has to be noticed that RNA/proteins of both nuclear and mitochondrial origins have been consistently identified in ELVs released from other cell types suggesting that SkM may also release EVs originated from different MVB subpopulations (Vesiclepedia: http://microvesicles.org/index.html). Indeed, it has been shown that cells contain different MVB-like structures and some of MVBs contain vesicles of different sizes (small vesicles can be inside larger ones) or filled with electron-dense material (Zabeo et al., 2017). Interestingly, it was shown that mitochondria were able to release small vesicles from their membranes to deliver specific mitochondrial contents into late endosome/MVBs (Soubannier et al., 2012; Sugiura et al., 2014; Cadete et al., 2016). In addition, the formation of nuclear vesicles was also reported as non-canonical pathway for mediating nucleo-cytoplasmic transport of ribonucleoprotein particles in *Drosophila* larvae muscle (Speese et al., 2012). All these data suggest a high level of material exchange between different types of MVB-like structures, which could result in the release of a heterogeneous population of SkM-ELVs and may explain the diversity of their protein contents. In line with this hypothesis, a new pathway was recently described to explain the presence of dsDNA and histones in small EVs. It would involve MVB and autophagy and the formation of an amphisome that, after fusion with the plasma membrane, would release dsDNA and histones in the extracellular space, as an exosome-independent mechanism (Jeppesen et al., 2019). It is now of utmost importance to characterize SkM-EVs subpopulations, to understand their cellular origin, and to determine whether they may have distinct biological activities on recipient cells (Lasser et al., 2018).

Composition of Skeletal Muscle-Released Extracellular Vesicles

Proteins

Proteomic analyses have identified two specific subsets of proteins for MPs and ELVs, released in the conditioned medium from human myotubes (**Figure 4A**; Le Bihan et al., 2012). MPs were enriched in genes coding for proteins involved in "protein synthesis, folding, and trafficking," "RNA post-transcriptional modifications," "translation modifications," and "amino acid metabolism." They were mainly cytosolic proteins, located in "endoplasmic reticulum," "Golgi vesicles," "mitochondria," and "cytoskeleton." Compared to MPs, human ELV-MT were enriched in proteins for "free-radical scavenging" and "cell-to-cell signaling and interactions." Conversely to MP proteins, ELV-MT proteins were localized in "subplasma-membrane cytoplasmic vesicles," "endosomes," "lysosome," and "plasma membrane" (Le Bihan et al., 2012), further suggesting that SkM would release small vesicles also through the budding of the plasma membrane (Romancino et al., 2013). These comparative proteomic analyses indicated that ELV-MT proteins are mainly those endocytosed and delivered into endosomes for incorporation into MVBs or for degradation into lysosomes. The selection process involved in the delivery of some membrane proteins to ILVs of MVBs for destruction while others remain outside is not well understood. It has been found that ubiquitination was necessary the partitioning of mainly, but not all, membrane proteins into invaginating MVBs (Reggiori and Pelham, 2001; Stringer and Piper, 2011). In this way,

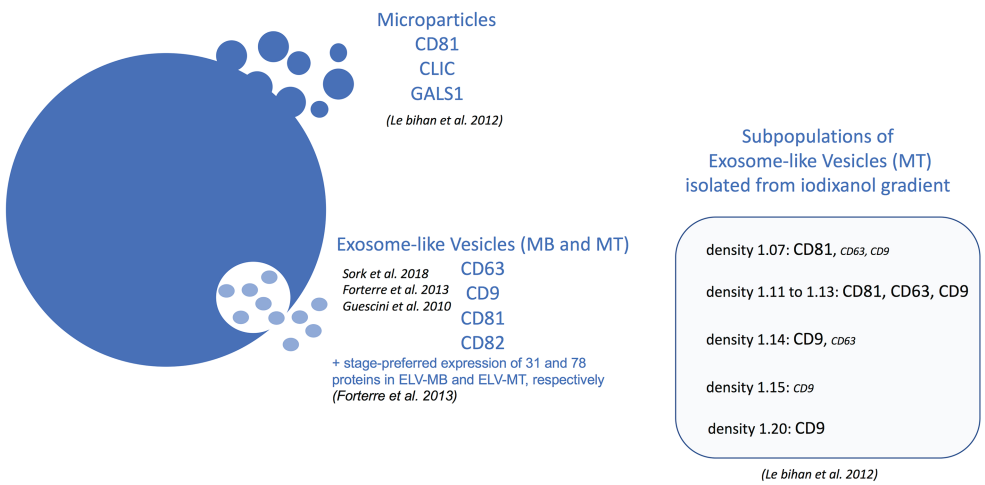

FIGURE 3 | Summary of all vesicles identified in the conditioned-medium from skeletal muscle (SkM) cells based on the detection of specific subsets of tetraspanins by proteomics or Western-blot.

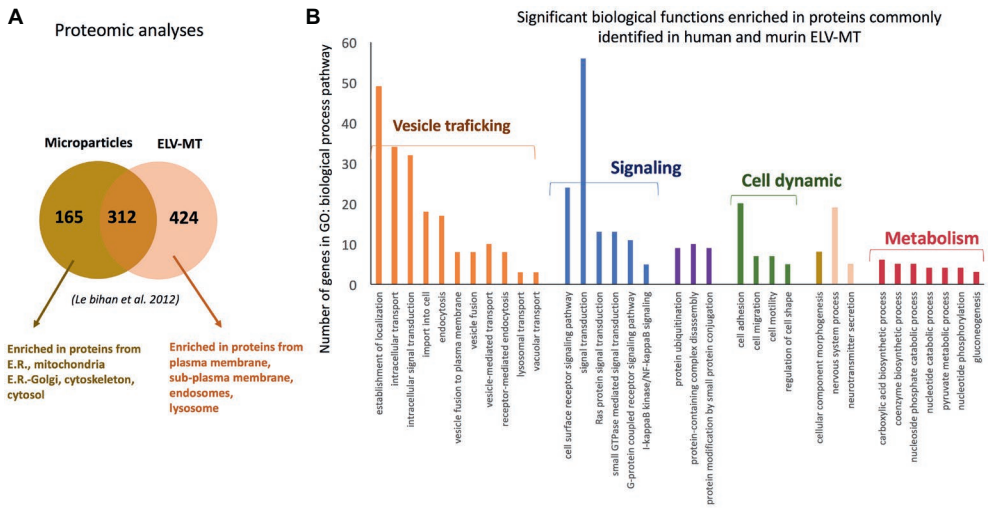

FIGURE 4 | (A) Proteomic analyses identified specific subsets of proteins in MPs and ELVs released from differentiated human SKM cells [full list of proteins are in Le Bihan et al. (2012)], protein are identified from protein fragments). **(B)** Significant GO biological functions enriched in genes commonly identified C2C12 myotubes- and primary human myotubes-ELVs isolated from conditioned medium. Proteomic data are from Forterre et al. (2014a) and Le Bihan et al. (2012) and were analyzed with PANTHER version 11 (Mi et al., 2017).

endosomes/MVBs regulate the composition of plasma membrane and thus play a pivotal role in a vast array of biological functions. For instance, the endosomal system functions like a digital-analogue computer that regulates the specificity and robustness of the insulin signaling response in the SkM (i.e., insulin induces translocation of key signaling proteins into endosome) (Balbis et al., 2000; Jethwa et al., 2015). The presence of proteins involved in gluconeogenesis, pyruvate metabolism, and biosynthesis of carboxylic acids (e.g., TPI1, PGK1, GAPDH, GPI, ENPP1, and ACLY) also suggests that ELV-MT release could represent an important pathway for the turnover of proteins involved in the regulation of SkM metabolism (**Figure 4B**). Gene enrichment analyses of the proteins commonly identified in ELV-MT from human (Le Bihan et al., 2012) and murine (Forterre et al., 2014a) SkM cells further confirmed that ELV-MT are enriched in proteins involved in exosome formation and intracellular trafficking (e.g., endocytosis, vesicle fusion, and transport, and signaling pathways involved in vesicle cellular localization) (**Figure 4B**). Interestingly, the majority of the proteins identified in ELV-MB was also found in ELV-MT (**Figure 5A**). This result indicates that although the organization of the cellular organelles and the plasma membrane of myoblasts changes dramatically during differentiation consequently to the formation of a single functional unit, SkM-ELV composition remains quite constant. This result further supports the concept that proteins sorting into ELVs appears to be selective and almost the same subset of proteins is exported whatever type of cell considered. **Figure 5B** shows the overlapping between three independent proteomic analyses from C2C12 myoblasts and the 59 proteins commonly identified are listed in **Table 1**. Of note, only CD63 was commonly identified among all tetraspanins (**Figure 3**) likely reflecting the protocol variabilities for SkM EV purifications (Jeppesen et al., 2019).

In order to determine the contribution of the ELV-associated proteins among the set of proteins isolated from muscle cell secretome, a functional enrichment analysis was performed by comparing the set of secreted proteins from C2C12 myoblasts (Henningsen et al., 2010) with the set of proteins identified from ELV-MB (**Figure 5C**; Forterre et al., 2014a). None of the GO categories previously found as significantly enriched in ELV proteins (**Figure 4B**) was identified in the 635 secreted proteins. By contrast, the C2C12 secretome was significantly enriched in GO term "cytokines." A similar result was obtained for human muscle cells (Le Bihan et al., 2012). These data indicated thus that SkM cell uses distinct pathways for the release of distinct protein subsets with likely distinct biological actions. One exception is the protein C1QTNF3 (Cartonectin) found in ELV-MT (Forterre et al., 2014a). Cartonectin is a new cytokine, paralog of adiponectin contained as a transmembrane protein in adipocyte-released EVs and in EVs isolated from serum (Phoonsawat et al., 2014). The function of this family of cytokines in ELVs is presently unknown.

Lipids

Lipids are essential constituent of ELV membranes, and several studies have described that specific lipids are enriched in ELVs compared with the parent cells (Skotland et al., 2017), i.e., a cholesterol, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and diacylglycerol. This specific lipid enrichment, their membrane high protein/lipid ratio and asymmetric distribution are associated with a higher membrane rigidity in ELVs in comparison with parent cells (Record et al., 2018). Lipidomic and proteomic analyses on ELVs and MPs released from three different cell lines have indicated that lipidomes of ELVs, MP and secreting cells were more similar to each

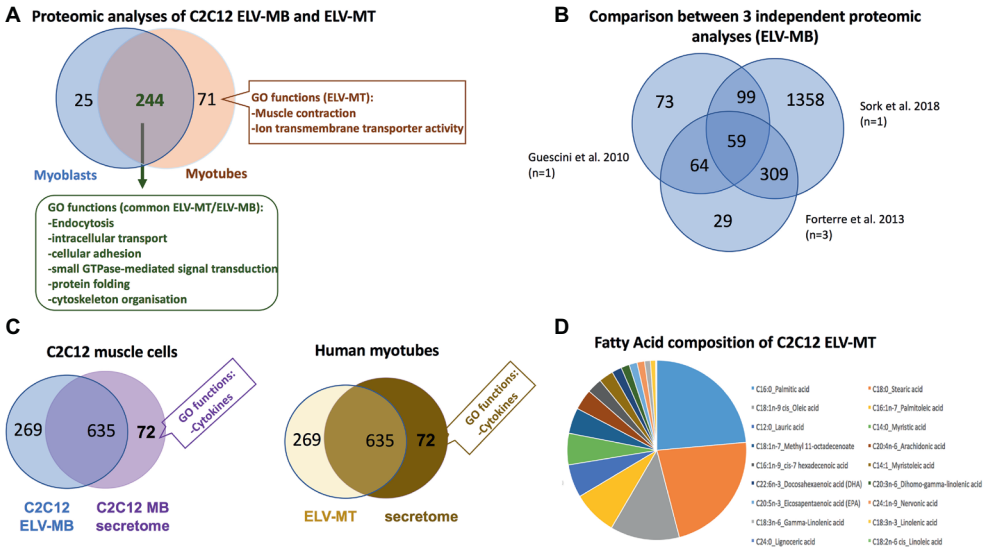

FIGURE 5 | (A) Overlap between the number of proteins from C2C12 ELV-MB and ELV-MT (see Forterre et al., 2014a for a full list of proteins, proteins are identified from protein fragments). **(B)** Overlap between three independent protomic analyses from C2C12 ELV-MB. **(C)** Left: overlap between the protein content of ELV-MB and the secretome from C2C12 myoblasts (Forterre et al., 2014a); right: overlap between the protein contents of ELV-MT and the secretome from human differentiated MT (Le Bihan et al., 2012). Only significant GO functions between the two sets of proteins are indicated. **(D)** Fatty acid composition of ELV-MT from C2C12 (Aswad et al., 2014).

other than their proteomes are (Haraszti et al., 2016). For muscle cells, only one study has determined the fatty acids (FA) composition of ELV-MT. As shown on **Figure 5D**, ELV-MT are enriched in palmitic acid, stearic acid, oleic acid, palmitoleic acid, and lauric acid compared to others FA (Aswad et al., 2014). These fatty acids are used for energy production or to produce phospholipids for phospholipid bilayers out of all cell membranes. Interestingly, ELVs released from C2C12 myotubes treated with palmitate were enriched in palmitate compared to BSA-treated cells (Aswad et al., 2014). As accumulation of intracellular fatty acids is associated with insulin resistance and/or an impaired glucose metabolism, the release of ELVs may represent a form of protection for the cell, to prevent accumulation of intracellular fatty acids.

RNA

ELV preparations from various cell types have demonstrated that they contain distinctive repertoires of RNA populations including transcripts (Valadi et al., 2007; Skog et al., 2008) and non-coding RNAs, i.e., long non-coding RNA, microRNAs (miRNAs), piwi protein interacting RNA (piRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), circular RNA (circRNA), sc RNA Y, natural antisense RNA (asRNA), ribosomal RNA (rRNA), and vault RNA (vRNA) (Nolte-'t Hoen et al., 2012). Interestingly, while cellular RNAs are enriched in full-length long RNA species, ELVs are enriched in shorter RNA species (≤200 nucleotides) including short transcripts, fragmented mRNAs (Batagov and Kurochkin, 2013), and small RNAs (i.e., miRNA, snoRNA, snRNA, Y RNA, and vault RNA). As the sequencing of these different populations often rely on separate libraries the direct comparisons between them are not always straightforward, but these analyses clearly indicated a differential profile of ncRNA subsets between parental cells and EVs (Guduric-Fuchs et al., 2012). In the case of SkM cells, it was shown that C2C12 ELV-MB were also enriched in small RNAs subsets compared with C2C12 parental cells (**Figure 6A**; Sork et al., 2018). Only the small RNA population has been further analyzed (Forterre et al., 2014b; Sork et al., 2018; Xu et al., 2018), and was found highly enriched in piRNAs and miRNAs (**Figure 6B**; Sork et al., 2018). For the majority of miRNAs, a good correlation was observed between miRNA levels in ELV-MB vs. MB in two independent studies (Forterre et al., 2014b; Sork et al., 2018), and between ELVs released from myofibers vs. myofibers (De Gasperi et al., 2017), suggesting that export of miRNAs into ELVs would permit to control their intracellular concentrations. *In silico* predictions of the most concentrated miRNAs in ELV-MB (Sork et al., 2018) indicated that they could collectively regulate important cellular pathways for muscle physiology, i.e., signaling pathways involved in the regulation of muscle mass (PI3K-Akt, insulin, MAPK, TGF-beta, proteolysis, and calcium), in the neuromuscular junction, in immune response, and in calcium signaling and cytoskeleton (**Figure 6C**). Interestingly, all studies reported also that individual miRNAs deviate from this relationship, i.e., some miRNAs expressed in muscle cells were not detected in ELV-MB (Forterre et al., 2014b) and/or ELV-MT (**Figure 6D**; Forterre et al., 2014b); other were found at higher rate in ELV-MB vs. myoblasts [miR-451, miR-6239, miR-6,240, miR-6236, miR-144, miR-223, miR-5112, miR-3062, miR-142a, and miR-2137 in Sork et al. (2018)]. Similarly, miR-720 was enriched in myofiber-derived ELVs compared with parent

TABLE 1 | Proteins identified in 3 independant proteomic analyses from C2C12 ELV-MB.

Gene symbols	Names
Aldoa	aldolase A, fructose-bisphosphate
Ldha	lactate dehydrogenase A
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Anxa1	annexin A1
Anxa2	annexin A2
Anxa3	annexin A3
Anxa4	annexin A4
Anxa5	annexin A5
Anxa6	annexin A6
Arhgdia	Rho GDP dissociation inhibitor (GDI) alpha
Cct3	chaperonin containing Tcp1, subunit 3 (gamma)
Cct4	chaperonin containing Tcp1, subunit 4 (delta)
Cct5	chaperonin containing Tcp1, subunit 5 (epsilon)
Cct6a	chaperonin containing Tcp1, subunit 6a (zeta)
Cct8	chaperonin containing Tcp1, subunit 8 (theta)
Cd63	CD63 antigen
Clic1	chloride intracellular channel 1
Cltc	clathrin, heavy polypeptide (Hc)
Des	desmin
Eef1a1	eukaryotic translation elongation factor 1 alpha 1
Eef2	eukaryotic translation elongation factor 2
Ehd1	EH-domain containing 1
Ehd4	EH-domain containing 4
Eif4a1	eukaryotic translation initiation factor 4A1
Ezr	ezrin
Fn1	fibronectin 1
Gnb2	guanine nucleotide binding protein (G protein), beta 2
Hspa8	heat shock protein 8
Hspb1	heat shock protein 1
Igsf8	immunoglobulin superfamily, member 8
Ipo5	importin 5
Itgb1	integrin beta 1 (fibronectin receptor beta)
Kpnb1	karyopherin (importin) beta 1
Msn	moesin
Myh9	myosin, heavy polypeptide 9, non-muscle
Pdcd6	programmed cell death 6
Pdcd6ip	programmed cell death 6 interacting protein
Psmc6	proteasome (prosome, macropain) 26S subunit, ATPase, 6
Ptgfrn	prostaglandin F2 receptor negative regulator
Ran	RAN, member RAS oncogene family
Rplp0	60S acidic ribosomal protein P0
Rps2	ribosomal protein S2
Rps3	Ribosomal Protein S3
Rps4x	ribosomal protein S4, X-linked
Rps8	ribosomal protein S8
Rpsa	ribosomal protein SA
Tpi1	triosephosphate isomerase 1
Tpt1	tumor protein, translationally-controlled 1
Tubb2b	tubulin, beta 2B class IIB
Tubb3	tubulin, beta 3 class III
Tubb5	tubulin, beta 5 class I
Tubb6	tubulin, beta 6 class V
Uba1	ubiquitin-like modifier activating enzyme 1
Vcl	vinculin
Vcp	valosin-containing protein
Vim	vimentin
Ywhae	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
Ywhaq	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide
Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

myofibers and inversely for miR-1 (De Gasperi et al., 2017). A third category was composed of miRNAs that were only found in ELVs and not detectable in the parental cells (**Figure 6D**). On the contrary, miR-147, miR-30b-3p, miR-467c, miR-615-3p, miR-669a, miR-677, miR-28a-3p, and miR-29-2-5p were never detected in C2C12-released ELV-MB or ELV-MT

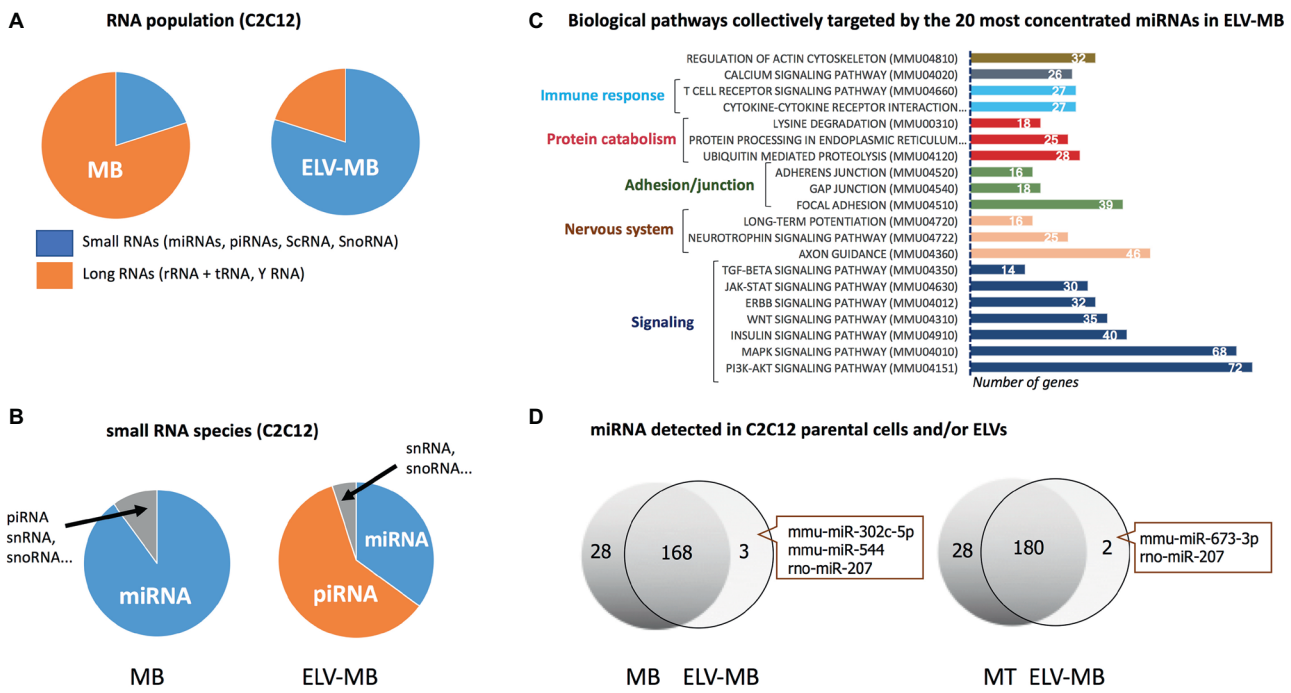

FIGURE 6 | (A–C) were built from the sequencing data published in Sork et al. (2018). **(A)** Percentage of small and large RNAs in C2C12 myoblasts and their released ELV-MB. **(B)** Number small RNA species in C2C12 myoblasts vs. ELV-MB expressed as percentages. **(C)** Functional enrichment analyses of the predicted target genes from ELV-MB miRNAs. Target predictions and the identification of significant biological pathways were performed with DIANA-microT-CDS and mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr). **(D)** Overlap between the miRNA species found in muscle cells and their respective ELVs (n = 3). The same quantity of total RNA was used as starting material for qRT-PCR (Forterre et al., 2014b).

(Forterre et al., 2014b). Taken together, these data indicated that the packaging of some specific miRNAs into muscle-released ELVs is selective. How miRNAs are loaded into ELVs is poorly understood and may be resulting from different mechanisms, i.e., (1) the 3′ end uridylation of miRNA directs these small RNAs to sites of ELV biogenesis, while adenylation appears to have the opposite effect (Koppers-Lalic et al., 2014); (2) the association of miRNAs with RNA-binding proteins which are targeted to MVBs through post-translation modifications (i.e., sumoylation of the ribonucleoprotein hnRNPA2B1, which recognizes the GGAG motif in a subset of miRNAs is associated with miRNA loading into MVB) (Villarroya-Beltri et al., 2013); (3) the association of miRNAs with proteins involved in their synthesis and their function, which are loaded into ELVs [i.e., AGO2 (Gibbings et al., 2009) and the elements of the RISC-loading (silencing) complex (ALIX) (Iavello et al., 2016)]; (4) the association with YBX1 localized in P-bodies which are closely juxtaposed to MVBs. It is hypothesized that YBX1 may complex with miRNAs whose mRNA targets are not expressed. This association would result in their sorting into MVBs for export by ELVs (Shurtleff et al., 2016).

Unlike miRNAs, there was no correlation between ELV-MB and cellular concentrations in myoblast for piRNAs (Sork et al., 2018). C2C12 ELV-MB also contained tRNAs (Sork et al., 2018) and Y RNA (mainly RNY1) (Forterre et al., 2014b; Sork et al., 2018). The function of these non-coding RNA in muscle-released ELVs has not been yet identified but based on recently published data from other cell types; they could participate in the biological action of SkM-ELVs (Cambier et al., 2017).

Messenger RNAs were also detected by using DNA microarrays, in ELVs and MPs released from human SkM cells (Le Bihan et al., 2012). Respectively, 185 and 4,431 transcripts were identified in ELVs and MPs. Hierarchical clustering showed that the mRNA cargos of ELVs and MPs were more closely related to each other than to the expression pattern of the secreting myotubes. The abundance of the 185 ELV mRNAs correlated with their abundance in myotubes, indicating that myotubes release a specific subset of transcripts in ELVs, and not only the most concentrated one. ELVs were significantly enriched in transcripts encoding receptors and ionic channels, and regulators of transcription in particular zinc-finger transcription factors (Le Bihan et al., 2012).

Biological Action of Skeletal Muscle-Released Extracellular Vesicles

Since the discovery that ELV cargos released from a specific cell type can be incorporated into various recipient cells (including a re-uptaking by the releasing cells) and by this way can regulate their fate (Valadi et al., 2007), numerous studies have demonstrated the involvement of EVs in health (i.e., embryogenesis and development, tissue homeostasis) and diseases (i.e., cancer metastasis, inflammatory diseases, and metabolic diseases) (**Figure 7**). In that context, it has been found that SkM-released EVs have important paracrine actions that would participate in muscle physiology and may have an impact at the whole-body homeostasis.

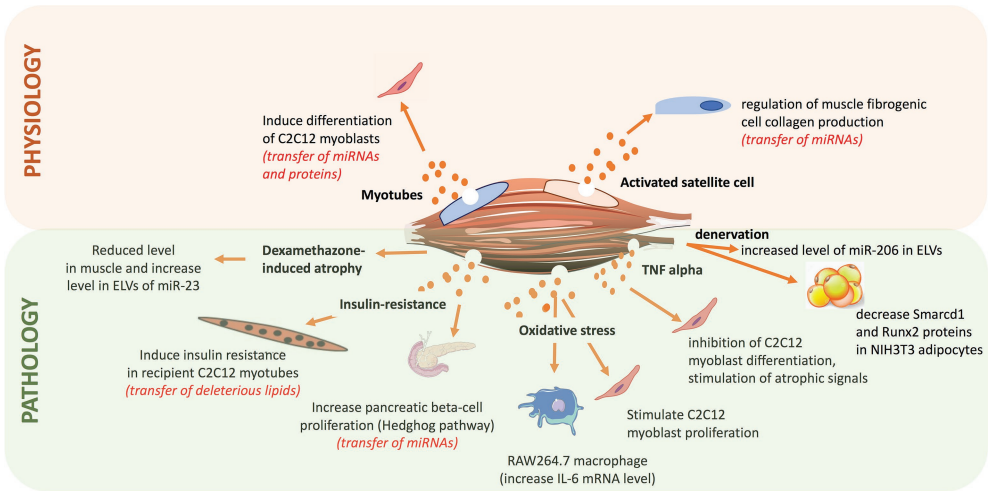

FIGURE 7 | Summary of the roles of exosome-like vesicles released from skeletal muscle (SkM) cells, published so far.

As protein and miRNA compositions were different between ELV-MB and ELV-MT, it was postulated that they might be involved in the process of myogenesis. Indeed, it was shown that both ELV-MT protein and miRNA contents were transferred into proliferating myoblasts (Forterre et al., 2014a,b). Treatment of myoblasts with ELV-MT but not ELV-MB resulted in a decrease in myoblast proliferation and in the induction of early markers of differentiation (e.g., myogenin mRNA) (Forterre et al., 2014a). Bioinformatic analyses revealed that predicted target genes of differentially expressed miRNAs between ELV-MB and ELV-MT were mainly involved in the control of signaling pathways, and particularly the Wnt signaling pathway known to be regulated during myogenesis (Tanaka et al., 2011). Additionally, it was demonstrated that ELV-MT miRNAs were enriched in miRNA species targeting the 3'-UTR of *sirt* 1, a gene involved in muscle cell proliferation. Therefore, through the ELV route MT-ELV miRNAs could reduce the level of endogenous Sirt1 in myoblasts thus inducing cell differentiation (Forterre et al., 2014b). Furthermore, it was found that removing ELVs from bovine serum of cell culture, affected C2C12 proliferation (i.e., decrease of Cyclin D1 and Sirt1 mRNA levels) and committed cells to differentiate prematurely (induction of myogenin mRNA before myoblast fusion) (Aswad et al., 2016). In addition to observations that bovine ELVs can transfer specific signals to cells from unrelated species (mice C2C12), this result also suggests that ELVs in mammalian serum might have unsuspected functions during embryogenesis and in the regulation of cellular adaptations that lead to muscle hypertrophy, hyperplasia, and metaplasia. In line with this hypothesis, proliferation of C2C12 myoblasts is increased after 24 h of treatment with serum ELVs from mice suffering from Duchenne muscular dystrophy (*mdx* mice) compared with ELV-depleted serum (Matsuzaka et al., 2016), thus suggesting an important role of blood ELVs on the regulation of myogenesis and muscle mass.

In adult, myogenesis is stimulated in response to muscle damage and a role of SkM-ELVs was suggested in the control of muscle mass. In the context of hypertrophy, SkM satellite cells are activated to give rise to myogenic progenitor cells (MPCs) within the extracellular matrix (ECM) located around the fibers. It was found that MPCs secrete ELVs containing miR-206, which repressed the ribosomal binding protein 1, a master regulator of collagen biosynthesis, in fibrogenic recipient cells. This would prevent excessive ECM deposition for optimal muscle remodeling in response to hypertrophic stimuli (Fry et al., 2017). These data have provided insights into how skeletal stem and progenitor cells interact with other cell types to actively control their extracellular environments for tissue maintenance and adaptation. They have also provided a new mechanism to explain the fibrotic pathogenesis associated with other SkM alterations (i.e., muscular dystrophy, aging) in which satellite cell activity is perturbated and thus likely the release/composition of ELVs.

In the context of atrophy, treatment of C2C12 myotubes with dexamethasone for 48 h reduced the intracellular level of miR-23a but increased its ELV-MT abundance. As dexamethasone did not alter the number of ELVs released these data suggested that atrophy-inducing conditions lead to a selective packaging of miRNAs into ELV-MT (Hudson et al., 2014), thus changing the delivered message to recipient cells. In line with this hypothesis, it was demonstrated that after denervation, muscle fiber released a new population of ELVs enriched in miR-206 able to downregulate the expression of *Smarcd1* and *Runx2* proteins into NIH3T3 adipocytes (De Gasperi et al., 2017). It was also found that myoblasts incubated with ELVs released from myotubes pre-treated with H_2O_2 that mimics an oxidative stress, leads to a reduction in myotube diameter and to a stimulation of myoblast proliferation (Guescini et al., 2017). Similar results were obtained when myotubes were treated with a pro-inflammatory cytokine mixture of TNF-alpha and INF-gamma (Kim et al., 2018), suggesting that in addition to myogenesis, ELV-MT would also participate in the maintenance and regeneration of SkM mass following injuries.

In the context of metabolic diseases, *in vivo* data showed that palmitate-induced SkM insulin-resistance triggered the release of a new population of ELV-MT enriched in palmitate,

in diet-induced obese mice (DIO) compared normal chow diet mice (NCD) (Aswad et al., 2014). It was demonstrated that palmitate contained in ELV-MT from DIO mice was transferred in recipient myotubes and resulted in the up-regulation of 240 genes involved in cell cycle and cellular adhesion. Among them, the pro-inflammatory cytokine IL-6 and the cell cycle regulator cyclin D1 were strongly upregulated, whereas Glut4 involved in glucose uptake was downregulated. In addition, muscle differentiation markers (myogenin, MyoD1, and CKMT2) were down-regulated showing that ELV-MT enriched in palmitate affected muscle phenotype. In addition to the release of a specific population of ELVs, mice fed with a palmitate-enriched diet released more ELV-MT (Aswad et al., 2014). It is likely that excessive concentration of circulating palmitate and saturated fatty acids associated with this specific high-fat diet had increased the concentration of intra-muscular ceramides that regulate ELV release from muscle (Matsuzaka et al., 2016). Interestingly, it has been found that intraperitoneal injections for 5–10 days of an inhibitor of ceramide synthesis improved muscle function and structure in *mdx* mice vs. untreated *mdx* animals (Matsuzaka et al., 2016). Although the composition and the biological action of SkM-ELVs from of these treated animals were not determined, it is a proof-of-concept that modulation of ELV release might be a therapeutic approach to regulate deleterious organ cross-talks associated with the transfer of deleterious EV cargos (e.g., the transfer of deleterious lipids during the development of metabolic diseases associated with high-fat diet).

Indeed, although communication between SkM and other tissues appears to be of importance in the development of various diseases, investigations to determine signals underlying these connections have been widely limited to the roles of cytokines. Recently, the possibility that SkM-ELVs could also act at systemic level has been studied in the context of type 2 diabetes associated with obesity (Jalabert et al., 2016). Intraperitoneal injections of fluorescent SkM-ELVs resulted in the fluorescent labeling of 9 organs in mice [i.e., brain, liver, heart, lungs, gastro-intestinal tract, spleen, pancreas, and kidney in addition to SkM (Aswad et al., 2014; Wiklander et al., 2015; Jalabert et al., 2016)]. ELVs collected from conditioned medium of SkM explants from DIO mice induced specific transcriptional signatures in pancreatic MIN6B1 recipient cells compared to SkM-ELVs from NCD mice. Microarray data analyses showed that the expressions of 460 mRNAs were significantly modulated including genes involved in immune responses known to be affected in pancreatic islets of diabetic patients (Esser et al., 2014; Jalabert et al., 2016). Additional bioinformatic analyses combined with *in vitro* experiments demonstrated that SkM-ELVs mediated the transfer of miRNAs from insulin-resistant muscles into MIN6B1 and affected the expression of proliferative genes such as *Ptch1* (Jalabert et al., 2016). These data suggested for the first time that SkM-released ELVs might transmit signals to the pancreas during the development of insulin-resistance. Because SkM-ELVs have no effect on insulin secretion, it would suggest that muscle-released ELVs would contribute to adaptations in beta cell mass occurring during insulin-resistance associated with obesity, at least in mice (Stamateris et al., 2013). As SkM is the unique organ for exercise, the variations of blood EV concentrations after physical activity have been studied (Frühbeis et al., 2015). The results indicate that a single bout of exhaustive exercise triggers the release of EVs with the size and marker profile of exosomes, which are cleared from the circulation during the early recovery period after cycling, but stay elevated after running exercise.

However, the clinical significance of these studies remains speculative until now and scientists have to be cautious not to over-interpret these data until the development of physiological models which would permit to follow EVs in blood vessels. Indeed, all results indicating a possible transfer of information between muscle cells and other tissues are based on *in vitro* data only. At current time, it not known whether SkM-ELVs could reach the blood circulation and have systemic actions. Recently, ELVs containing alpha-sarcoglycan (SGCA) a component of the dystrophin-glycoprotein complex involved in the stability of muscle fiber membranes, were found in human plasma (Guescini et al., 2015). These ELVs were also positive for CD81, TSG101 and miR-206, known to be highly expressed in SkM. Therefore, the authors of this study suggested that these SGCA positive ELVs might originate from SkM-ELVs and could be used as SkM liquid biopsies. Although we exclude this possibility, it has to be mentioned that SGCA and miR-206 are expressed in mainly other tissues (Mitchelson and Qin, 2015[2]) suggesting that the origin of the circulating pool of SGCA-positive ELVs is more complex and not only the result of SkM ELV secretion. In an attempt to follow muscle-released ELVs in blood, fluorescent ELVs were injected in the right *tibialis anterior* (TA) from mouse (Jalabert et al., 2016). Twenty-four hours after injection, fluorescence was detected in the left TA and in the right *quadriceps* too, suggesting again a paracrine-like action of muscle-released ELVs. Although these data strongly suggest that SkM can release ELV in blood, the generation of transgenic animals expressing GFP in SkM-released ELVs would help to quantify the impact of SkM-ELVs *in vivo* in the cross talks between SkM and key metabolic organs. Such model would also permit to identify all subtypes of EVs released from SkM cells in order to decipher their mode of biogenesis, and to understand how to modulate their release at the cellular level and to impact on their target tissues at the whole-body level.

CHALLENGES AND FUTURE DIRECTIONS

The release of EVs with biological properties from muscle cells is an exciting new discovery given the importance of SkM in the regulation of glucose homeostasis and as organ of locomotion. Further studies are now required to determine the exact roles of these SkM-EVs at the whole-body level. In addition, it is important to identify the pathophysiologic conditions that influence their biogenesis, their compositions and fate. In the context of metabolic diseases or muscle-related pathologies, it is important to determine whether MVB biogenesis and EVs release might be new targets for therapy. Presently, this aspect is more or less considered by pharmaceutical companies to treat alterations of glucose/lipid metabolism, but the consequences

[2]www.genecards.org

on EV release/composition are not known, e.g., statins, used to reduce cholesterol level in patients and to prevent cardiovascular diseases target chemokines to MVBs in endothelial cells (Hol et al., 2012); metformin, the well-known antidiabetic drug affects the endosomal pathway by modulating pH levels in recycling endosomes and MVB (Kim et al., 2016; Kim and You, 2017). How these drugs affect EV release and compositions and participate in the side effects of the treatments is presently unknown, and has never been suggested. Beside to the use of drugs to modulate EV release, recent data suggest that EVs from healthy subjects might be used in the treatment of obesity, T2DM, and aging-associated metabolic disorders. Indeed, injections of adipose tissue macrophage-derived EVs isolated from lean mice improved glucose tolerance and insulin-sensitivity of diet-induced obese mice (Ying et al., 2017). Similarly, the injection of 3-month-old mice blood EVs to 18-month-old mice reversed the expression aging-associated biomarkers (Lee et al., 2018). The injection of MSC-derived EVs was found more efficient than the use of MSC in treating stroke (Chang et al., 2018). Based on these pre-clinical data, we can speculate that blood EVs isolated from athletes following exercise would transfer some of the beneficial systemic effects of exercise in patient suffering from metabolic disorders or SkM alterations (Frühbeis et al., 2015; Lovett et al., 2018). For example, it was demonstrated that endurance exercise induced the release of procoagulant MPs from blood cells in healthy subjects (Sossdorf et al., 2010). The authors of this study suggested that they would participate in the positive effect of exercise on vessel repair and wound healing, both affected in T2DM patients. Whether SkM-EVs from healthy subjects might be used to treat metabolic alterations or SkM dysregulation has never been envisaged.

AUTHOR CONTRIBUTIONS

SR drafted and wrote the manuscript. AF, MM, and KB critically advised and reviewed the manuscript.

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frontiers in Physiology

BRIEF RESEARCH REPORT
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Irisin Exerts Inhibitory Effect on Adipogenesis Through Regulation of Wnt Signaling

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Irisin is an exercise-induced myokine known to induce adipocyte browning through induction of uncoupling protein 1. Recent studies have reported that irisin is also an adipokine. However, there is limiting evidence on the role of endogenous irisin from adipocytes. In this study we aim to elucidate the expression and secretion pattern of irisin during adipocyte differentiation and the role of endogenous and exogenous irisin on the adipogenic process. As such, recombinant irisin, plasmid expressing FNDC5 and small interfering RNA were utilized. Our results show that the gene expression of irisin precursor FNDC5 and irisin secretion increases at the early stage of adipogenesis. Both recombinant irisin treated cells and FNDC5-overexpressed cells resulted in inhibition of adipogenesis evidenced by downregulated C/EBPα, PPARγ, and FABP4 expression and reduced lipid accumulation. Further data showed that the inhibitory effect of irisin on adipogenesis is mediated though potentiation of Wnt expression, which is known to determine the fate of mesenchymal stem cells and regulate adipogenesis. Conversely, FNDC5 knockdown cells showed downregulated Wnt expression, but failed to further induce adipogenesis. This study suggests that both exogenous and endogenous irisin is able to inhibit adipogenesis and that activation of Wnt and subsequent repression of transcription factors is partly involved in this process. This provides a novel insight on the local effect of irisin on adipocytes and additional benefit to protect against obesity-related metabolic disorders.

Keywords: adipogenesis, FNDC5, irisin, Wnt, myokine

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INTRODUCTION

Obesity is an epidemic rapidly increasing worldwide. Obesity leads to various medical problems such as type 2 diabetes, cardiovascular disease, and cancer (Keller and Lemberg, 2003; Wahba and Mak, 2007). The classical role of adipose tissue is to store energy but an imbalance between food intake and energy expenditure leads to accumulation of excess lipid in adipose tissue, causing dysregulation of adipocyte metabolism (Choe et al., 2016). Development of obesity involves two routes, the increase in the size of adipocytes (hypertrophy) or the increase in the number of adipocytes (hyperplasia) (Jo et al., 2009). The latter is achieved by increased adipogenesis, the process involving differentiation of preadipocytes into mature adipocytes. Adipogenesis is induced by a complex network of transcription factors, including peroxisome proliferator-activated receptor (PPARγ), CCAAT/enhancer binding proteins (such as C/EBPα), and fatty acid binding protein 4

(FABP4), which are under the control of epigenetic modifiers through the Wnt/β-catenin signaling (Ross et al., 2000; Rosen and MacDougald, 2006). PPARγ and C/EBPα are the master regulators involved in the early differentiation process of adipocytes. FABP4, on the other hand, is considered to be involved in mature adipocyte formation (Moseti et al., 2016). In contrast, the canonical Wnt/β-catenin signaling pathway, stimulated by ligands such as Wnt6, Wnt10a, and Wnt10b, has been shown to inhibit the early stages of adipogenic differentiation (Cawthorn et al., 2012). With this regard, regulation of Wnt signaling has been suggested as a therapeutic strategy to combat obesity and related metabolic disorders.

It is well-known that adipocytes possess endocrine function by secreting various factors, so-called adipokines (Kershaw and Flier, 2004). Adipose tissue enlargement causes adipokine dysfunction which implies an imbalance between pro-inflammatory and anti-inflammatory factors. The extension of adipose tissue increases secretion of pro-inflammatory factors such as IL-6, IL-8, IL-18, and TNFα (Makki et al., 2013). On the other hand, anti-inflammatory factors such as IL-10 and adiponectin are decreased (Fasshauer and Bluher, 2015). Dysregulation of adipokines results not only in development of insulin resistance and inflammation in local adipocyte environment but also affect the metabolism and function of other peripheral tissues thereby causing systemic metabolism impairment (Jung and Choi, 2014).

Exercise is an effective strategy to overcome obesity and metabolic diseases. In addition to the direct enhancement of muscle strength, exercise has many benefits on whole body metabolism, thereby protecting the body from the risk of metabolic disorder development (So et al., 2014; Schnyder and Handschin, 2015; Stanford and Goodyear, 2016). Interestingly, recent studies have suggested that similar to adipocytes, skeletal muscle can also secrete hormones, known as myokines. Myokines are induced through exercise, which suggests that myokines are responsible for the crosstalk between muscle and other tissues (Pedersen and Febbraio, 2012). Therefore, myokines are suggested to counteract the increased pro-inflammatory adipokines during obesity (Leal et al., 2018). The exercise-induced myokines discovered so far include irisin, IL-15, SPARC, BDNF, FGF-21, etc. (Huh, 2018). These myokines are reported to modulate glucose uptake and improve glucose tolerance, regulate fat oxidation, and reduce fat accumulation in adipocytes (So et al., 2014). Among these, irisin is a myokine dependent on activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which is a major mediator of the effect of exercise in muscle (Lukaszuk et al., 2015). It was found that PGC1α overexpression leads to increased expression of fibronectin type III domain-containing protein 5 (FNDC5), which is cleaved to produce irisin and then secreted by exercise (Bostrom et al., 2012; Gouveia et al., 2016). The major role of secreted irisin is suggested to be browning of adipocytes through upregulation of uncoupling 1 (UCP1) expression (Bostrom et al., 2012), which has advantages in increasing energy expenditure and thus leads to improvement in metabolic health (Bartelt and Heeren, 2014; Panati et al., 2016). In addition, numerous studies have reported diverse action of irisin on the metabolism. Irisin is reported to increase mitochondrial content and function in cultured myocytes (Vaughan et al., 2014). Also, irisin treatment on human adipocytes inhibited lipid accumulation by increasing adipose triglyceride lipase (ATGL) and decreasing fatty acid synthase (FAS) (Huh et al., 2014).

Recent studies have suggested that irisin is not only a myokine but also an adipokine (Moreno-Navarrete et al., 2013; Roca-Rivada et al., 2013). Albeit low levels compared to muscle, expression and secretion of irisin/FNDC5 was confirmed in both mouse and human adipose tissue and cultured adipocytes. However, little is known about the functional role of adipocyte-derived irisin on metabolism. In this study, we investigated how irisin is regulated during adipocyte differentiation and then the role of endogenous and exogenous irisin in regulation of adipocyte differentiation was elucidated. Our results show that irisin and its precursor FNDC5 are expressed at early stages of adipogenesis and that ectopic expression of FNDC5 inhibits adipocyte differentiation. Moreover, we revealed that exogenous irisin led to activation of Wnt ligand expression. These findings suggest that irisin/FNDC5 disturbs adipogenic differentiation partially through regulation of Wnt signaling pathway.

MATERIALS AND METHODS

Cell Culture

3T3-L1 mouse preadipocytes were purchased from ATCC (Manassas, VA, United States). For maintenance of preadipocytes, 100 mm culture plates were used. For gene expression analysis and Western blotting the cells were seeded in 6 well plates, whereas 96 well plates were used for cell viability assay and Oil Red O staining. The cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% FBS (HyClone, Australia) and 1% penicillin/streptomycin (Carlsbad, CA, United States) and incubated at 37°C in 5% CO_2. 3T3-L1 cells were treated with differentiation media (MDI; methylisobutylxanthine, dexamethasone, insulin) to induce adipogenesis as previously described (Huh et al., 2012). To examine the effect of irisin on adipogenesis, 100 ng/mL recombinant irisin was treated every other day for 6 days, starting from 2 days before changing to MDI. Recombinant irisin was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, United States). Plasmid encoding FNDC5 gene and small interfering RNA were also used to examine the effect of genetic manipulation of irisin on adipocyte differentiation, as described below.

Cell Viability Assay

Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium-bromide (MTT) assay reagent. When the 3T3-L1 preadipocytes reached 50% confluence, recombinant irisin was treated in various concentrations for 24 and 48 h. Then MTT assay reagent was added to a final concentration of 0.5 mg/mL. The cells were then incubated for 2 h until the purple formazan crystals are displayed under the microscope. Then, DMSO was added and incubated for 10 min before measuring the absorbance at 540 nm.

Gene Expression Analysis

Total RNA was extracted from 3T3-L1 using TRI Reagent (MRC, Cincinnati, OH, United States). cDNA was synthesized using TOPscript^{TM} RT DryMIX (Enzynomics, South Korea). mRNA levels were measured by real-time PCR using Rotor-Gene Q (QIAGEN) with 20 μL reaction volume consisting of cDNA transcripts, primer pairs, and TOPreal SYBR Green PCR Kit (Enzynomics, South Korea). The gene expressions were normalized to 18S rRNA levels.

Western Blotting

Cells were harvested and lysed with RIPA buffer (Thermo Scientific, Rockford, IL, United States). Prepared protein sample were separated by SDS-PAGE and transferred to PVDF membrane. Primary antibodies were incubated at 4°C overnight. After overnight incubation, secondary antibody was incubated at room temperature for 1 h and the blots were detected with LAS-4000 (Fuji Photo Film, Tokyo, Japan). PPARγ (#2435), C/EBPα (#8178), and FABP4 (#3544) antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). Wnt10a antibody (#sc-376028) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States). FNDC5 antibody (#SAB1301655) was obtained from Sigma-Aldrich (St. Louis, MO, United States). β-tubulin antibody (#MA5-16308) was obtained from Thermo Scientific (Rockford, IL, United States).

Enzyme-Linked Immunosorbent Assay

Irisin secretion in the culture medium of adipocytes was measured with commercially available ELISA kits (Phoenix Pharmaceuticals, Burlingame, CA, United States), according to the manufacturer's instruction. Culture medium was harvested every 2 days starting from day 0 of adipocyte differentiation.

Cell Transfection

For the expression of irisin precursor FNDC5 gene, a plasmid expressing FNDC5 was generated using a modified pVAX1 mammalian expression vector. The gene encoding murine FNDC5 were genetically optimized for enhanced expression, including codon and RNA optimization, and a highly efficient immunoglobulin E leader sequence was added to facilitate expression. The construct was synthesized commercially, sequence verified, and then the optimized gene was subcloned into a modified pVAX1 vector under control of the cytomegalovirus immediate-early promoter. Sequence analysis was done by using GeneArt program from Life Technologies (**Supplementary file**). The DNA plasmid was designated as pVAX1-mFNDC5. 3T3-L1 cells were transfected with pVAX1-mFNDC5. Turbofect transfection reagent (Thermo Scientific, United States) and 1 μg of plasmid DNA were treated when the cells reached 80% confluence (4 days before adipocyte differentiation). Four days after transfection, transfected cells were differentiated according to the differentiation protocol. A modified pVAX1 mammalian expression vector was transfected as a negative control.

Commercially available FNDC5 small-interfering RNA (siRNA) and non-targeting siRNA as a negative control (Bioneer, South Korea) were used at 25 nmol/L. FNDC5 siRNA sequence is as follows: sense CAAGGUGCACCUUUGCAAA (dTdT), antisense UUUGCAAAGGUGCACCUUG (dTdT). Opti-MEM transfection media and lipofectamine (both from Invitrogen, Paisley, United Kingdom) were used for transfection, following the manufacturer's protocol. Transfection was performed on 3T3-L1 2 days before induction of adipogenesis. One day before transfection, the growth media was replaced with 10% FBS media without penicillin/streptomycin. Cells were treated with silencing reagent/siRNA mixture for 48 h and were harvested to examine the transfection efficiency. For the effect of silencing on Wnt expression, the cells were harvested 1 day after MDI treatment.

Oil Red O Staining

Oil Red O (ORO) staining was performed 8 days after induction of differentiation. Briefly, the cells were fixed with 10% formalin for 1 h. Then the cells were stained with 0.3% ORO solution (Sigma-Aldrich, St. Louis, MO, United States) for 2 h. After staining, the cells were washed with distilled water and representative pictures were taken under the microscope. For quantification, 100% isopropanol was added and the absorbance was measured at 490 nm.

Statistical Analysis

All statistical analysis was performed using Statview software. Mean values obtained from three independent experiments were compared by ANOVA with subsequent Fisher's significant difference method for *post hoc* paired comparisons. *P*-value of < 0.05 was used as the criterion for a statistically significant difference.

RESULTS

Changes in FNDC5 Gene Expression and Irisin Secretion During Adipocyte Differentiation

Previous studies have suggested that irisin is not only a myokine but also an adipokine (Moreno-Navarrete et al., 2013; Roca-Rivada et al., 2013; Huh et al., 2014). However, there is limiting evidence on the changes in its expression during adipogenesis. As such, the gene expression of FNDC5 during adipocyte differentiation was examined. The significant increase in PPARγ mRNA expression between days 1 to 8 during 3T3-L1 differentiation is shown as a positive marker of adipogenesis (**Figure 1A**). Concurrently, the gene expression of FNDC5 was the highest at day 0 (before induction of adipocyte differentiation) and significantly decreased after adipogenic stimulation by MDI treatment (**Figure 1B**), where the level was lowest on day 2 and gradually increased thereafter. This implies that the adipogenic cocktail is a strong stimulant for downregulation of FNDC5 gene expression. Next, the secretion of irisin during adipocyte differentiation was examined by performing ELISA in the conditioned medium. As a result, significantly increased secretion of irisin was observed between days 2 and 4 of adipocyte differentiation (**Figure 1C**), which is

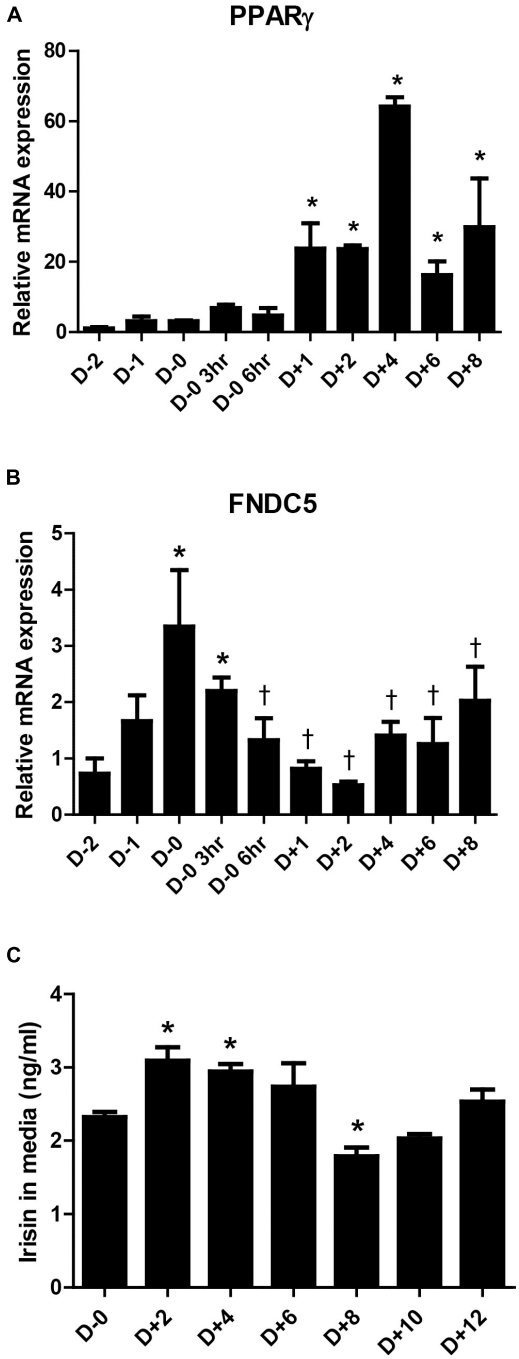

FIGURE 1 | Irisin gene expression and secretion during 3T3-L1 adipogenesis. **(A,B)** Gene expressions of PPARγ and FNDC5 were examined by real-time PCR during day D–2 to D+8 of adipogenesis. Quantifications were normalized to 18S in each reaction. $*P < 0.05$ compared with D–2, $\dagger P < 0.05$ compared with D–0. **(C)** Irisin secretion in media during adipogenesis was analyzed by ELISA. $*P < 0.05$ compared with D–0.

later period than increased expression of FNDC5 gene. Similar to the pattern of mRNA expression, secretion of irisin was highest at early stage (day 2) and then gradually decreased until day 8. These results confirm that irisin is an adipokine, where its mRNA expression and secretion are increased at early stage of adipocyte differentiation.

Endogenous and Exogenous Irisin Both Induce Suppression of Adipocyte Differentiation

First, the effect of recombinant irisin on cell viability was tested. Preadipocytes were treated with recombinant irisin at concentrations 50, 100, and 200 ng/ml for 24 and 48 h. The results showed that recombinant irisin does not exert any toxic effect on preadipocytes (**Supplementary Figure S1**). As previously reported, FABP4, C/EBPα, and PPARγ are the key regulators of adipogenesis responsible for the formation of mature adipocytes (Moseti et al., 2016). To evaluate the role of exogenous irisin on adipocyte differentiation, adipocytes were treated with 100 ng/mL recombinant irisin during adipogenesis. Gene expressions of FABP4, C/EBPα, and PPARγ were increased in time-dependent manner at day 4 and day 6 of differentiation period, whereas irisin treatment significantly downregulated the expression of these transcription factors at both days (**Figures 2A–C**).

To further validate the role of endogenous irisin on adipocyte differentiation, FNDC5 was overexpressed in 3T3-L1 preadipocytes. Transfection efficiency was confirmed by the significantly increased FNDC5 mRNA expression compared with control (**Supplementary Figure S2A**). Also, protein levels were assessed by using anti-FNDC5 antibody. Similar to the pattern shown in previous findings (Perez-Sotelo et al., 2017; Zhang et al., 2017), both bands corresponding to the full-length FNDC5 protein and the cleaved irisin peptide were detected (**Supplementary Figure S2C**). Interestingly, while both FNDC5 and irisin were increased in FNDC5-overexpressed preadipocytes, only the increase in irisin levels was statistically significant (**Supplementary Figure S2D**). In line with the results from recombinant irisin treatment, gene expressions of FABP4, C/EBPα, and PPARγ were significantly lower in FNDC5-overexpressed cells compared with control at day 5 and day 8 (**Figures 2D–F**). Consistently, protein levels of FABP4, C/EBPα, and PPARγ were also significantly lower in FNDC5-overexpressed cells (**Figures 2G,H**). Moreover, Oil red O staining performed at day 8 showed a significant decrease in the number of mature adipocytes in FNDC5-overexpressed cells compared to control, which resulted in less accumulation of lipids (**Figures 2I,J**). These results imply that irisin endogenously produced from adipocytes possesses the ability to inhibit adipocyte differentiation through regulation of transcription factors, corresponding to the effect of recombinant irisin treatment.

Wnt Expression Is Induced by Recombinant Irisin Treatment During Adipocyte Differentiation

Previous studies have shown that Wnt signaling influences the fate of many cell types and cell differentiation (Logan and Nusse, 2004; Rosen and MacDougald, 2006). Especially, Wnt

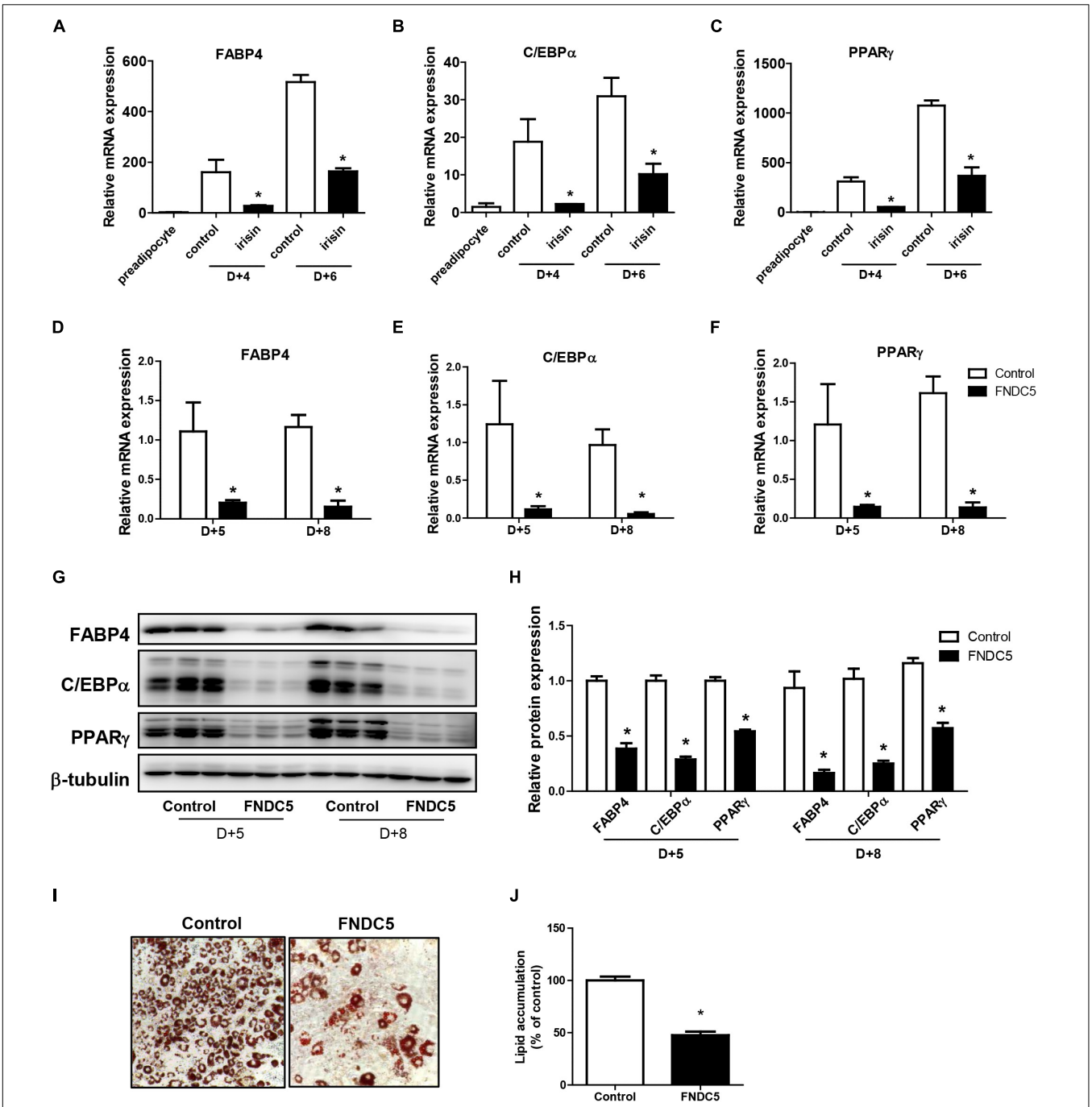

FIGURE 2 | Effect of recombinant irisin and FNDC5 overexpression on adipogenesis. **(A–C)** 3T3-L1 cells were treated with or without 100 ng/ml irisin from D–2 to D+6 of adipogenesis. Gene expressions of transcription factors were measured by real-time PCR and normalized to 18S rRNA. **(D–J)** 3T3-L1 preadipocytes were transfected with control or plasmid expressing FNDC5. Two days confluent cells were induced to differentiate and were harvested at day + 5 and day + 8. **(D–F)** Gene expressions of PPARγ, CEBPα, and FABP4 were analyzed by real-time PCR and normalized to 18S. **(G,H)** Western blot analysis of transcription factors. **(I,J)** Representative pictures and quantification of Oil red O staining in FNDC5-overexpressed cells at day 8. $^*P < 0.05$ compared with control at each time-point.

signaling is reported to be involved in inhibition of adipocyte differentiation (Rosen and MacDougald, 2006). Since numerous reports have shown varying patterns in Wnt expression during adipogenesis (Shen et al., 2011; Cawthorn et al., 2012), we first monitored the gene expressions of various Wnt ligands during adipocyte differentiation. The gene expressions of Wnt6, 10a, and 10b were markedly reduced from day 2 compared with day 0 (**Figures 3A–C**). To gain further insight into the pattern of Wnt decrease, changes in the gene expression from 2 days before initiation of adipogenesis (D−2) until day 2 was analyzed in

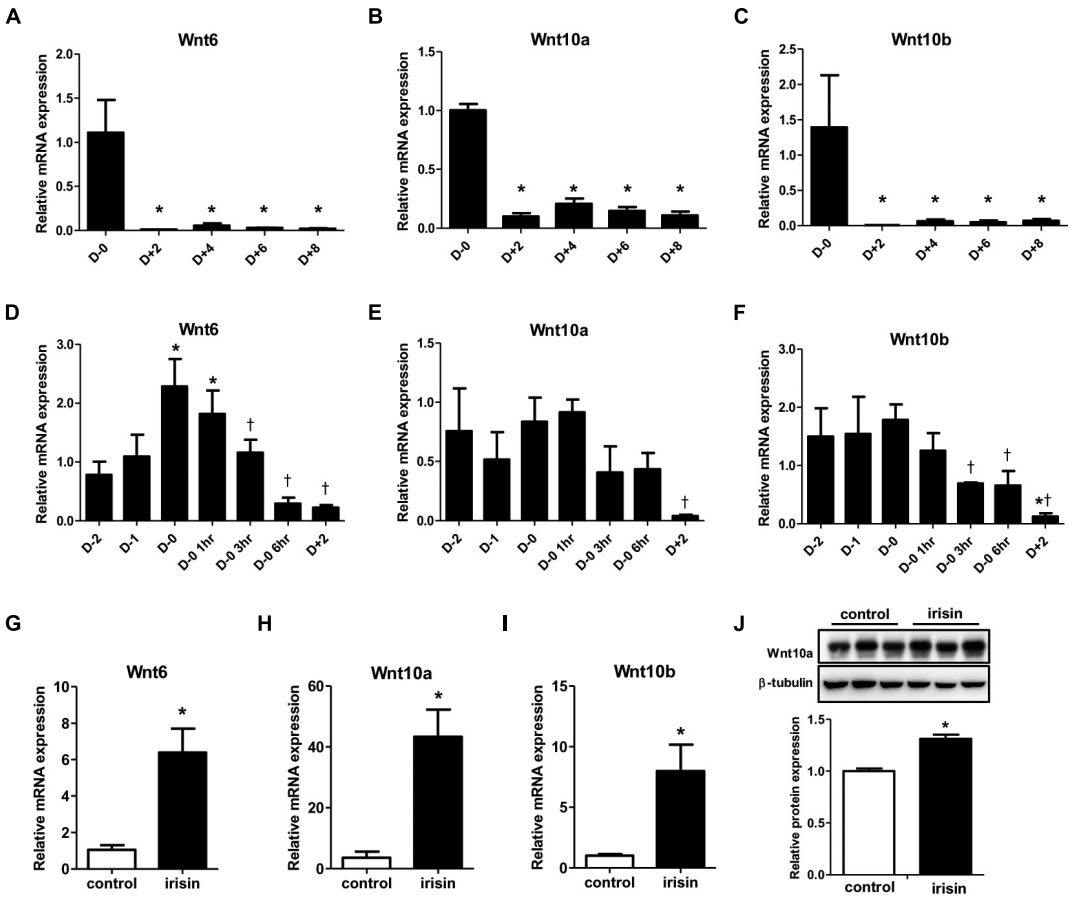

FIGURE 3 | Effect of recombinant irisin treatment on Wnt expression. **(A–C)** Gene expression analysis of Wnt ligands during 3T3-L1 adipocyte differentiation by real-time PCR. Quantification were normalized to 18S rRNA $^*P < 0.05$ compared with D–0. **(D–F)** Gene expression of analysis of Wnt ligands at early stage of adipogenesis by real-time PCR. $^*P < 0.05$ compared with D–2, †$P < 0.05$ compared with D–0. **(G–J)** 3T3-L1 cells were treated with 100 ng/ml recombinant irisin at D–2. **(G–I)** Wnt gene expressions were measured by real-time PCR and normalized to 18S rRNA. **(J)** Protein expression of Wnt10a with or without recombinant irisin treatment were analyzed by Western blot, $^*P < 0.05$ compared with control.

detail. The expression of Wnt6 was highest at day 0 and started to decrease from 3 h after changing the media to adipogenic cocktail (**Figure 3D**), which is similar to the mRNA expression pattern of FNDC5. Wnt10a and Wnt10b had similar pattern where the expression level was stable until day 0, and then the expression decreased from 3 h after MDI treatment (**Figures 3E,F**). Taken together, it was confirmed that Wnt expression is dramatically reduced by adipogenic stimulation.

Subsequent to the previous results on the effect of irisin on transcription factors, we investigated whether the inhibitory effect of irisin on adipogenesis is associated with regulation of Wnt. In 3T3-L1 preadipocytes, recombinant irisin was treated from 2 days before initiation of differentiation (D−2). Interestingly, the gene expressions of Wnt6, 10a, and 10b on day 2 of adipogenesis were significantly restored by irisin treatment compared to untreated control (**Figures 3G–I**). The protein expression of Wnt10a was also significantly increased by recombinant irisin treatment (**Figure 3J**), although to a lesser extent than observed with the change in gene expression. In summary, above results imply that irisin induces the expression of Wnt, which would subsequently disrupt adipogenesis through controlling transcription factors.

Effect of FNDC5 Silencing on Adipogenesis

Next, we sought to examine whether reduction in endogenous irisin expression would lead to the opposite effect. To verify, gene silencing was conducted using FNDC5 siRNAs in 3T3-L1 cells. Significant decrease in FNDC5 gene expression confirmed the transfection efficiency of siRNA (**Supplementary Figure S2B**). As expected, gene expressions of Wnt6 and Wnt10a were significantly decreased in FNDC5 knockdown (FNDC5-KD) cells compared to preadipocytes treated with negative siRNA (**Figures 4A,B**). Wnt10b gene expression was also marginally decreased (**Figure 4C**).

Since alteration in endogenous FNDC5 level led to changes in Wnt expression, we further evaluated whether FNDC5 silencing leads to regulation of adipogenesis. In contrast to the effects observed in FNDC5 overexpressed cells, Oil red O staining

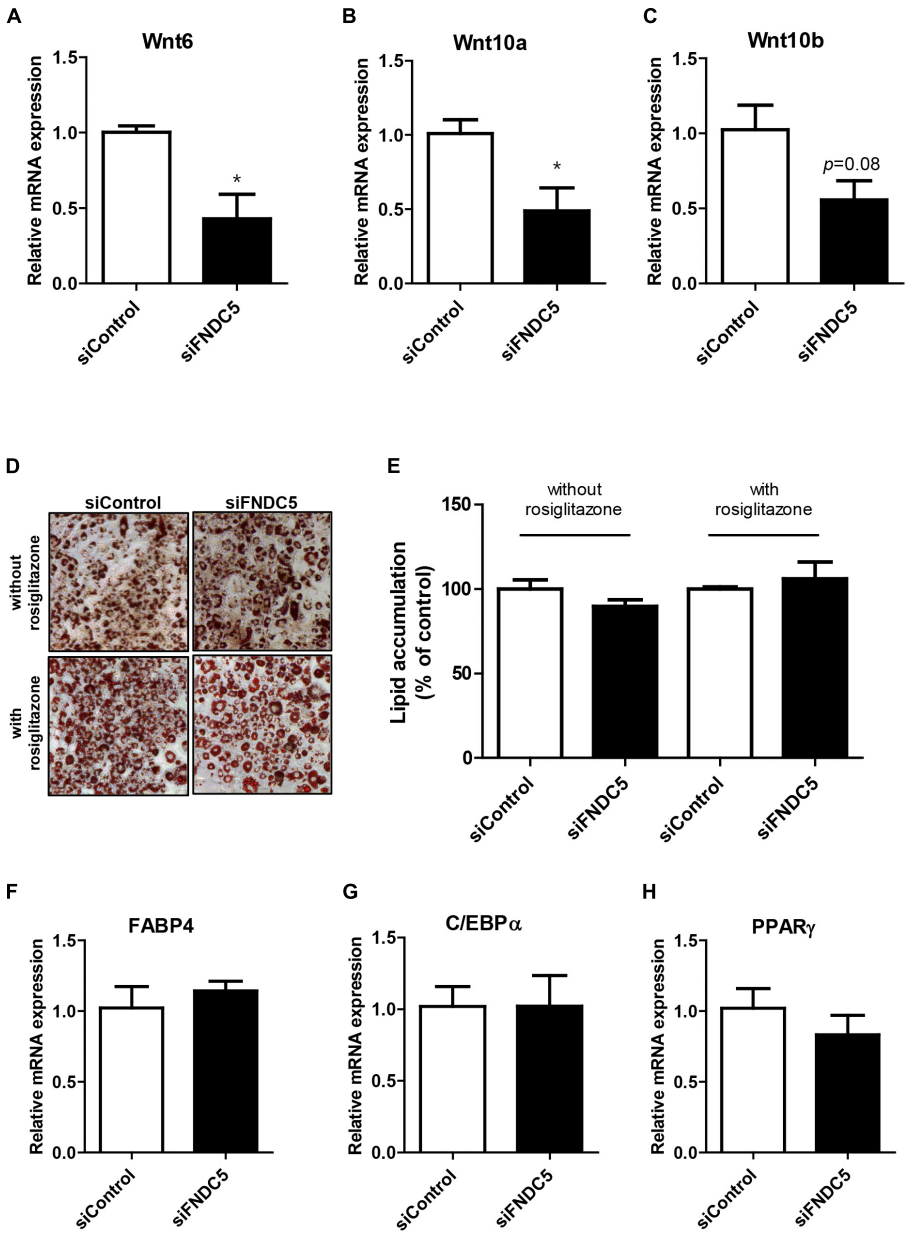

FIGURE 4 | Effect of FNDC5 silencing on Wnt gene expression and adipogenesis. 3T3-L1 preadipocytes were transfected with FNDC5 siRNA and negative control at 25 nmol/L for 48 h. **(A–C)** Cells were harvested 1 day after MDI treatment. Gene expression of Wnt ligands were examined by real-time PCR and normalized to 18S in each reaction. **(D,E)** Representative pictures and quantification of Oil red O staining after siFNDC5 transfection. The adipogenesis was induced by conventional MDI treatment or with addition of PPARγ agonist, rosiglitazone, and harvested on day 8. **(F–H)** Real-time PCR analysis of transcription factors after siFNDC5 transfection. Data is shown as $^*P < 0.05$ compared with siControl.

performed at day 8 of adipogenesis showed no difference in the extent of adipocyte differentiation between FNDC5-KD and control cells as evidenced by similar levels of lipid accumulation (**Figures 4D,E**). The results were similar whether the cells were differentiated using conventional MDI or with addition of PPARγ agonist rosiglitazone. In line with this observation, the gene expressions of adipogenic transcription factors were also unaltered in response of FNDC5 silencing (**Figures 4F–H**). These data suggest that although Wnt expression is partially affected by inhibition of FNDC5 levels, it was not sufficient to regulate the differentiation of adipocytes.

DISCUSSION

Irisin has received a significant amount of attention since its discovery as adipocyte browning factor (Bostrom et al., 2012). Recent findings show pluripotent role of this exercise-induced

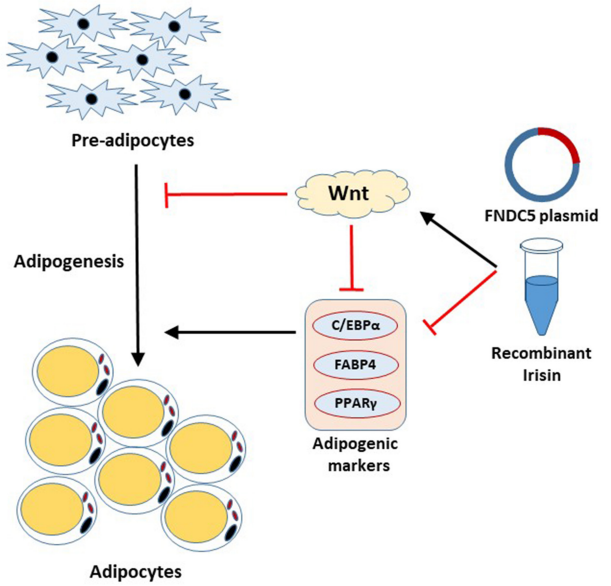

FIGURE 5 | Schematic diagram of the effect of irisin on adipogenesis.

hormone in various tissues, which emphasizes the beneficial role of exercise on whole body metabolism (Perakakis et al., 2017; Askari et al., 2018; de Oliveira Bristot et al., 2019; Farmer, 2019). The present study unveiled the significant role of FNDC5/irisin on adipogenesis. This study, for the first time, reports that irisin exhibits inhibitory effects on adipogenesis through regulation of Wnt signaling. The important aspects of our findings are; (1) FNDC5 mRNA expression and irisin secretion are increased at the early stage of adipogenesis, (2) Endogenous as well as exogenous irisin regulates the adipocyte differentiation through downregulation of the transcription factors, (3) Irisin inhibits adipocyte differentiation by inducing Wnt expression, (4) FNDC5 silencing in adipocytes alters the Wnt expression but shows no significant effects on adipocyte differentiation and lipid accumulation. These results provide evidence on the intrinsic role of irisin in adipocytes (**Figure 5**).

Previous studies have shown the potential role of exercise-induced irisin in the treatment of various diseases. These reports have highlighted the role of irisin as a myokine, mediating the beneficial effects of exercise on adipose tissue, liver, bone, brain, etc. through endocrine effect (Huh et al., 2014; Zhang et al., 2017; Bi et al., 2019; Lourenco et al., 2019). Although muscle is responsible for the majority of secreted irisin in circulation, reports are continuously appearing suggesting the local expression of irisin in other organs (Colaianni et al., 2015; Wahab et al., 2019). Similar to other myokines reported to date, irisin is also reported as an adipokine (Moreno-Navarrete et al., 2013; Roca-Rivada et al., 2013; Huh et al., 2014; Zhang et al., 2016; Perez-Sotelo et al., 2017). It is interesting to note that in the case of obesity, irisin seems to be over-secreted from adipose tissue which may be an adaptive response to counterbalance metabolic dysregulation (Moreno-Navarrete et al., 2013; Roca-Rivada et al., 2013). However, the functional role of adipocyte-derived irisin has not been studied in detail. We and others have previously reported that in addition to the role of irisin in inducing adipocyte browning, irisin has the ability to inhibit adipocyte differentiation (Huh et al., 2014; Zhang et al., 2016). In the current study, we found that FNDC5-overexpressed preadipocytes are inhibited from adipogenic differentiation, suggesting the intrinsic role of adipocyte-derived irisin. Interestingly, both results from recombinant irisin treatment and FNDC5 overexpression show that there are significant inhibitory effects during the differentiation process (days 4–5) and after the adipogenesis is completed (days 6–8). ORO staining at day 8 shows a contrast in number of mature adipocytes compared to control. We further found that the inhibitory effect of irisin on adipogenesis is mediated via activation of Wnt, which is known to repress adipogenesis by blocking the induction of PPARγ and C/EBPα, as shown in our results (Kennell and MacDougald, 2005; Cawthorn et al., 2012). It remains to be studied whether downstream signaling of Wnt such as β-catenin is also involved in this process. Furthermore, there is immense need to discuss the effect of Wnt agonist and inhibitors on the anti-obesity and anti-diabetic effects of irisin.

Canonical Wnt signaling is known to control the balance between myogenesis, osteogenesis, and adipogenesis (Christodoulides et al., 2009; Cawthorn et al., 2012). Accordingly, it has been recently reported by several groups that irisin regulates bone metabolism through increasing osteoblastogenesis and mineralization (Colaianni et al., 2015; Zhang et al., 2017). Not surprisingly, this effect was mediated through downregulation of sclerotin, a Wnt/β-catenin pathway inhibitor. Therefore, the activation of Wnt by irisin seems to exert dual effects by enhancing bone strength while limiting the expansion of adipose tissue through inhibition of adipocyte formation. Similarly, other myokines are also reported to regulate the Wnt signaling. For example, myostatin is known to inhibit osteoblastic differentiation (Qin et al., 2017) and SPARC is known to inhibit adipocyte differentiation and adipose tissue turnover (Nie and Sage, 2009) through regulation of Wnt pathway, which suggest a common role of exercise-induced myokines.

In accordance with our data on the effect of ectopic expression of FNDC5, a recent study has reported that adipocytes lacking FNDC5 gene expression show increased accumulation of lipids during adipogenesis (Perez-Sotelo et al., 2017). In our study, although we have observed significantly reduced gene expression of Wnt ligands by knockdown of FNDC5, the rate of adipocyte differentiation did not differ between FNDC5-KD and control cells. In the study by Perez-Sotelo et al. (2017), FNDC5 was knocked down using shRNA with lentiviral infection which resulted in over 90% reduction in gene expression, whereas in our data the reduction rate was around 70–80%. In addition, the discrepancy may derive from the difference in the cell lines used (3T3-L1 versus C3H10T1/2 murine mesenchymal stem cells). This idea is supported by the fact that the two studies show difference in the expression profile of FNDC5 during adipocyte differentiation. Perez-Sotelo et al. (2017), showed a gradual increase in FNDC5 expression from day 2 to 10, paralleling the expression of PPARγ. In contrast we observed the increase in both FNDC5 gene expression and irisin secretion at the

early stage of adipogenesis. This is in line with our previous report where the FNDC5 mRNA level in mature adipocytes was significantly lower than stromal vascular cells in humans (Huh et al., 2014). Of note, the mRNA expression pattern of FNDC5 during adipogenesis showed similarity to that of Wnt6, providing a plausible relationship between the two factors that negatively regulate adipogenesis. It is also possible that since the basal FNDC5 levels in preadipocytes are very low [several 100-fold lower compared to myocytes (Moreno-Navarrete et al., 2013; Huh et al., 2014)], silencing has minor influence on the cells. Further studies are needed at *in vitro* and *in vivo* level to resolve the controversy.

Irisin is produced as a result of cleavage of its precursor, FNDC5 (Bostrom et al., 2012) Studies have shown that increase in FNDC5 is directly involved with elevated circulating irisin levels (Xiong et al., 2015; Geng et al., 2019). In our study, we have shown that similar outcome is brought by recombinant irisin treatment and induction of endogenous FNDC5 expression. This partly suggests that regulation of FNDC5 expression leads to changes in irisin secretion. It is important to note that the protease responsible for cleavage of FNDC5 is currently unknown. Therefore, it is possible that irisin secretion could be induced by induction of FNDC5 cleavage not expression *per se*. Following secretion, irisin is expected to exert autocrine effect through recently identified irisin receptor aV integrin (Kim et al., 2018). This receptor is reported to exist in fat and is responsible for the effect of irisin in inducing thermogenic genes. Whether this receptor is also responsible for the effect of irisin on inhibition of adipogenesis should further be studied in the future.

In conclusion, we found that FNDC5/irisin levels are induced at early process of adipocyte differentiation, and this leads to inhibitory effect on adipogenesis through regulation of Wnt. Moreover, we have demonstrated that endogenous expression of FNDC5/irisin is able to act on adipocytes in an autocrine/paracrine manner. In addition to the browning effect of irisin on mature adipocytes, our data provide evidence on its ability to limit expansion of adipose tissue, which further potentiates its therapeutic potential in obesity-related metabolic disorders.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

EM, NS, MJ, and JH conceived and designed the study, and drafted and critically revised the manuscript. EM, NS, and JH performed the experiments and analyzed the data. All authors 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.2019.01085/full#supplementary-material

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Examination of the Myokine Response in Pregnant and Non-pregnant Women Following an Acute Bout of Moderate-Intensity Walking

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Background: It is recommended that women accumulate 150-min of weekly moderate-intensity physical activity (MPA) when pregnant. Engaging in regular physical activity (PA) confers many health benefits to both the mother and the fetus. However, the molecular mechanisms by which these health benefits are bestowed are not well understood. One potential factor that may be contributing to the observed benefits is myokines, which are small peptides secreted by skeletal muscles. In the non-pregnant population, myokines are believed to be involved in the molecular mechanisms resulting from PA. The objective of this study was to characterize and compare the myokine profile of pregnant and non-pregnant women, after an acute bout of MPA.

Methods: Pregnant (n = 13) and non-pregnant (n = 17) women were recruited from the Ottawa region to undergo a treadmill walking session at moderate-intensity (40–60% heart rate reserve). Pre- and post-exercise serum samples were taken, and a set of 15 myokines were analyzed although only 10 were detected. IL-6 was analyzed using a high-sensitivity assay, while FGF21, EPO, BDNF, Fractalkine, IL-15, SPARC, FABP-3, FSTL-1, and oncostatin were analyzed using various multiplex assays.

Results: The pregnant and non-pregnant groups did not differ in terms of age, height, non/pre-pregnancy weight, BMI, and resting heart rate. Baseline levels of EPO and oncostatin were higher in the pregnant group while FGF21 was higher in the non-pregnant group. Circulating levels of three myokines, FGF21, EPO, and IL-15 significantly increased in response to the acute exercise in the pregnant group. Non-pregnant women exhibited an increase in three myokines, FABP-3, FSTL-1, and oncostatin, while one myokine, EPO, decreased post-exercise. SPARC, fractalkine and BDNF were shown to increase post-exercise regardless of pregnancy status while the response for BDNF was more pronounced in the non-pregnant group.

Conclusion: This is the first study examining myokine response following an acute bout of PA in pregnancy. Moderate intensity PA, which is recommended during pregnancy, elicited an increase in four myokines post-compared to pre-exercise in the pregnant group. Further research is warranted to understand the role of myokines in pregnancy.

Keywords: pregnancy, myokines, physical activity, exercise, gestational weight gain

INTRODUCTION

Contrary to outdated beliefs, evidence shows that engaging in regular physical activity (PA) during pregnancy is associated with a plethora of health benefits for both the mother and the fetus (Nascimento et al., 2012; Mudd et al., 2013). The 2019 Canadian guidelines for PA throughout pregnancy recommend that pregnant women without contraindications engage in at least 150-min moderate-intensity PA per week (Mottola et al., 2018). A combination of resistance and aerobic exercises has been deemed safe and is highly recommended to achieve greater benefits (Mottola et al., 2018). Systematic review evidence illustrates that pregnant women who are physically active compared to those that are not experience less musculoskeletal pain, gestational weight gain (GWG), postpartum weight retention, urinary incontinence as well as gestational diabetes and insulin resistance (Nascimento et al., 2012; Davenport et al., 2018a,b; Ruchat et al., 2018). Exercise interventions during pregnancy have been shown to decrease the odds of developing gestational hypertension and pre-eclampsia (Davenport et al., 2018c). Additionally, habitual PA while pregnant can decrease depressive symptoms during pregnancy and the postpartum period (Robledo-Colonia et al., 2012; Vargas-Terrones et al., 2018) and improve quality of life (Victoria et al., 2010). Alternatively, PA engagement during pregnancy may have positive downstream effects for the health of the infant (Ferraro et al., 2012). These findings are in line with the developmental origins of health and disease (DOHaD) hypothesis, suggesting that the intrauterine environment plays a critical role in determining health outcomes later in life (Wadhwa et al., 2009). Being physically active during pregnancy could contribute to optimizing the development of the fetus' immune and central nervous systems, thereby decreasing the risk of developing neurodevelopmental and psychiatric disorders (Marques et al., 2015). It is possible that maternal-fetal health benefits linked to PA may be accorded through changes in development and function of the placenta. Regular participation in PA throughout pregnancy is associated with improved placental function by way of optimizing nutrient transport to the fetus due to an increase in intervillous space blood volume (Jackson et al., 1995; Clapp, 2003).

Although it is well established that habitual PA is beneficial during pregnancy, the molecular mechanisms by which PA acts on different organs and body systems in pregnant women remain to be fully understood. It has been suggested that myokines, peptides that are synthesized by skeletal muscles and most often released in the body as a result of contraction (Pedersen et al., 2007), are responsible, in part, for the crosstalk between muscles and various organs and tissues in the body (Pedersen, 2013). The investigation of myokine response as a result of PA has been mainly conducted using male subjects. To date, the circulating myokine profile resulting from PA has yet to be examined in the context of human pregnancy. Dubé et al. (2017) postulate that myokines released during PA may play a role in the optimization of fetal and placental growth outcomes. Hundreds of these peptides have been identified, including, IL-6, IL-15, and fibroblast growth factor 21 (FGF21) (Leal et al., 2018). Myokines are involved in paracrine and endocrine signaling pathways (Pedersen, 2013), while some myokines, such as myostatin and leukemia inhibitory factor (LIF) exert their actions in an autocrine fashion upon the same muscle that synthesizes them (Pedersen, 2013; Carson, 2017). Most myokines are not exclusively produced and secreted by muscle fibers but can also be expressed or derived from other tissues or organs in the human body, such as bone, liver, adipose tissue and macrophages (Ishimi et al., 1990; García et al., 1999; Kakoti and Goswami, 2013; Salminen et al., 2017). Collectively, the myokine secretome has various functions attributable to the unique activity of each myokine. For instance, IL-6, the most well-characterized myokine, has been recognized as a key mediator of glucose metabolism as it increases insulin sensitivity (Steensberg et al., 2000). Although the function of each myokine is specific, it is suggested that there is one commonality in their roles, that of being mediators to the benefits and protective effects observed as a result of engaging in PA (Benatti and Pedersen, 2015; Whitham and Febbraio, 2016). However, the characterization of the myokine profile and thus its potential effects, in pregnant women, has yet to be investigated. This study aimed to compare the myokine response to moderate-intensity exercise between non-pregnant and pregnant women.

Abbreviations: BDNF, brain-derived neurotophic factor; BMI, body mass index; EPO, erythropoietin; EV, extracellular vesicles; FABP-3, fatty acid binding protein 3; FGF21, fibroblast growth factor 21; FSTL-1, follistatin-like 1; GDM, gestational diabetes mellitus; GLT, godin leisure time; GWG, gestational weight gain; HR_{max}, heart rate maximum; HR, heart rate; HRR, heart rate reserve; IOM, Institute of Medicine; LIF, leukemia inhibitory factor; MPA, moderate-intensity physical activity; PA, physical activity; RHR, resting heart rate; SPARC, secreted protein acidic and rich in cysteine.

MATERIALS AND METHODS

Ethics Approval and Informed Consent

This study was approved by the Research Ethics Board at the University of Ottawa (file number: H-06-18-634), and all aspects conform to the Declaration of Helsinki. Written

informed consent was obtained from each participant willing and eligible to participate.

Participants

Pregnant and non-pregnant women were recruited from the Ottawa region (ON, Canada) via recruitment flyers posted at the University of Ottawa and on social media platforms. Eligibility was confirmed via telephone by the researchers. Inclusion criteria were as follows: between 18 and 40 years of age, having a self-reported non/pre-pregnancy body mass index (BMI) classified as normal or overweight (18.5–29.9 kg/m^2) with no contraindication to exercise. Those with hypertension, diabetes, or untreated thyroid disease were excluded. Pregnant women in their second trimester (13–28 weeks gestation) were included. Participants were excluded if they were not able to complete the exercise session or if a blood sample was not obtained either pre- or post-exercise. Anthropometric measurements such as height and body weight were recorded at the time of the visit using a Tanita HR-200 wall-mounted stadiometer (Lachine, QC) and a Tanita BWB-800 scale, respectively. In the pregnant group, GWG was calculated by subtracting the weight measured at the study visit by the self-reported pre-pregnancy weight. Based on the Institute of Medicine (IOM) recommendations for GWG, women should gain a maximum of 9.7 kg in their first trimester, regardless of BMI (American College of Obstetricians and Gynecologists, 2013). Thereafter, a maximum of 2.2 and 1.5 kg of weight gain per week is recommended for women with a BMI classified as normal and overweight, respectively (American College of Obstetricians and Gynecologists, 2013). The GWG at the time of the visit in addition to gestational age of participants were used to calculate the percentage of upper-limit of weight gained in accordance to the IOM guidelines.

Exercise Protocol

Participants were asked to fast for 8 h and refrain from any PA for 12 h before the study visit. Upon arrival, participants were provided with, and asked to consume a standardized snack of approximately 340 kcal. The snack consisted of a fruit juice (orange or cranberry), a granola bar and a small fruit (apple or pear). However, we could not force a participant to eat if they chose not to. Following the snack, a 10-min seated resting phase began during which heart rate (HR) was monitored continuously and was recorded at 1-min intervals using a Polar V800 (Lachine, QC) heart rate monitor. Resting HR (RHR) was determined from the average of the last 5-min of measurements. The acute bout of exercise was conducted using a Woodway Pro XL 27 treadmill (Woodway USA, Waukesha, WI, United States) following the resting phase. Initially, participants underwent the acclimation phase starting with a warm-up for 3-min at 2.0 mph followed by an increase in the speed of 0.2 mph every minute, until the calculated moderate intensity, or 40–60% heart rate reserve (HRR), was achieved. The incline was set at 6% throughout both the acclimation and the acute bout of exercise. HRR was calculated using the Karvonen formula (Eqs. 1 and 2) (She et al., 2014).

$$\%\mathrm{HRR} = [[\mathrm{HR_{max}} - \mathrm{RHR}] * \%\mathrm{intensity}] + \mathrm{RHR} \quad (1)$$

$$\mathrm{HR_{max}} = 220 - age \quad (2)$$

Once the target HR intensity was met, the speed was kept constant for 30-min. HR was monitored throughout to ensure the target HR intensity zone was maintained. If HR was either below or above the desired range, the speed was adjusted by 0.2 mph accordingly. The rate of perceived exertion (RPE) was measured every 1-min during the acclimation phase and every 5-min during the acute exercise bout using the Borg Scale (Borg, 1982).

Blood Collection and Processing

A blood sample was taken pre- and post-exercise from the medial cubital vein and collected in serum blood collection tubes (#367820; BD Biosciences, Franklin Lakes, NJ) immediately before and after the completion of the exercise protocol. Serum was left to clot at room temperature for 30-min after which it was centrifuged for 15-min at 4°C at a speed of 1000 × *g* using an Eppendorf 5702R centrifuge (Thermo Fisher Scientific Inc., Mississauga, ON, Canada). Serum samples were stored at −80°C until further analysis.

Human Myokine Assays

Serum samples from the pregnant and non-pregnant participants were assayed in duplicate for 15 myokines: apelin, brain-derived neurotrophic factor (BDNF), erythropoietin (EPO), fatty acid binding protein 3 (FABP-3), follistatin-like 1 (FSTL-1), fibroblast growth factor 21 (FGF21), fractalkine, interleukin-6 (IL-6), interleukin-15 (IL-15), irisin, LIF, myostatin, oncostatin M, osteocrin and SPARC. Of the 15 myokines analyzed, 10 were detected in our samples. Serum samples were shipped overnight, on dry ice to Eve Technologies (Calgary, AB) for analysis using the MILLIPLEX MAP Human Myokine Magnetic Bead Panel (HMYOMAG-56K, Millipore Sigma, Oakville, ON, Canada). Only four of the fifteen myokines were within the range of detection of the Milliplex assay: oncostatin, FABP-3, FSTL-1, and SPARC. Apelin, BDNF, EPO, FGF21, fractalkine, IL-6, IL-15, irisin, IF, myostatin and osteocrin were undetected in our samples. A custom high sensitivity IL-6 assay (Millipore Sigma) was conducted by Eve Technologies. Additionally, a U-PLEX Assay from Meso Scale Discoveries (MSD, Rockville, MD, United States) was used to analyze five myokines: BDNF, EPO, FGF21, Fractalkine, IL-15 while SPARC was re-analyzed using an R-PLEX assay (MSD, Rockville, MD, United States).

Statistical Analysis

The Student's *t*-test and the Mann-Whitney *U* test were used, as appropriate, to compare demographic variables and exercise session indices between pregnant and non-pregnant women. Based on the distribution of the data, either a parametric or non-parametric test was chosen. The main analysis was performed using a 2-way mixed ANOVA to assess changes in myokine

concentrations in pregnant compared to non-pregnant women following an acute bout of moderate-intensity walking (**Figure 2**). Data shown to deviate from normality following a Shapiro-Wilk test for normality were transformed using the natural logarithm. A Bonferroni *post hoc* correction for multiple comparisons was performed. Myokine data were excluded if they were not in the assay's detectable range. Pearson correlations were performed between delta change in myokine levels and BMI and age, respectively (data not shown), in addition to the exercise session indices and baseline characteristics of participants (**Figure 3**). For all statistical analyses, $p \leq 0.05$ was considered significant. Data are presented as mean ± standard deviation (SD). Data presented in **Table 1** and **Figure 3** were analyzed using GraphPad Prism Software (version 8.0.0, San Diego, CA, United States) while SPSS Software (version 13, Armonk, NY, United States) was used to analyze data in **Table 2** and **Figures 1-3**.

RESULTS

Baseline Characteristics and Exercise Parameters

In total, 13 pregnant and 17 non-pregnant women met the inclusion criteria and were included in the analysis. Baseline characteristics, such as age, height, non/pre-pregnant BMI, weight, and HR, did not differ between groups (**Table 1**). The RPE scores were compared between the pregnant and non-pregnant groups and did not differ. Thus, the perceived intensity of the exercise session was viewed equally in both groups and corresponded to moderate-intensity (Norton et al., 2010; Berghella and Saccone, 2017). Based on the same relative moderate-intensity exercise session, non-pregnant women were able to reach a significantly higher maximal ($p = 0.006$) and average speed ($p = 0.009$) compared to their pregnant counterparts (**Table 1**). Additionally, the duration of the exercise session was longer for non-pregnant compared to pregnant women ($p = 0.006$) (**Table 1**).

Comparison of Baseline Serum Myokine Levels Between Pregnant and Non-pregnant Women

The 2-way mixed ANOVA revealed differences in baseline serum myokine concentrations between the pregnant and non-pregnant groups. Of the 10 myokines, EPO ($p \leq 0.001$) and oncostatin ($p = 0.02$) were significantly increased in the pregnant group while FGF21 ($p = 0.001$) was higher in the non-pregnant group (**Figure 1**).

Myokine Serum Levels Pre- versus Post-exercise in Pregnant and Non-pregnant Groups

The 2-way mixed ANOVA revealed a significant interaction between time (pre- and post-exercise) and pregnancy status (pregnant and non-pregnant) for FGF21 ($F = 11.25$, $p = 0.002$), EPO ($F = 18.45$, $p < 0.0001$), oncostatin ($F = 20.56$, $p < 0.0001$), FABP-3 ($F = 20.29$, $p < 0.0001$), FSTL-1 ($F = 20.29$, $p = 0.002$), IL-15 ($F = 10.18$, $p = 0.003$), and BDNF ($F = 35.89$, $p < 0.0001$) (**Figure 2**). Bonferroni corrections were applied to multiple comparisons. Briefly, for the pregnant group, FGF21 ($p \leq 0.001$), EPO ($p = 0.004$), IL-15 ($p = 0.018$), and BDNF ($p = 0.025$) increased post-exercise (**Table 3**). Whereas, oncostatin ($p \leq 0.001$), FABP-3 ($p \leq 0.001$), FSTL-1 ($p \leq 0.001$), and BDNF ($p \leq 0.001$) increased post-exercise in the non-pregnant group, while EPO ($p = 0.002$) decreased post-exercise (**Table 3**). The main effect of time on SPARC ($F = 9.60$, $p = 0.005$) and fractalkine ($F = 20.6$, $p < 0.0001$) was significant: women, regardless of pregnancy status, exhibited an increase in both myokines post-exercise (**Tables 2**, **3**).

Correlations Between Maximum Speed Reached, and Weight Gained

The following indices were examined to help understand the difference in maximal and average speed reached during the exercise session in the pregnant and non-pregnant groups: age,

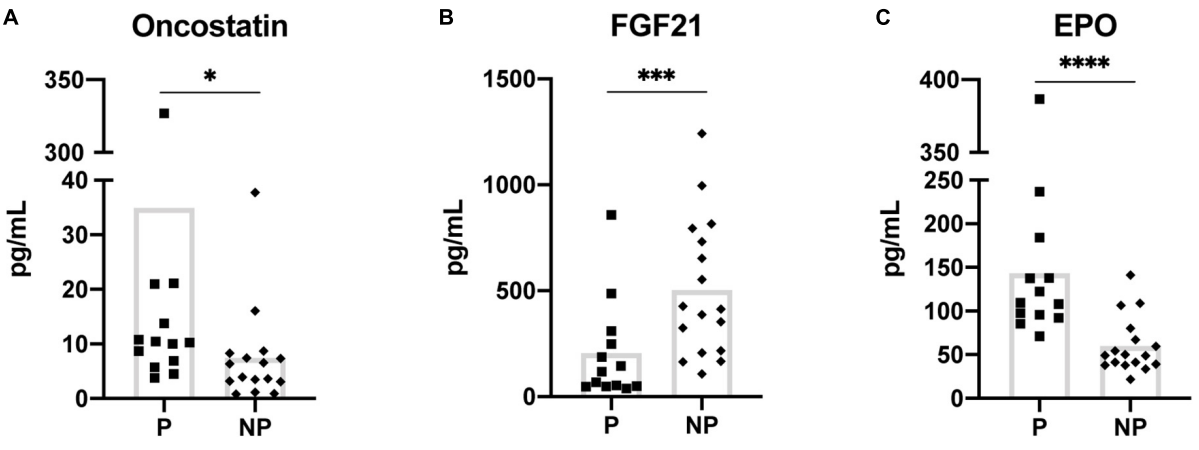

FIGURE 1 | Myokines showing a statistically significant difference in baseline (pre-exercise) serum concentration between pregnant and non-pregnant women **(A–C)**. Means are shown with box on graphs. P, Pregnant; NP, Non-pregnant. *$p \leq 0.05$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

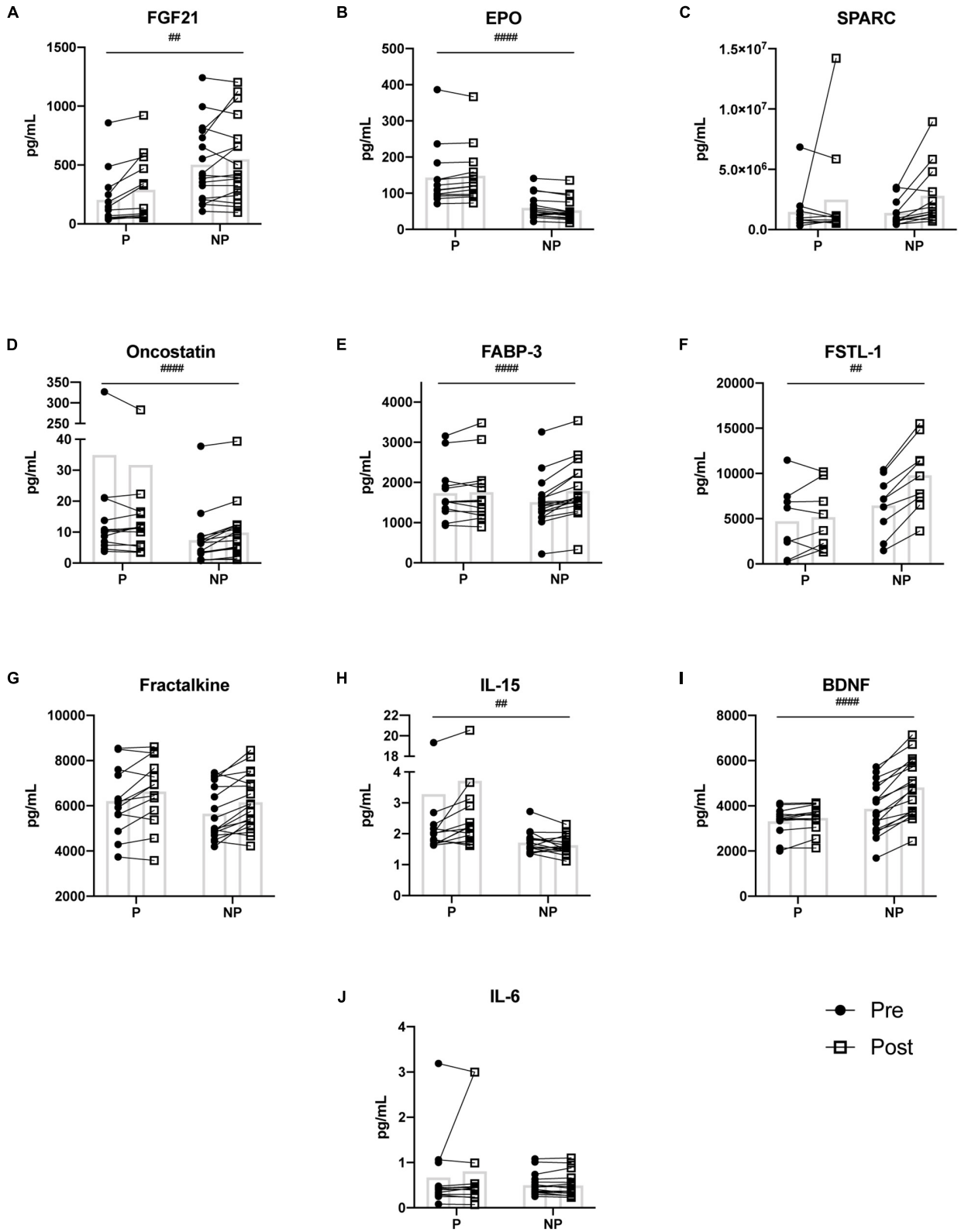

FIGURE 2 | Pre- and post-exercise serum concentrations of ten myokines **(A–J)** measured in serum of pregnant and non-pregnant women. Statistically significant pregnancy status by time interactions are depicted with '#' symbol. Means are shown with box on graphs. P, Pregnant; NP, Non-pregnant; Pre, Pre-exercise serum; Post, Post-exercise serum. ##$p \leq 0.01$; ###$p \leq 0.001$; ####$p \leq 0.0001$.

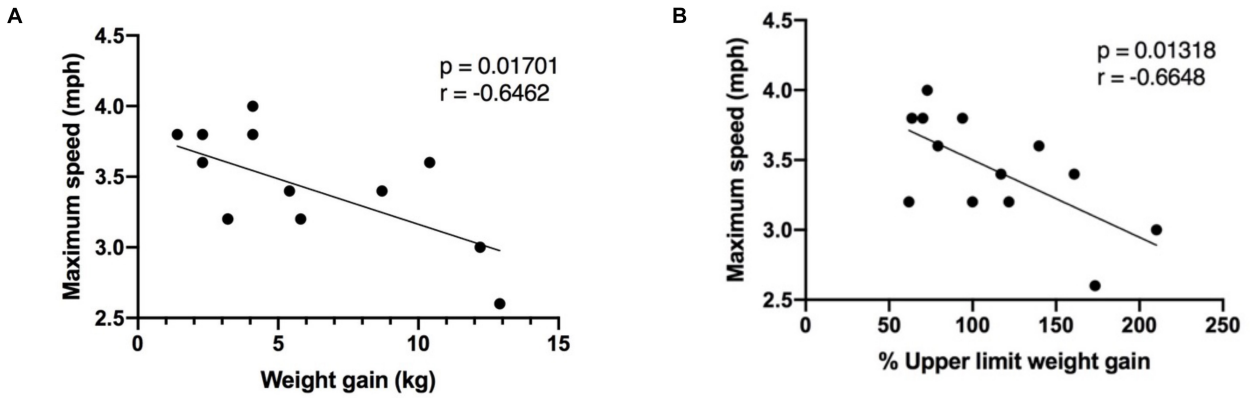

FIGURE 3 | Correlation between maximum speed reached during the exercise session, in pregnant women (n = 13), and **(A)** the weight gained at the time of the visit; **(B)** the percentage of upper limit weight gained in accordance to IOM gestational weight gain guidelines. Mph, miles per hour; kg, kilograms.

TABLE 1 | Study population demographics and exercise session indices.

	Pregnant N = 13	Non-pregnant N = 17	p-value
Age (years)	31.2 ± 3.5	30.2 ± 4.3	0.48
Gestational age (weeks)	20.1 ± 5.0	–	–
Gestational weight gain at time of session (kg)	5.8 ± 3.9	–	–
Height (cm)	166.7 ± 5.4	166.3 ± 6.3	0.87
Non/pre-pregnant BMI (kg/m^2)	23.7 ± 3.6	21.8 ± 2.3	0.09
Non/pre-pregnant body weight (kg)	63.7 ± 9.5	60.0 ± 8.4	0.27
Resting heart rate (bpm)	81.4 ± 14.6	74.3 ± 9.3	0.11
Rate of perceived exertion (Borg Scale)	12.7 ± 1.2	12.7 ± 1.3	0.90
Maximal speed reached (mph)	3.4 ± 0.4	3.8 ± 0.3	0.006*
Average speed (mph)	3.2 ± 0.5	3.6 ± 0.3	0.009*
Duration of exercise session (min)	40 ± 2.1	42 ± 1.6	0.006*

*Values are presented as mean ± standard deviation. BMI, body mass index; bpm, beats per minute; mph, miles per hour; min, minutes; *, indicates significance.*

RHR, pre-pregnancy BMI, gestational age and weight gained at the time of the visit in the pregnant group. There was an inverse correlation between weight gained (expressed both as absolute weight gained, and the percentage of upper limit weight gained according to IOM GWG guidelines) and maximal speed reached (**Figure 3**). Age, RHR, BMI, and gestational age were not significantly correlated with maximal speed reached or average speed achieved during the exercise session.

DISCUSSION

Following an acute bout of moderate-intensity walking, the concentration of three myokines; FGF21, EPO, and IL-15 significantly increased in pregnant women. The myokines FABP-3, FSTL-1, and oncostatin exhibited an increase in the non-pregnant group for the same relative intensity exercise. In contrast, EPO was the only myokine to decrease significantly post-exercise in the group of non-pregnant women. SPARC, fractalkine and BDNF were found to increase post-exercise in all women, regardless of pregnancy status. However, BDNF showed a stronger response in non-pregnant women. To our knowledge, this is the first study examining the myokine profile in pregnant women after an acute bout of exercise. Maternal, fetal, and placental health is optimized in women that engage in PA throughout their pregnancies, yet, how PA confers these observed benefits remains to be elucidated. Myokines may be one of the many mediators at play, hence, characterizing the myokine response post-exercise is an essential first step in understanding whether myokines may facilitate the health benefits resulting from PA engagement in pregnancy.

One of the myokines found to differ, FGF21, is a protein thought to regulate energy metabolism by increasing insulin sensitivity and glucose uptake in skeletal muscle and adipocytes

TABLE 2 | Myokines for which there was no statistically significant pregnancy by time interaction, but there was a statistically significant main effect of time.

			Pregnant		Non-pregnant	
Myokine	**p-value**	**F-value**	**Pre-exercise mean ± SD**	**Post-exercise mean ± SD**	**Pre-exercise mean ± SD**	**Post-exercise mean ± SD**
SPARC	0.005	9.6	1465435 ± 1846666	2491219 ± 4172003	1390862 ± 1083435	2809113 ± 2511117
Fractalkine	<0.0001	20.6	6210 ± 1481	6639 ± 1514	5658 ± 1165	6165 ± 1256

Pre- and post-exercise mean concentrations of myokines presented in pg/mL. SD, standard deviation.

TABLE 3 | Post-exercise response of myokines detected in serum of pregnant and non-pregnant women following the acute bout of treadmill walking.

Myokine	Pregnant women	Non-pregnant women	All women (regardless of pregnancy status)
FGF21	↑	–	**n/a (i)**
EPO	↑	↓	**n/a (i)**
SPARC	*n/a (n/i)*	*n/a (n/i)*	↑
Oncostatin	–	↑	**n/a (i)**
FABP-3	–	↑	**n/a (i)**
FSTL-1	–	↑	**n/a (i)**
Fractalkine	*n/a (n/i)*	*n/a (n/i)*	↑
IL-15	↑	–	**n/a (i)**
BDNF	↑	↑	**n/a (i)**

Post-exercise response of myokines with a statistically significant pregnancy status and time interaction with subsequent multiple comparison analysis (FGF21, EPO, Oncostatin, FABP-3, FSTL-1, IL-15, and BDNF) are depicted in bold font. Myokines without a significant interaction but with a statistically significant time effect, pre- versus post-exercise (SPARC and fractalkine), are depicted in italic font. ↑, increase; ↓, decrease; –, no significant change; n/a (i), not applicable because of statistically significant interaction; n/a (n/i), not applicable because absence of statistically significant interaction.

(Ge et al., 2011; Mashili et al., 2011; Salminen et al., 2017). Based on our data illustrating an exercise-induced increase in circulating FGF21 levels in pregnant women, we hypothesize that this myokine may enhance glucose uptake and be an important mediator in decreasing the risk of developing gestational diabetes mellitus (GDM; glucose intolerance and insulin resistance in pregnancy). In support of this hypothesis, research shows that exercise interventions decrease the odds of developing GDM (Davenport et al., 2018c).

Increases in circulating EPO, a molecule known to stimulate erythropoiesis (Byts and Sirén, 2009), as a result of exercise in pregnancy, may be playing a role in blood volume adaptation. Blood volume can increase up to 50% during pregnancy compared to the pre-pregnancy period (Soma-Pillay et al., 2016). Thus, elevated EPO may be necessary to induce an increase in red blood cell production. On the contrary, BDNF is a molecule that plays an important role in the regulation of the nervous system as it is in part responsible for the maintenance, development, and survival of neuronal cells (Pedersen, 2009). Low circulating levels of BDNF have been associated with a plethora of diseases such as obesity, type 2 diabetes, depression, and cognitive impairments (Pedersen, 2009). In the context of pregnancy, low serum BDNF has been associated with antenatal depression (Fung et al., 2015) and increased risk of low birth weight (Christian et al., 2016). Increased levels of BNDF, made possible by exercise, may be important to promote the health of the nervous system.

Of the other myokines shown to increase in pregnant women post-exercise, IL-15 is known to increase trophoblast invasion and migration (Zygmunt et al., 1998). Moreover, IL-15 has been shown to play a role in muscle growth (Brandt and Pedersen, 2010) and the reduction of adipose tissue mass (Nielsen and Pedersen, 2008). Thus, increased IL-15 following exercise may help facilitate appropriate GWG corresponding to the IOM guidelines (American College of Obstetricians and Gynecologists, 2013). In summary, the increase in certain myokines following exercise may contribute to the optimization of maternal-fetal health and future work should aim to explore this hypothesis.

Brisk walking was chosen as the exercise modality for this study as it is a recommended moderate-intensity PA during pregnancy (Mottola et al., 2018). Since the walking session was of 'relative' intensity and our groups did not differ based on anthropometric and baseline characteristics (**Table 1**), it is particularly interesting that both groups demonstrated different myokine response profiles. A possible explanation as to why pregnant women are not exhibiting a change in certain circulating myokines compared to the non-pregnant group following a bout of MPA is the speed at which they were walking. Non-pregnant women were able to reach a higher maximal speed and averaged a higher speed than their non-pregnant counterparts, for the same relative intensity (**Table 1**). Furthermore, as illustrated in **Figure 3**, maximal speed reached in the pregnant group was inversely correlated to both GWG and percentage of upper-limit of recommended GWG, based on the IOM guidelines (Committee on Obstetric Practice, 2014). These results suggest that although the exercise session was of comparable relative intensity, and that the pregnant and non-pregnant groups did not differ in variables that would influence target HR zone intensity, such as age and RHR, the weight that pregnant women gain across pregnancy is likely hindering their ability to reach the higher speeds. Thus, it is possible that lower speed would translate to an insufficient muscle fiber recruitment or stimulation needed to elicit an observable change in a more substantial number of circulating myokines, in the pregnant participants. It is also conceivable that a higher intensity is necessary to stimulate myokine synthesis and release. For instance, circulating IL-6 is consistently shown to increase post-exercise, however, in this study, it remains unchanged in both the pregnant and non-pregnant groups following the walking session which may be attributed to the intensity of the exercise (Leal et al., 2018; Garneau and Aguer, 2019).

The myokine secretome is vast, complex, and the release of distinct myokines seem to be dependent on specific muscular stimulus. In light of this complexity, studies examining the same myokine report contradictory results. For instance, serum secreted protein acidic and rich in cysteine (SPARC) levels have been showed to increase following a 30-min aerobic bout of cycling in healthy young men (Aoi et al., 2013). In contrast, reports indicate no change in serum SPARC levels following a brief bout of supramaximal cycle sprint (Songsorn et al., 2017). These discrepancies are likely related to variations in experimental protocols. Exercise type or modality, intensity, and duration are all factors that could influence the rate of synthesis and thus, the release of detectable myokines in the bloodstream (Leal et al., 2018). Variables such as nutritional status, environmental conditions (Rai and Demontis, 2015) and disease states (Kurdiova et al., 2014) may also be other elements influencing myokine response. The duration of the exercise session in this study is one variable that may be contributing to the differing myokine response post-exercise between both groups. Pregnant women exercised for a significantly shorter

period (40-min vs. 42-min) compared to non-pregnant women (**Table 1**). Although this result was not by design, it indicates that the acclimation phase, during which the speed is increased every 1-min interval until the target heart rate range is met, was shorter for pregnant women who reached the desired intensity more rapidly. Thus, some myokines may require a longer duration rather than a higher intensity of exercise in order to be synthesized and released into the bloodstream.

While exercise session parameters may account for a proportion of the difference observed in the myokines released by the pregnant compared to the non-pregnant women, pregnancy-specific responses could also be at play. For instance, FGF21 increases exclusively in the pregnant group following the acute bout of exercise. Among the many physiological adaptions during pregnancy, cardiac hypertrophy occurs thereby accommodating the transient increase in blood volume (Li et al., 2012; Redondo-Angulo et al., 2017). Redondo-Angulo and colleagues explain that FGF21 is a key molecule responsible for cardiac remodeling during pregnancy. Thus, the increase of some and lack of change in other myokines in the pregnant compared to the non-pregnant women may have implications related to the physiological adaptations required as a result of pregnancy.

We also compared baseline myokine levels in the pregnant and the non-pregnant participants, as differences in baseline concentrations could potentially help clarify the main result. Of the 10 myokines studied, three demonstrated differences between pregnant and non-pregnant at baseline. Interestingly, pregnant participants had higher baseline levels of EPO and oncostatin, while FGF21 was lower. Regarding oncostatin, our results are consistent with research by Ogata et al. (2000) who demonstrated a higher level of serum oncostatin in pregnant versus non-pregnant women, attributed to production by decidual and chorionic tissues (Ogata et al., 2000). In line with these findings, oncostatin has been identified as a member of a cytokine family that plays a role in cellular differentiation (Rose and Bruce, 1991), a vital cellular process during pregnancy (Malassiné and Cronier, 2002) which could clarify the increased levels observed in pregnant individuals. Also, the known elevation in blood volume during pregnancy provides logical reasoning for higher levels of EPO in pregnant women. In brief, although myokines are synthesized and secreted by skeletal muscle; similar molecules are also regulated via other tissues and organs, regardless of PA engagement. Likewise, cardiovascular adaptions during pregnancy are particularly relevant when considering serum myokine levels. Blood volume increases disproportionately during pregnancy as plasma volume exhibits a more significant increase compared to red blood cell mass, creating a concept known as hemodilution (Costantine, 2014; Soma-Pillay et al., 2016). Thus, it is possible that blood volume adaptations and fluctuations in the serum proteome across gestation may influence observed myokine levels in pregnant women (Romero et al., 2017).

Pregnancy is undoubtedly a critical period for the health of both the fetus and the mother. While it is well established that maternal PA is essential for the short- and long- term health of mom and baby, the pathways and mediators involved in the crosstalk between skeletal muscle, the placenta and subsequently the fetus are mostly unknown. The results of this study propose four possible mediators: FGF21, EPO, BDNF, and IL-15. Recognizing the preliminary nature of this work focusing on a panel of well-characterized myokines, other myokines are likely being released during pregnancy that were not examined, and various exercise parameters may elicit differing circulatory responses in this particular population.

Limitations

Although our study had a similar sample size to other studies examining myokines post-exercise, such as six (Steensberg et al., 2002) and nine males (Aoi et al., 2013), given the inter-individual variability in our pregnant group, a larger sample size could potentially translate to differences in myokines that remained unchanged. However, the lack of information and published results on myokines in pregnant women did not allow us to perform a power calculation to determine an appropriate sample size. Additionally, a measure of fitness would have been helpful in providing insight as to the role of chronic exercise on myokine release. However, obtaining such a measure in our pregnant population would have required multiple visits and in turn render the study less feasible.

Future Directions

Our results warrant further investigation of the role of myokines in pregnancy. As this was an initial characterization of the myokine profile following a single bout of moderate-intensity walking, follow-up work is needed to understand whether myokine signaling is a vital part of placental and fetal health optimization. Future studies should aim to investigate whether different exercise modalities, such as cycling, swimming, or resistance training, which are all deemed safe during pregnancy, influence the myokine response in pregnant women. The exercise duration chosen for this study was based on current guidelines for PA during pregnancy (Mottola et al., 2018) and ranged from 36 to 43 min for the pregnant participants. Longer exercise durations or bouts of higher intensity may elicit different responses. Examination of the relationship between objectively measured PA level (volume) during pregnancy and the myokine response would also be valuable. Further exploration of these variables would allow us to identify if prior fitness level or chronic exposure to exercise influences circulating myokines following PA.

CONCLUSION

This novel study found that walking at moderate-intensity between 36 and 43 min elicited a change in four of the ten myokines measured in the pregnant participants and five of the ten myokines in non-pregnant controls, while two myokines increased post-exercise regardless of pregnancy status. Future studies should aim to explore whether the myokines shown to

be elevated post-exercise in the pregnant group of this study are involved in the molecular mechanism by which maternal, fetal and placental health is optimized as a result of PA engagement.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Research Ethics Board at the University of Ottawa (file number: H-06-18-634) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by Research Ethics Board at the University of Ottawa.

AUTHOR CONTRIBUTIONS

KH drafted the manuscript. KH and SM primarily performed data collection and designed the study. KM, LG, and CA secondarily performed the data collection. All authors contributed to the design of the study, revised and edited the manuscript, and read and approved the final version of the manuscript.

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REVIEW
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Neuromuscular Electrical Stimulation: A New Therapeutic Option for Chronic Diseases Based on Contraction-Induced Myokine Secretion

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Myokines are peptides known to modulate brain neuroplasticity, adipocyte metabolism, bone mineralization, endothelium repair and cell growth arrest in colon and breast cancer, among other processes. Repeated skeletal muscle contraction induces the production and secretion of myokines, which have a wide range of functions in different tissues and organs. This new role of skeletal muscle as a secretory organ means skeletal muscle contraction could be a key player in the prevention and/or management of chronic disease. However, some individuals are not capable of optimal physical exercise in terms of adequate duration, intensity or muscles involved, and therefore they may be virtually deprived of at least some of the physiological benefits induced by exercise. Neuromuscular electrical stimulation (NMES) is emerging as an effective physical exercise substitute for myokine induction. NMES is safe and efficient and has been shown to improve muscle strength, functional capacity, and quality of life. This alternative exercise modality elicits hypertrophy and neuromuscular adaptations of skeletal muscles. NMES stimulates circulating myokine secretion, promoting a cascade of endocrine, paracrine, and autocrine effects. We review the current evidence supporting NMES as an effective physical exercise substitute for inducing myokine production and its potential applications in health and disease.

Keywords: electrotherapy, cytokines, transcutaneous electrical nerve stimulation, physical exercise, skeletal muscle contraction

INTRODUCTION

Functions classically attributed to skeletal muscle are movement and maintenance of posture, protection of vital organs, stimulation of blood and lymphatic circulation, and activation of metabolic pathways as the consequence of the large amount of energy consumed. More recently, this perspective has broadened, as contracting skeletal muscles have been shown to release molecules responsible for signal transmission to other tissues (Hawley et al., 2014). These molecules

were referred to as "the work stimulus," "the work factor" or "the exercise factor" (Pedersen et al., 2003). It was originally hypothesized that this exercise factor could be potassium ions, lactic acid, adenosine, interleukin (IL)-6 or other metabolites, until Pedersen et al. (2003) suggested in 2003 that cytokines – produced and released by skeletal muscles contraction and exerting their effects on other organs – should be named "myokines."

Myokines are recognized as potential candidates to manage metabolic diseases through their capacity to modulate fuel oxidation, hypertrophy, angiogenesis, inflammatory processes, and extracellular matrix regulation (Leal et al., 2018; Piccirillo, 2019). Myokines may also influence the onset and the course of other diseases through their endocrine functions, as they interplay with body weight regulation, inflammation, insulin sensitivity, tumor growth, and cognitive function (So et al., 2014; Carson, 2017; Hoffmann and Weigert, 2017). Thus, myokines may exert beneficial effects on metabolic syndrome-related disorders such as obesity, insulin resistance and type-2 diabetes, dyslipidemia; cardiovascular diseases such as hypertension and coronary heart disease; breast and colon cancer; and neuropsychiatric diseases such as Alzheimer's, Parkinson's and depression (Sanchis-Gomar and Perez-Quilis, 2014; Sanchis-Gomar et al., 2014; Pareja-Galeano et al., 2015).

Myostatin was the first myokine identified in 2008 (Allen et al., 2008), and IL-6 was the first myokine found to be secreted into the bloodstream in response to muscle contraction (Pedersen and Febbraio, 2008). Since then, several hundred myokines have been described, including cytokines, small proteins (∼ 5–20 kDa) and proteoglycan peptides produced and released by contracting muscle cells via secretion of proteins that signal between muscle and the rest of the body (Pedersen et al., 2007; Catoire et al., 2014). Thus, these peptidic molecules are expressed, produced, and released by muscle fibers which thus exert autocrine, paracrine, and/or endocrine effects (Pedersen et al., 2003). The autocrine, paracrine and/or endocrine (systemic) actions of myokines occur at picomolar concentrations (Pedersen and Febbraio, 2012; Pedersen, 2013). The autocrine and paracrine effects of myokines are mainly involved in the regulation of muscle physiology, muscle growth or lipid metabolism. However, myokine receptors have been identified in different tissues and organs, including the muscle itself, adipose tissue, liver, pancreas, bone, brain, heart, vessels, and immune cells, thereby modulating a myriad of functions (Pedersen et al., 2007; Lee and Jun, 2019).

Plasma levels of most myokines depend on the amount of contracted muscle mass and are hence strongly related to the amount of muscle mass exercised (Pedersen and Febbraio, 2008; Hody et al., 2019). For instance, IL-6 production is sensitive to exercise intensity (Ostrowski et al., 2000), an indirect measure of the muscle mass involved in contractile activity (Pedersen and Febbraio, 2008). Contracting skeletal muscle is an important source of plasma IL-6 (Steensberg et al., 2000; Fischer et al., 2004), and exercise involving a limited muscle mass (e.g., upper limb muscle) may be insufficient to significantly increase plasma IL-6 levels (Nosaka and Clarkson, 1996; Hirose et al., 2004; Bergfors et al., 2005; Pedersen and Febbraio, 2008). The sharpest increase in plasma IL-6 is typically observed in running, which involves several large muscle groups (Fischer, 2006; Pedersen and Fischer, 2007). On the other hand, although IL-8 mRNA increases up to 10-fold in response to exercise and up to twofolds with a pharmacological cocktail (palmitate, forskolin, and ionomycin) to mimicking exercise-stimulated contractions *in vitro* (Covington et al., 2016), circulating IL-8 increases only transiently after exhaustive exercise, suggesting that this myokine acts only locally in an autocrine/paracrine fashion (Nielsen and Pedersen, 2007). In this regard, neuromuscular stimulation of cultured human primary skeletal muscle cells (hSkMCs) increases IL-8 secretion by muscle cells (Scheler et al., 2013). The production of the myokine brain-derived neurotrophic factor (BDNF) is stimulated by some types of physical exercise. Acute aerobic exercise leads to increased BDNF plasma levels in an intensity-dependent manner, whereas acute strength exercise does not elicit this effect (Knaepen et al., 2010; Huang et al., 2014). Thus, rest periods between efforts, relative intensity and a limited amount of muscle mass mobilized and contracted simultaneously during strength exercise could limit the production of this and other myokines (**Table 1**).

Active skeletal fibers produce and release several myokines that act as hormones (Carson, 2017). These myokines released into the bloodstream exert well defined specific endocrine effects in different organs. This endocrine function of skeletal muscle may underlie numerous health benefits such as maintaining adequate body weight, reducing low-grade inflammation typical of chronic diseases, improving insulin sensitivity, protecting from tumor growth, and improving cognitive function. Therefore, physical exercise in which large muscle groups are mobilized at sufficient intensity and duration may produce benefits from a modulation of circulating myokines (He et al., 2018).

TABLE 1 | List of myokines potentially induced by muscle contraction and regular exercise (So et al., 2014; Schnyder and Handschin, 2015; Lightfoot and Cooper, 2016; Garneau and Aguer, 2019).

- Angiopoietin-like 4 (ANGPTL4)
- Apelin
- β-aminoisobutyric acid (BAIBA)
- Brain-derived neurotrophic factor (BDNF)
- Chemokine ligand and chemokine (C-X-C motif) ligand family
- Decorin
- Fibroblast growth factor 21 (FGF21)
- Interleukin-6 (IL-6)
- IL-8
- IL-10
- IL-13
- IL-15
- IL-18
- Irisin (FNDC5)
- Musclin
- Myonectin - C1q tumor necrosis factor a-related protein isoform 5 (C1QTNF5)
- Myostatin
- Leukemia inhibitory factor (LIF)
- Secreted protein acidic rich in cysteine (SPARC)
- Tumor necrosis factor-alpha (TNF-α)

Exercise also increases circulating small vesicles and exosomes as well as extracellular vesicles-packaged proteins involved in several biological functions (Whitham et al., 2018). Importantly, extracellular exosomes and extracellular matrix proteins might also be classified as myokines, particularly the extracellular matrix protein tenascin C, which is produced after electrical stimulation (Crameri et al., 2007) and affect muscle healing and regeneration (Fluck et al., 2008). However, the physical and metabolic limitations of some individuals will prevent them from undertaking physical exercise of sufficient intensity or duration to trigger such a myokine response.

Neuromuscular electrical stimulation (NMES) is based on applying in the transcutaneous electrical currents to a group of muscles, stimulating it to contract (Veldman et al., 2016). This method is usually employed as a passive "substitute" of dynamic training and acts as an "exercise emulator." In fact, NMES can activate PGC-1α (the master regulator of mitochondrial biogenesis activated by endurance exercise) as well as the target of rapamycin (mTOR), which in turn activates insulin and IGF-1 receptors (Atherton et al., 2005). Accordingly, this strategy could be particularly useful in patients with paraplegia, tetraplegia, obesity and limited mobility, frail elderly, or any person needing prolonged bed rest.

This review describes the most relevant *in vivo* research findings linking NMES and endocrine myokine expression, revealing NMES as an ergo-mimetic agent, and discusses how this method of stimulating the production of plasma myokines can exert a beneficial effect on the pathophysiology of several conditions in patients with limited mobility. We have particularly focused on myokines for which sufficient scientific evidence is currently available.

MYOKINE PRODUCTION THROUGHOUT NEUROMUSCULAR ELECTRICAL STIMULATION: A REVIEW OF *IN VIVO* EVIDENCE

Neuromuscular electrical stimulation may act as an efficient protector of muscle competence when subjects are unable or unwilling to engage in resistance or aerobic training programs. In accordance with this hypothesis, superimposed muscle contraction produced by electrical stimulators was found to enhance functional capacity in heart failure (HF) patients tested using the 6-min walk test (Nuhr et al., 2004; Karavidas et al., 2006). Similar results emerged from a meta-analysis of functional electrical stimulation in patients with chronic HF (Sbruzzi et al., 2010). Thus, NMES improved functional capacity (measured as VO_{2peak}) in HF patients to a similar extent as conventional aerobic training. The greatest improvements in the NMES group were detected in patients with a lower exercise capacity (Deley et al., 2008). A Cochrane systematic review (Jones et al., 2016) considered NMES a valid therapeutic intervention to improve muscle weakness in adults with conditions such as chronic obstructive pulmonary disease (COPD), chronic respiratory disease, chronic HF, or thoracic cancer. Likewise, NMES could be useful during recovery from injury (Caggiano et al., 1994; Wall et al., 2015), since it increases antioxidant capacity and decreases redox imbalance caused by disuse (Gondin et al., 2011a,b; Pellegrino et al., 2011).

INTERLEUKINS

NMES behaves as a powerful stimulus to skeletal muscle with systemic consequences when undertaking considered at low to moderate workload. Compared to groups in which the only intervention was active cycling or passively applied NMES, cycling plus NMES produced the greatest increases in plasma IL-6 levels immediately and 30 and 60 min after the intervention, showing a significant interaction effect (intervention*time) (partial $\eta^2 = 0.55$; power = 0.99) (all $p < 0.001$) (Wahl et al., 2015).

The duration of exercise seems to be the most critical factor regulating the amplitude of the systemic IL-6 response. Since the intervention time was exactly the same for the three groups, these results may arise from the larger amount of muscle mass engaged in the intervention combining NMES and active cycling (Fischer, 2006).

Increased peripheral blood levels of IL-6 were measured after a single 30-min NMES session in healthy participants receiving bilateral lower extremity muscle stimulation in the quadriceps, tibialis anterior and gastrocnemius. This intervention produced a significant increase in peak IL-6 from the mean pre-NMES value [0.65 (0.89) to 1.04 (0.89) pg ml^{-1}, $P = 0.001$], and a significant decrease in interleukin-10 (IL-10) [0.08 (0.07) to 0.02 (0.02) pg ml^{-1}, $P = 0.041$] and TNF-α [2.42 (0.54) to 2.16 (0.59) pg ml^{-1}, $P = 0021$]. Significantly higher mean values of IL-6 were also observed after NMES throughout the full 120-min period (Truong et al., 2017).

Although the magnitude of change was not impressive, these results are in line with similar responses to exercise observed in healthy adults (Greiwe et al., 2001; Steensberg et al., 2002). Truong et al. (2017) demonstrated a clear relationship between exercise-induced release of IL-6 and TNF-α, thus supporting its putative anti-inflammatory role. Specifically, IL-6 seems to exert its anti-inflammatory actions during low to moderate exercise, through induction of IL-1ra transcription, which in turn inhibits the pro-inflammatory cytokine IL-1. Moreover, IL-6 increases the production of IL-10, which inhibits lipoprotein saccharide-stimulated production of the pro-inflammatory cytokines TNF-α, IL-1α, and IL-1β (Steensberg et al., 2003; Fischer, 2006).

Chronic conditions such as COPD may lead to a pro-inflammatory state. In these situations, IL-6 may be increased in the presence of increased TNF-α expression. Akar et al. (2017) studied COPD exacerbation during intubation in hospital, and found significantly reduced plasma IL-6 levels after a rehabilitation program based on NMES and active mobilization [5.70 (1.70–13.70) – 1.20 (0.50–2.70) ($P = 0.015$)], and on NMES alone [3.35 (0.70–14.18) – 1.20 (0.50–6.70) ($P = 0.068$)]. The intervention group only undergoing active mobilization showed increased IL-6 levels without significant differences (Akar et al., 2017). Similarly decreased levels of IL-8 were

TABLE 2 | Summary of the different NMES protocols most commonly used to evaluate circulating myokine's secretion in humans.

Study	Type	Frequency	Pulse width	Intensity	Time	Total number of sessions	Stimulated zone	Others
Dirks et al., 2014	Biphasic symmetrical rectangular-wave pulses	Warm-up: 5Hz Stimulation period: 100Hz Cooling down phase: 5Hz	Warm-up: 250 μs Stimulation period: 400 μs Cooling down phase: 250 μs	Subjects set the intensity of the stimulation to a level at which full contractions of m quadriceps femoris were visible and palpable	Warm-up: 5′ Stimulation period: 30' Cooling down phase: 5′	10 sessions (2 sessions per day/5-day period)	Self-adhesive electrodes placed on the distal part at the m. rectus femoris and the m. vastus lateralis	Volunteers were subjected to 5 days of one-legged knee immobilization
Wahl et al., 2015	Bipolar rectangular pulse continuously applied	60 Hz	400 μs	Progressively increased at maximum tolerated	60′	One unique session	Circular electrodes around the thigh and the calf and laminar electrodes in the gluteal zone	The electrical stimulation was continuous and independent from the cycling
Akar et al., 2017	Symmetrical biphasic squared waveform, pulsed (ramp- up 1.5 s, 6 s duration contraction and 0.75 ramp-down; the rest time was not reported and the duty-cycle rest was unknown)	50 Hz	Not reported	Until visible contraction was obtained, 20-25 mA (depending on patient tolerance).	Not reported	20 sessions (5 days per week, 4 weeks).	bilateral upper extremity (deltoid) and bilateral lower extremity (quadriceps).	Volitional contraction was not allowed for the patients
Dalise et al., 2017	Interference wave at an amplitude-modulated frequency of 20 Hz, on-off ratio 4.5:4.5 s (ramp-up 1 s and ramp-down 0.5 s)	2000 Hz	50 μs	Progressively increase to the highest tolerated intensity during the experiment	20′	One unique session	Bilateral lower limbs (quadriceps) were stimulated alternately	–
Truong et al., 2017	Pulsed asymmetrical biphasic waveform (2 s ramp-up, 5 s duration contraction and <1 s ramp-down, 8/18 duty-cycle)	50 Hz	400 μs for quadriceps and 250 μs for tiabialis anterior and gastrocnemius	Until visible contractions were obtained	30′	One unique session	Quadriceps, tibialis anterior and gastrocnemius	Electrical stimulation of the tibialis anterior and gastrocnemius alternated to stimulate physiologic volitional contraction in order to prevent discomfort

(Continued)

TABLE 2 | Continued

Study	Type	Frequency	Pulse width	Intensity	Time	Total number of sessions	Stimulated zone	Others
Maekawa et al., 2018	Pulsed rectangular waveform (3 s stimulation time and 7 s rest period Duty-cycle: 3/10)	100 Hz	1000 μs	3 to 5 V	10 contractions per set, 5 sets with an interval of 3'	One unique session	Triceps surae muscle.	–
Miyamoto et al., 2018	Pulsed rectangular monophasic wave form	4 Hz	250 μs	Progressively increased until maximum tolerated	30' (single bout of NMES) and 40' for the 8 weeks protocol	One unique session and 5 days per week, for 8 weeks (40 sessions), respectively	Circular electrodes were used around the thigh and the calf and laminar electrodes were placed in the gluteal zone	–
Kimura et al., 2019	Interference wave at an amplitude-modulated frequency of 20 Hz, on-off ratio 4.5:4.5 s (ramp-up 1 s and ramp-down 0.5 s)	2000 Hz	0.05 ms	Progressively increase to the highest tolerated intensity during the experiment	20'	One unique session	Bilateral lower limbs (quadriceps) were stimulated alternately	–

found in a NMES and active mobilization group [13.64 (1.47–23.70) – 2.35 (0.80–17.63) (P = 0.017)], and a NMES alone group [6.13 (2.35–25.00) – 3.92 (0.80–17.63) (P = 0.017)]. This time, the active mobilization intervention group showed no significant differences in IL-8. Although no significant difference was reported, NMES and active mobilization, as well as NMES alone, were effective in increasing IL-10, whereas the active mobilization alone group showed reduced IL-10 values. This downregulation of IL-6 and IL-8, and upregulation of IL-10, could be consequent to a reduced proinflammatory state mediated by muscle contraction.

BRAIN-DERIVED NEUROTROPHIC FACTOR

Miyamoto et al. (2018) carried out several studies in healthy subjects and those with type 2 diabetes mellitus. These authors reported significantly improved plasma BDNF concentrations after an 8-week period of NMES training and after a single bout of NMES, respectively. In this study, a single 30 min bout of NMES significantly increased plasma BDNF levels (pre-NMES: 150.5 ± 126.7 vs. post-NMES: 250.5 ± 131.1 pg mL^{-1}; $p = 0.017$); this effect was similar to that observed in subjects completing a 30-min cycling ergometer exercise test at 60% VO_{2peak} (post-NMES: 250.5 ± 131.1 vs. post-exercise: 268.6 ± 123.8 pg · mL^{-1}; $p = 0.908$). However, this acute response was not associated with an improvement in cognitive function (Miyamoto et al., 2018). The 8-week protocol of NMES training in subjects with type 2 diabetes was able to induce a significant increase in plasma BDNF (pre-NMES: 117.0 ± 40.4 vs. post-NMES 245.5 ± 51.2 pg/ml; $p = 0.026$) compared to participants allocated to the control group and who showed a decline in plasma BDNF during the 8-week period without NMES. The NMES intervention also induced a greater reduction in the body fat percentage and fasting glucose concentrations than in the control group (Miyamoto et al., 2018).

Kimura et al. (2019) compared voluntary exercise and NMES-induced muscle contraction with the same integrated force measured using electro-myographic technology, and observed that the increase in serum BDNF in the NMES group was higher than that in the voluntary exercise group (18625.6 ± 4173.5 pg/ml, $p = 0.003$ vs. 15103.0 ± 4177.9 pg/ml, $p = 0.004$). NMES could therefore be even more effective than active exercise using the same integrated force to increase serum BDNF.

Several animal experiments have also examined the relationship between BDNF and exercise. In 2017, Dalise et al. (2017) obtained surprising results in Sprague-Dawley rats. These authors compared serum myokines such as vascular endothelial growth factor-A (VEGF-A), insulin-like growth factor-1 (IGF-1), Klotho (i.e., an anti-aging single-pass membrane protein), and BDNF produced in response to different intensities (low, medium, or high) of active exercise and analogous NMES interventions. NMES did not modify IGF-1 levels yet led to a modest increase in plasma Klotho concentrations in the low- and high intensity interventions. Notably, after a medium-intensity session of NMES, serum BDNF underwent a dramatic eightfold increase ($p = 0.01$).

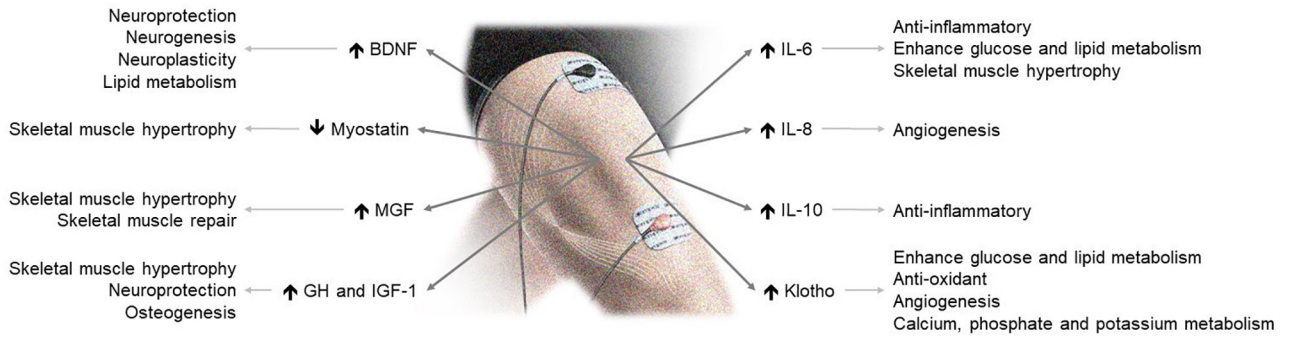

FIGURE 1 | NMES-induced myokine production and its effects on health. BDNF, brain-derived neurotrophic factor; GH, growth hormone; IGF-1, insulin-like growth factor 1; MGF, mechano-growth factor.

Maekawa et al. (2018) found that 50 repeated maximal electrically evoked-isometric contractions in unconscious rats were effective at increasing BDNF protein expression and activate its hippocampus receptor. These findings could provide reliable evidence of an alternative means of communication between muscle and different organs, additional to endocrine interactions.

MYOSTATIN

Myostatin is an inverse modulator of muscle mass in animals and humans (McPherron and Lee, 1997; Schuelke et al., 2004; Mosher et al., 2007), inhibiting mTOR signaling (Rodriguez et al., 2011). Wall et al. (2012) showed that the expression of myostatin mRNA declined significantly after 60 min of NMES in the lower limbs, coinciding with a significant increase in MyoD mRNA expression. These results are consistent with an anabolic stimulus following a bout of resistance exercise. Dirks et al. (2014) reported that the expression of myostatin mRNA was lower after NMES compared to baseline or a control group not receiving electrical stimulation. The expression of MyoD mRNA was also increased after NMES compared to values recorded at baseline and in the non-NMES group.

GH AND IGF-1 SIGNALING

In an experimental study in rats subjected to sciatic neurectomy to reproduce adverse effects such as disuse amyotrophy and cortical bone loss (Feng et al., 2016), 30-min NMES sessions 5 days per week for 9 weeks downregulated mRNA expression levels of myostatin and upregulated those of mechano-growth factor (MGF) and insulin-like growth factor 1 (IGF-1).

In a treadmill study in Sprague-Dawley rats, resistance training (based on superimposed NMES-induced isometric contraction) led to increased and maintained IGF-1 and GLUT-4 translocation compared to aerobic exercise, suggesting a potential role of NMES resistance training as a regulator of glucose metabolism (Kido et al., 2016).

The growth hormone (GH) response to exercise is a driving force of anabolic protein synthesis linked to strength and muscle mass enhancement. NMES is a valid and efficient tool to stimulate a hormone response in healthy subjects (Aldayel et al., 2010; Wahl et al., 2014). Collectively, evidence so far suggests that NMES mediates a protective effect on muscular structural integrity and functional capacity via autocrine and paracrine mechanisms.

DOSE-EFFECT RELATIONSHIP BETWEEN CONTRACTION INTENSITY AND MYOKINES

Unfortunately, no information exists on a minimal contraction and/or number of sessions needed to stimulate myokines' secretion. However, although at present there are no precise data about the dose-response relationship between NMES-induced contraction intensity and myokines secretion, higher intensities of muscle contraction provoked by NMES improved muscle function in patients after an anterior cruciate ligament reconstruction (Snyder-Mackler et al., 1994). There is also a linear dose-response relationship between the increase in energy expenditure and the intensity of NMES in healthy subjects (Hsu et al., 2011) as well as with muscle function in patients with rheumatoid arthritis (Almeida et al., 2018). Likewise, a linear dose-response relationship was observed between NMES intensity and quadriceps strength and voluntary activation in subjects who received NMES after total knee arthroplasty, although there was no evidence of an association with muscle cross-sectional area (Marmon and Snyder-Mackler, 2011). Therefore, it is likely that there is a dose-effect relationship between NMES intensity and other variables, but additional investigations are still needed to elucidate the dose-effect relationship between NMES intensity-frequency-duration-muscle/s and myokines' secretion.

POTENTIAL DRAWBACKS AND LIMITATIONS OF USING NMES

NMES-provoked muscle damage characterized by histological alterations in muscular and connective tissue, creatine kinase

(CK) activity increases, declines in muscle strength, and delayed onset muscle soreness has been recently reported (Nosaka et al., 2011). Several cases of rhabdomyolysis induced by NMES have been reported (Guarascio et al., 2004; Kastner et al., 2015; Johannsen and Krogh, 2019). NMES might increase myostatin (also named GDF-8; growth/differentiation factor 8), an inhibitor of skeletal muscle growth, and GDF-15 (growth differentiation factor 15), suggesting thus that excessive NMES could damage muscles (Bloch et al., 2014). Nevertheless, it seems that pre-conditioning muscles by isometric contractions or submaximal eccentric might attenuate NMES-provoked muscle damage (Nosaka et al., 2011).

On the other hand, a rehabilitation program including NMES must be accompanied by functional task to guarantee the eventual success of the intervention (Azman and Azman, 2017). Although there is controversy regarding the additional benefits of "superimposed" NMES in trained subjects vs. voluntary exercise alone, the former could be effective in untrained subjects or in patients who cannot mobilize appropriately (Paillard et al., 2005). Superimposed NMES seems more effective than voluntary exercise alone for the prevention of muscle atrophy, maintenance of muscle oxidative capacity and prevention of strength loss, and to recover knee function and gait kinematics after ligament surgery (Eriksson and Haggmark, 1979; Snyder-Mackler et al., 1991). A large muscle mass and a higher strain to skeletal muscles than normal are needed to increase the secretion of certain myokines (Wahl et al., 2015): this is the reason why superimposed NMES might be more effective to induce a higher local muscle stimulus for myokine secretion. Finally, voluntary exercise is at least as beneficial as superimposed NMES, although the latter could produce additional benefits in weaker muscles (Hartsell, 1986).

MOST COMMONLY NEUROMUSCULAR ELECTRICAL STIMULATION PROTOCOLS USED IN HUMANS TO EVALUATE CIRCULATING MYOKINE'S SECRETION

Table 2 summarizes the most commonly NMES protocols used in human investigations. In general, NMES sessions last ∼20 to 60 min, with a stimulation frequency between 4 and 2000 Hz, pulse (biphasic rectangular pulses) duration of 50–1000 μs, with the highest tolerable intensity to maximize force production, performed every day in patients with activation neural deficits, and alternate days to produce hypertrophy in the affected muscle when the neural deficits have improved (Maffiuletti et al., 2018).

CONCLUSION

The available data show that NMES is safe and efficient to improve muscle strength, functional capacity and quality of life. Evidence mounts that NMES stimulates the secretion of circulating myokines with clinically relevant endocrine, paracrine and autocrine consequences (**Figure 1**). Accordingly, NMES may have many potential effects and applications in health and disease. Although further data are needed, including measurements of other central myokines (**Table 1**), current evidence suggests that NMES may be a useful physical exercise substitute for myokine modulation eliciting skeletal-muscle hypertrophy and neuromuscular adaptations.

LIMITATIONS

This review was not planned to be a comprehensive, systematic, and/or cumulative review of existing evidence regarding NMES pros and cons, but a narrative review aiming to give a broad overview on NMES clinical use and address whether there is enough scientifically valid evidence to recommend NMES as an effective physical exercise substitute or complement to induce myokine production, and delineate the potential applications of NMES in health and disease. Therefore, there was not a predefined protocol-based search method of the bibliography which might involve subjective selection bias. Unfortunately, there are not many scientific studies that specifically evaluate the effects of NMES on circulating myokine levels. For that reason, we have limited this review to certain myokines for which sufficient scientific evidence is currently available. Therefore, additional studies would be needed for particularly assessing the potential role of NMES in regulating the expression of a wider variety of myokines.

AUTHOR CONTRIBUTIONS

FS-G conceived and coordinated the writing of the manuscript. FS-G, SL-L, CR-M, NM, GL, and HP-G contributed to the writing of the manuscript. HP-G prepared the figure.

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Myokine Regulation of Insulin Secretion: Impact of Inflammation and Type 2 Diabetes

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Skeletal muscle (SkM) secretes protein factors (myokines) that can exert multiple actions. To study the control of myokine regulation of β-cell function, SkM biopsies were taken from non-diabetic (ND) and Type 2 diabetic (T2D) subjects and satellite cells cultured to myotubes (MT). MT were also treated with lipopolysaccharide (infectious inflammation – II) or a combination of glucose (10 mM), insulin (120 pM), and palmitate (0.4 mM) (metabolic inflammation – MI) to model the inflammatory and metabolic conditions seen *in vivo* with T2D. Conditioned media (CM) was collected from MT after 24 h and used to treat INS-1 cells for 24 h. Cell viability, total insulin content, glucose-stimulated insulin secretion (GSIS) and maximal (IBMX-stimulated) IS (IS_{max}) were monitored. Under baseline conditions, CM from ND and T2D MT had no effects on INS-1 cell viability, insulin content, GSIS, or IS_{max}. After exposure to II, CM from ND-MT augmented GSIS in INS-1 cells by 100 ± 25% over control ($p < 0.05$); T2D-CM had no effect. After exposure to MI, T2D-CM suppressed GSIS by 35 ± 5% ($p < 0.05$); ND-CM was without effect. Under either of these conditions cell viability, total insulin content and IS_{max} were unaffected. Effects of CM on GSIS were lost after CM was boiled. Both augmentation of GSIS by ND-CM from II-treated MT, and suppression by T2D-CM from MI-treated MT, were inhibited by wortmannin, Ro 31-8220, and SB203580. In summary: (1) ND-MT are able to augment GSIS when stressed, (2) T2D-MT responding to a diabetic-like environment secrete myokines that suppress GSIS, (3) Unknown protein factors exert effects specifically on GSIS, possibly through PI-3K, PKC, and/or p38 MAPK. In T2D, both insulin resistance and a suppression of adaptive increased insulin secretion are intrinsic properties of SkM that can contribute to the full T2D phenotype.

Keywords: inflammation, myokines, skeletal muscle, type 2 diabetes, insulin secretion

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INTRODUCTION

As the major insulin target tissue and the predominant site of post-prandial glucose disposal, insulin resistance in skeletal muscle (SkM) is a defining feature of type 2 diabetes (T2D) (DeFronzo and Tripathy, 2009). Beyond its role in regulated glucose disposal, evidence accumulated over the past 10–15 years has revealed that SkM can also be viewed as a secretory organ, releasing multiple

factors (reviewed in De Rossi et al., 2000; Whitham and Febbraio, 2016). Proteomic analysis of the SkM secretome has identified several hundred potential protein myokines, as well as metabolites (Rai and Demontis, 2016), miRNAs (Massart et al., 2016), and other factors, often delivered as cargo in exosomes (Jalabert et al., 2016). While some of these secreted factors are unique to SkM (e.g., myostatin), many are cyto- and chemokines and growth factors produced and released from multiple tissues (reviewed in Raschke and Eckel, 2013). These muscle-secreted factors have been demonstrated to exert autocrine, paracrine and endocrine actions on multiple tissues (reviewed in Pedersen and Febbraio, 2012).

One such endocrine action of myokines and other muscle secreted factors could be to influence islet health and function, as shown by the impact of exercise. Exercise training programs improved both insulin sensitivity and glucose responsiveness of insulin secretion in sedentary individuals, with the nature of the response depending on the intensity of effort (Rohling et al., 2016). Given the fact that the expression and secretion of multiple factors, including myokines are regulated by exercise (reviewed in Pedersen and Febbraio, 2012), it is reasonable to expect that some of these factors may be involved in the β-cell response to exercise, though the specific factors and pathways involved remain to be investigated. One mechanism by which exercise can improve β-cell function was revealed by the work of Ellingsgaard et al. (2011), which showed that IL-6, either administered directly or increased following exercise, acted on intestinal L cells and pancreatic α-cells to stimulate GLP-1 secretion, ultimately elevating insulin secretion. A similar β-cell protective role of IL-6 was seen in exercising mice (Paula et al., 2015). Conversely, many myokines are pro-inflammatory and could contribute to islet inflammation. Evidence for such direct communication between muscle and the β-cell was provided by Bouzakri et al. (2011), where insulin resistance was induced in human myotubes (MTs) by treatment with TNFα. Exposure of isolated β-cells to conditioned media (CM) from these cells both increased apoptosis and reduced glucose-stimulated insulin secretion (GSIS) (Bouzakri et al., 2011), creating a model of β-cell failure. Only the later response was dependent on TNFα, suggesting that other muscle-secreted factors were responsible for modulating β-cell mass.

Seeing that the secretion of multiple myokines, e.g., TNFα (Saghizadeh et al., 1996), MCP1 (DiGregorio et al., 2005), and IL6 (Munoz-Canoves et al., 2013), as well as miR-29 (Massart et al., 2017) by T2D muscle or myotubes have been reported to be altered compared to healthy individuals or cells derived from them (Ciaraldi et al., 2016) (reviewed in Garneau and Aguer, 2019), it is possible that some of these factors could contribute to regulation of β-cell mass and function in T2D. The importance of insulin resistance in adipose tissue and SkM as initial "hits" in the pathogenesis of T2D cannot be overstated. However, since preserving β-cell function is also crucial to preventing diabetes (DeFronzo and Abdul-Ghani, 2011), it is critically important to understand how insulin resistant fat and muscle can contribute to β-cell failure and, conversely, how "healthy" fat and muscle may help preserve normal β-cell mass and function. With that background in mind, the current study was designed to address two specific questions: (1) How might the demonstrated differences in the secretome of SkM from T2D individuals impact β-cell function and mass, and (2) How might the environment that SkM is exposed to in T2D influence that secretome and it's effects on the β-cell? We investigated these questions employing myotubes cultured from healthy and T2D individuals, which display differences in secretion of myokines and other factors, while also evaluating the impact of conditions designed to partially model the hormonal/metabolic milieu characteristic of T2D and the chronic, low-grade inflammation also observed in T2D (Olefsky and Glass, 2010).

MATERIALS AND METHODS

Cell culture materials were purchased from Irvine Scientific (Irvine, CA, United States) except for SkM growth medium and supplements, which were obtained from Lonza (Walkersville, MD, United States).

All other chemicals were reagent grade and purchased from Sigma Chemical (St. Louis, MO, United States). Electrophoresis reagents were from Bio-Rad (Richmond, CA, United States) or Invitrogen (Carlsbad, CA, United States). Primary antibodies were obtained from the following sources: IkBα (catalog #9242), phosphop38 MAPK (#9216), p38 MAPK (#8690), phospho-p44/42 MAPK (#4370), p44/42 (#4695), caspase 3 (#9665), phospho-S^{473}-Akt (#4051), Akt (#4685) (Cell Signaling Technology, Beverly, MA, United States); phospho-JNK (#sc-6254), JNK (#sc-571) (Santa Cruz Biotechnology, Santa Cruz, CA, United States); β-actin (#NB600-503) (Novusbio, Littleton, CO, United States). Fluorescently labeled secondary antibodies and blocking buffer were obtained from Licor (Licor, Lincoln, NE, United States). Protein controls for caspase 3 (Jurkat cell extracts treated ± cytochrome C) were from Cell Signaling.

Subjects

Samples of SkM were collected from 20 non-diabetic (ND) subjects and 22 T2D subjects. General inclusion criteria included: weight stable (± 2 kg) for 1 month and medication use stable for at least 3 months. Use of steroids and anti-depressants were cause for exclusion. Subjects were classified as ND based on fasting [glucose] < 100 mg/dL on screening and HbA1c < 5.7% within 2 months of biopsy. None of the subjects from the ND group had a family history of T2D. None of the women were taking hormonal replacement therapy. Blood was collected after an overnight (10–12 h) fast, serum prepared and stored at -80°C before analysis. Percutaneous needle biopsies of vastus lateralis muscle were performed and muscle tissue was immediately processed for culture.

Cell Culture and Treatments

Skeletal Muscle Cells

The procedure for the isolation, propagation and differentiation of SkM cells has been detailed elsewhere (Ciaraldi et al., 1995). Briefly, after enzymatic isolation, muscle satellite cells were grown in serum-free SkGM (Lonza) supplemented with

the bullet kit, omitting insulin. Cells were passed once into the required formats. After attaining 80–90% confluence, cells were fused for 5 days in α-MEM containing 2% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. After three washes with PBS, the media was replaced with serum-free α-MEM (0.1% BSA) containing glutamine, antibiotics and the indicated treatments. The infectious inflammation (II) condition was induced by treatment with lipopolysaccharide (LPS, Sigma #4391), added from a 1 mg/mL stock in serum-free α-MEM to attain a final concentration of 1 μg/mL. The metainflammation (MI) mix was made fresh from stocks of D-glucose ([final] = 10 mM), human recombinant insulin ([final] = 120 pM) and palmitate conjugated to fatty acid-free BSA (Sinha et al., 2004) ([final] = 400 μM), diluted in serum-free α-MEM. CM was collected after 24 h in culture, centrifuged (800 × *g*, 10 min, 4°C) to remove debris and stored at −80°C. Fresh media and treatments were added to the muscle cells and these were cultured an additional 24 h before protein extraction (see below).

INS-1 Cell Culture

INS-1 cells were grown in RPMI 1640, supplemented with 10% FBS, 10 mM HEPES, 2 mM L-Glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were split when they reached 100% confluence, every 3–4 days. For experiments, INS-1 cells were seeded in 24-well plates, and were grown to 100% confluence. MT-CM was concentrated 2-fold using a Centricon filter (mw cut-off 3000), and a 3:1 mixture of RPMI:MT-CM (glucose supplemented to a final concentration of 11 mM) was used to treat INS-1 cells for 24 h before further experimentation. Individual wells were exposed to CM collected from a single subject, performed in duplicate.

Media Controls

Given that MT-CM was generated with a different medium (serum-free αMEM) than that in which INS-1 cells are routinely cultured (RPMI 1640-10%FCS), it was necessary to determine the conditions under which INS-1 cells could be exposed to MT-CM. Twenty four hour exposure of INS-1 cells to a 3:1 mixture of RPMI 1640 and s/f-α-MEM concentrated two-fold (glucose supplemented to attain a final concentration of 11 mM), resulted in no change in overall cell viability, as monitored by total cell protein, LDH release, or caspase 3 cleavage, when compared to RPMI 1640 alone (**Supplementary Figure S1**). More importantly, while there was a modest increase in total insulin content, there were no changes in insulin release in the presence of either low or high [glucose] (not shown). Consequently, there were no differences in GSIS or maximally stimulated IS (IS_{max}) (**Supplementary Figure S1**). Moving forward, treatment of INS-1 cells with MT-CM refers to exposure to a 3:1 mix of RPMI1640 and two-fold concentrated s/f-α-MEM conditioned by MT. The control for this situation would be RPMI: αMEM (3:1) not conditioned by MT. For **Figures 7**, **8**, the controls would be either RPMI: αMEM + II (3:1) not conditioned by MT or RPMI: αMEM + MI (3:1) not conditioned by MT. The nature of the controls specific to each set of experiments is also described in the figure legends. The protocol for the generation of CM and treatment of INS-1 cells is presented in **Figure 1**.

LDH Release Assay

Media was collected from MT and INS-1 cells after exposure to control or CM, centrifuged and stored at −80°. LDH release into the media was quantified using the "*In vitro* toxicology assay kit" (Sigma) following the manufacturer's instructions.

Insulin Secretion

INS-1 cells were washed in HEPES-buffered salt solution (HBSS) and incubated for 1 h in HBSS containing 2.5 mM glucose. Cells were then stimulated with 2.5 mM, 16.5 mM glucose, or 16.5 mM glucose + 100 μM IBMX (for IS_{max}). Each condition, high or low [glucose], was performed in duplicate, for each specific treatment. After 1 h the HBSS was collected, and the cells were lysed in extraction buffer (Ciaraldi et al., 2007). Insulin concentration in the media was calculated as a percentage of the total insulin content in that well. Insulin secretion index = $[insulin]_{highglucose}/[insulin]_{lowglucose}$.

Protein Extraction

After the collection of CM, muscle cells were rapidly washed 5× with 4°C PBS and then lysed in extraction buffer (Ciaraldi et al., 2007). Proteins were extracted from INS-1 cells as described in the insulin secretion protocol above. Protein concentration was determined by the Bradford assay and extracts stored at −80°C until analyzed.

Western Blotting

Cell protein extracts were resolved on 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked overnight at 4° with Odyssey block (LI-COR Biosciences). Incubation with anti-bodies was for 3 h at RT. Detection and quantification of band intensity was performed using Odyssey Infrared Imaging System and Image Studio analysis software (version 3.1.4).

Assay of Circulating and Secreted Proteins

Serum insulin levels of the human subjects were determined with a human specific RIA kit (Millipore Corp, Billerica, MA, United States); sensitivity was 2 μU/mL, with inter- and intra-assay coefficients of variation (CV) of 6 and 4%, respectively, cross-reactivity with rat insulin = 0.1%. Secreted and cellular insulin from the rat INS-1 cells were detected with a rat specific RIA (Millipore); sensitivity of 0.1 ng/mL, CVs of 9 and 4%, cross-reactivity with human insulin = 100%. Selected myokines in serum and CM were analyzed with MILLIPLEX MAP kits (Millipore) using a BioPlex 200 instrument (Bio-Rad Corp, Hercules, CA, United States). Sensitivities (in pg/mL), inter- and intra-assay CVs for each analyte are as follows: IL1β (0.4, 7%, 6%), IL6 (0.3, 12, 8), IL8 (0.2, 12, 7), IL10 (0.3, 9, 5), IL15 (0.4, 10, 7), GRO (10.1, 12, 5), and VEGF (5.8, 8, 6). BDNF (sensitivity = 20 pg/mL, intra-assay CV = 4%) and TGFβ1 (sensitivity = 15.4 pg/mL, intra-assay CV = 3%) were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN, United States). Wnt 3a was measured with an ELISA kit

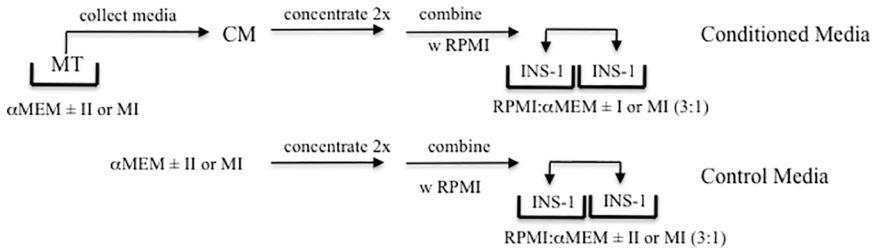

FIGURE 1 | Experimental Design. Schematic representation of the production of MT conditioned (CM, top) and control (lower) media, and treatment of INS-1 cells.

purchased from Lifespan Biosciences (Seattle, WA, United States) (sensitivity = 1.56 ng/mL, intra-assay CV = 5.4%), while Wnt 4 was measured by an ELISA kit from Ray Biotech (Norcross, GA) (sensitivity = 1.22 ng/mL, intra-assay CV < 10%).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad, San Diego, CA, United States). Data were analyzed by *t*-test or 1 way ANOVA with Tukey's *post hoc* test where appropriate. Data were tested for normality with the Kolmogorov–Smirnov test. For results that were not normally distributed, data were log-transformed for statistical analysis and then back-transformed and reported in original units as mean ± SEM. Statistical significance was accepted as $p < 0.05$. The number of individual determinations for each measurement is indicated in the Figure body or legend. An individual determination is a measurement made on MT or MT-CM from one individual subject, performed in duplicate (as indicated in the Figure legends).

RESULTS

Subjects

Subjects were recruited from the general population by advertisement; their characteristics are presented in **Table 1**. All of the women but one were post-menopausal, that subject was biopsied during the early follicular phase of her cycle. None of the women were taking hormone replacement therapy. Assignment to the T2D group was made on the basis of an existing clinical diagnosis with the limitation of [HbA1c] = 7.5–9.5%. The time since diagnosis ranged from 1–15 years. T2D subjects continued on their prescribed medications up to the day of biopsy; sample collection was performed before morning medication. Anti-diabetic medication use included: insulin alone ($n = 1$), metformin alone (7), metformin + insulin (6), glipizide + metformin (1), glipizide + metformin + linagliptin (1), glipizide + metformin + insulin (1), canagliflozin + metformin (1), glipizide + metformin + insulin + linaglutide (1), liraglutide + metformin (1), liraglutide + metformin + insulin (1). One subject was controlled without medication.

The groups were similar in age, and BMI. The T2D subjects were significantly more insulin resistant and displayed reduced steady-state β-cell function (**Table 1**).

Myokine Secretion

The release of selected myokines from untreated MT, including several reported to influence β-cell mass or function (Nunemaker et al., 2014; Kozinski et al., 2016)(reviewed in Barlow and Solomon, 2018), was measured (**Figure 2**). While we reported previously that the secretion of GRO and IL8 was elevated from T2D-MT (Ciaraldi et al., 2016), and they both also tended to be higher with the current cohort, these differences did not attain statistical significance ($p = 0.079$ and 0.105, respectively), Neither Wnt3a nor Wnt4 could be detected in CM.

Impact of ND and T2D Secretomes on INS-1 Cells and Insulin Secretion

Myotubes-Conditioned media would be expected to contain a stew of proteins, metabolites and other factors. CM collected from ND and T2D MT, when processed as described in section "Materials and Methods" had no negative impact on the measures of INS-1 cell viability over 24 h (**Figures 3A,B** and **Supplementary Figure S1C**) when compared to either RPMI 1640 (control 1) or the 3:1 mixture of RPMI+non-conditioned s/f-α-MEM (control 2). While total insulin content was reduced in cells exposed to either ND- or T2D-CM, compared to non-conditioned media, neither ND-CM nor T2D-CM altered other aspects of INS-1 cell function, including insulin secretion in the presence of either low or high [glucose] (**Figure 3D**). Consequently, GSIS and IS_{max} were comparable to what was seen in the presence of non-conditioned media (**Figures 3E,F**). There were no differences between the effects of CM collected from either ND or T2D-MT on any of these outcomes, including

TABLE 1 | Subject characteristics.

	ND	T2D
n (F/M)	20 (2/18)	22 (7/15)
age (year)	57.0 ± 2.7	57.2 ± 1.7
BMI (kg/m^2)	30.20 ± 1.04	33.27 ± 1.28
Fasting [Glucose] (mM)	5.30 ± 0.16	9.41 ± 0.81†
Fasting [Insulin] (pM)	55 ± 8	114 ± 19†
HOMA2-IR	1.61 ± 0.20	2.93 ± 0.55†
HOMA-%B	92.5 ± 8.7	63.3 ± 7.2*

$p < 0.05$ vs. ND; †$p < 0.01$ vs. ND. Ave ± SEM.

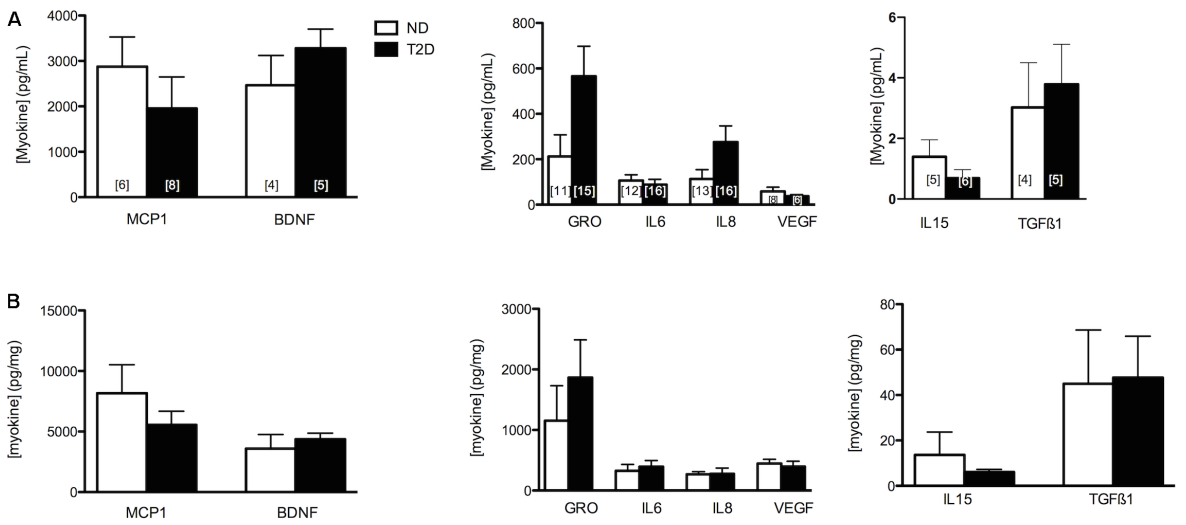

FIGURE 2 | Myokine Secretion. Secretion of selected MK from ND- and T2D-MT. CM collected from untreated MT after 24 h in culture. Results presented as absolute value **(A)** or normalized to cell protein **(B)**, Ave + SEM. The numbers of sets of MT from individual subjects evaluated is presented within each bar, measured in duplicate.

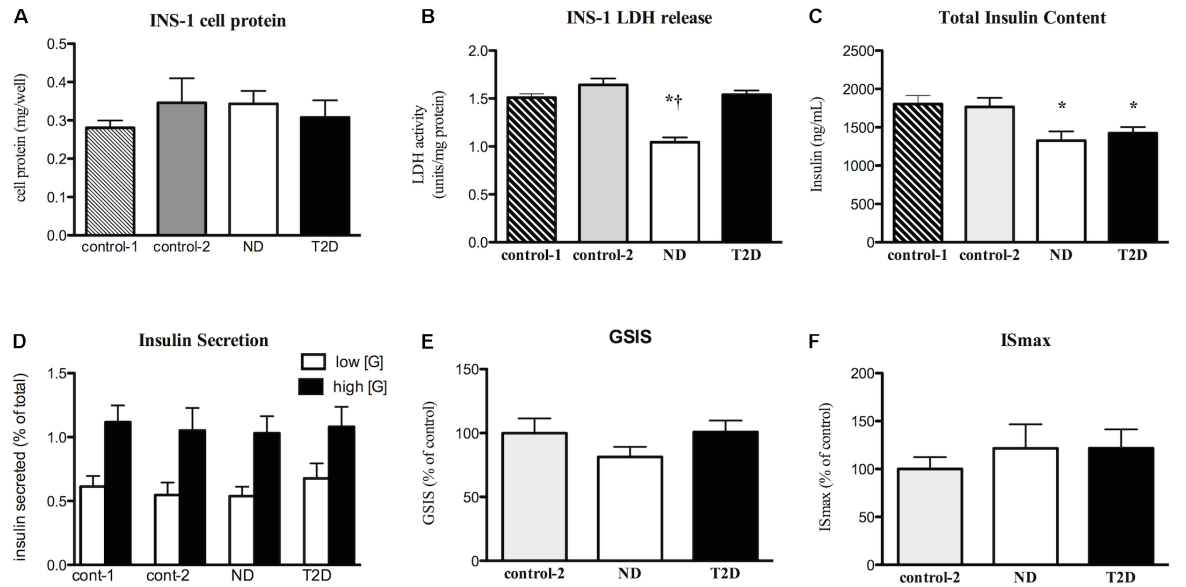

FIGURE 3 | Effects of myotube (MT) conditioned media (CM) on INS-1 cell viability and function. Serum-free CM collected after 24 h and processed as described in the section "Materials and Methods." INS-1 cells treated with the indicated media for 24 h before harvest or assay. **(A)** Total cell protein (n = 12). **(B)** LDH release (n = 12). **(C)** Total insulin content (n = 20). **(D)** Insulin secretion in the presence of low (2.5 mM) and high (16.5 mM) glucose (n = 11/11/16/12). **(E)** GSIS (n = 20). **(F)** Maximal, IMBX-stimulated, insulin secretion (IS_{max}, n = 8). Results presented as absolute value **(A–D)** or as a percentage of Control-1 media **(E,F)**, Ave + SEM. Control 1 = RPMI, Control 2 = RPMI: αMEM (3:1) not conditioned by MT. "n" represents the number of independent determinations, CM from MT from separate individuals, each performed in duplicate. *$p < 0.05$ vs. controls, †$p < 0.05$ vs. T2D.

insulin content, but for LDH release, which was actually reduced by ND-MT-CM (**Figure 3B**).

Impact of Infectious and Metabolic Inflammation on Myotubes

The control condition under which MT were differentiated reflects the euglycemia (5 mM) and normo-insulinemia (∼ 20 pM with 2% FCS) characteristic of healthy individuals. In studying the impact of inflammation on metabolic regulation, treatment with LPS has been used *in vitro* (Frisard et al., 2010; Kewalramani et al., 2010) to induce an inflammatory state. The doses of LPS most frequently used (0.1–1.0 μg/mL) are more reflective of those present during infection (infectious inflammation – II); we employed a similar dose. We also wished to determine the effects of a metabolic environment at least partially resembling

that chronically present in the circulation of individuals with T2D. To establish these conditions, we determined the circulating fasting glucose, insulin and FFA levels of 24 consecutive T2D volunteers studied on our unit. None of these subjects were included in the present study. The average values were used to define the milieu used to induce metabolic inflammation (MI): [glucose] = 10 mM, [insulin] = 120 pM, [FFA] = 550 μM. Since palmitate represents the most abundant circulating FFA, we settled on 400 μM as a properly representative concentration, a level that has also been shown to induce insulin resistance *in vitro* (Hommelberg et al., 2011). That the fasting glucose and insulin concentrations of the current cohort of T2D subjects (**Table 1**) are similar to those in the MI mix validates this approach.

Exposure of either ND or T2D-MT for 24 or 48 h to either II or MI under serum-free conditions had no significant effect on total cell protein or cleavage of caspase 3 compared to s/f α-MEM lacking the additions (**Supplementary Figure S2**).

Possible Inflammation in MT

When cultured in s/f-αMEM with no additions there were no differences between ND and T2D-MT with regard to protein expression of IkBα, a marker of inflammatory state (**Figure 4B**). Similarly, there were no significant differences between the groups for either the protein content or phosphorylation of a number of key kinases involved in inflammatory signaling, p38-MAPK, p44/42-MAPK, and JNK (**Figure 4B**).

Exposure of ND-MT to the II condition for 48 h had no effect on the protein expression of IkBα, p38, p44/42, or JNK (**Figures 4A,C**). Phosphorylation of p44/42 in ND-MT was modestly increased by II (**Figure 4C**), while that of p38 and JNK were unaltered. T2D-MT displayed a different response to the II condition; p38 protein was down-regulated (**Figure 4C**), while its phosphorylation was modestly, but significantly ($p = 0.044$), increased.

Exposure of ND-MT to MI conditions had no significant effects on the protein expression or phosphorylation of the markers of inflammation (**Figure 4C**). T2D-MT showed greater sensitivity to MI, as protein expression of both p38 and p44/42 were reduced slightly, but significantly (**Figure 4C**), even as phosphorylation of both kinases was increased modestly.

There were several instances where responses of the cells from the same individual to II and MI conditions differed. In ND-MT, p44/42 phosphorylation increased after II and decreased after MI exposure ($p < 0.01$).

Insulin Signaling in Myotubes

Under control (no additions) conditions Akt protein expression was similar in ND- and T2D-MT (6.32 ± 0.65 vs. 7.97 ± 1.47 AU). In agreement with previous reports (Cozzone et al., 2008; Kase et al., 2015), both basal and insulin-stimulated phosphorylation of Akt on S^{473} were lower in T2D-MT (**Figure 5B**), though only the difference for basal activity attained statistical significance.

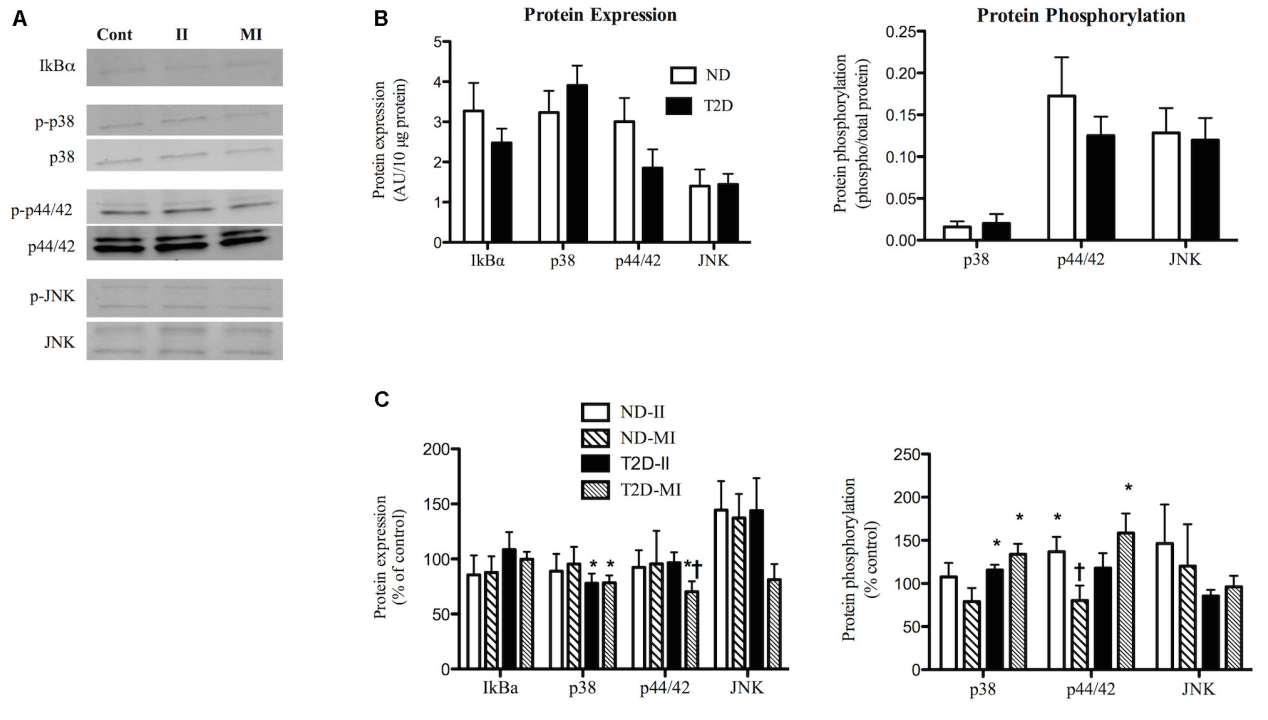

FIGURE 4 | Effects of II and MI conditions on markers of inflammatory signaling in ND- and T2D-MT. **(A)** Representative western blots for IkBα, total and phosphorylated p38, p44/42, and JNK after MT treated for 48 h. **(B)** Quantification of western blots under control conditions (n = 10–13). **(C)** Quantification of western blots after treatment (n = 10–13). Results presented as absolute value **(B)** or as a percentage of the appropriate control media **(C)**, Ave + SEM. Control = MT from the same individual w/o treatment. *$p < 0.05$ vs. matched control, †$p < 0.05$ vs. matched II.

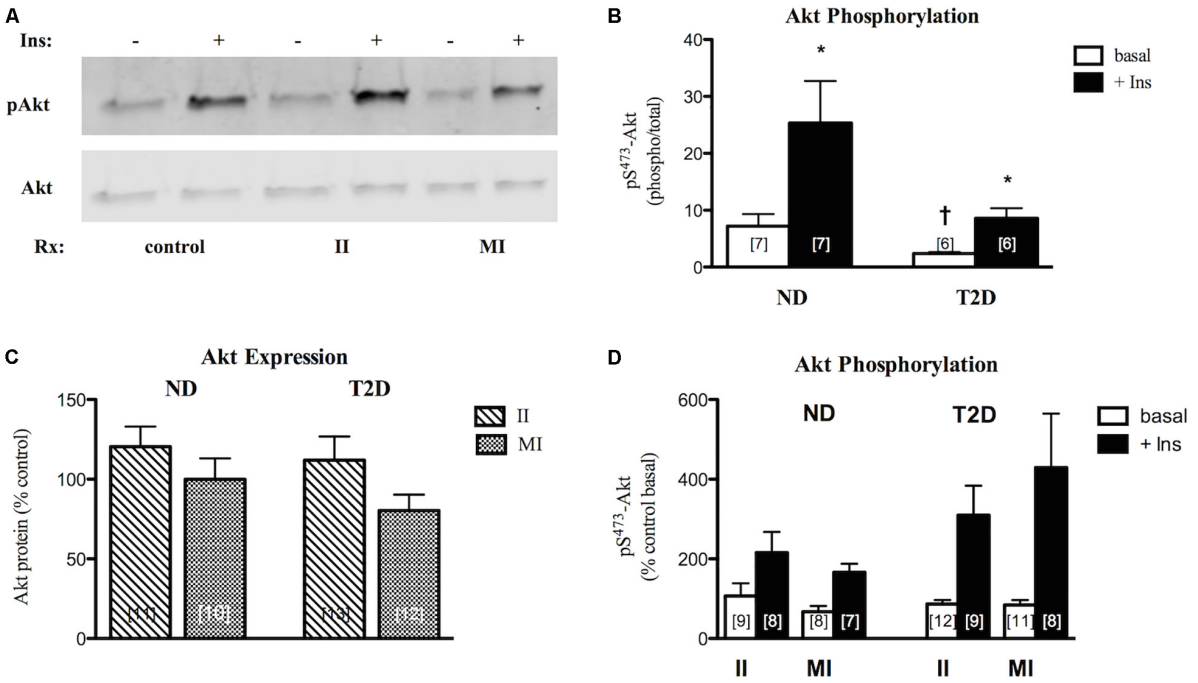

FIGURE 5 | Effects of II and MI conditions on MT function. **(A)** Representative western blots for total and phosphorylated (S^{473}) Akt after MT treated for 48 h. **(B)** Quantification of western blots for pS-Akt under control (w/o treatment) conditions. **(C)** Regulation of Akt protein expression by treatment. Results presented as a percentage of MT from the same individual w/o treatment. **(D)** Regulation of Akt phosphorylation. Results presented as a percentage of basal activity (phospho/total) in MT from the same individual w/o treatment, Ave + SEM. *$p < 0.05$ vs. basal, †$p < 0.05$ vs. ND.

In ND-MT, neither II nor MI conditions had a statistically significant effect on Akt protein expression (**Figure 5C**). Again, T2D-MT seemed more sensitive to the MI condition, as the response of Akt protein expression to the two treatments did differ significantly ($p = 0.019$ for II vs. paired MI).

Both basal and insulin-stimulated Akt phosphorylation were unaltered in ND-MT treated under II conditions; insulin action was retained ($p = 0.024$ basal vs. insulin). Meanwhile, insulin stimulation was reduced, and no longer significant, after MI treatment ($p = 0.056$ basal vs. insulin) (**Figure 5D**), indicating an induction of insulin resistance. Neither basal nor insulin-stimulated Akt phosphorylation in T2D-MT were significantly influenced by either II or MI treatment; significant insulin responsiveness was retained ($p = 0.016$ and 0.023, basal vs. insulin, respectively).

Regulation of Myokine Secretion by Infectious and Metabolic Inflammation

In contrast to the relatively modest effects of the II condition on markers of inflammation and insulin action in ND-MT, the same treatment over 24 h induced large increases in the secretion of selected myokines (**Figure 6**). Meanwhile, MI conditions did not significantly alter secretion of any of the same factors but for TGFβ1. T2D-MTs also responded to II treatment with stimulation of myokine secretion, significantly so for GRO, but with tendencies for IL6 ($p = 0.0696$) and IL8 ($p = 0.0681$). The MI condition was without effect on T2D-MTs for secretion of the myokines measured.

Impact of Infectious and Metabolic Inflammation on INS-1 Cells

Before evaluating the impact of II and MI stress-induced secreted factors on β-cell function, it was necessary to investigate the direct effects of the II and MI conditions, since those elements would still be present in the MT-CM. INS-1 cells were treated for 24 h with RPMI + αMEM containing either LPS (II) or the glucose/insulin/palmitate mix (MI), processed in the same way as MT-CM. Neither the II nor MI media had a significant effect on total cell protein or caspase 3 cleavage, and only a modest increase in LDH release, while function (total insulin content, insulin release in the presence of either low or high [glucose], GSIS, or IS_{max}) was unaltered (**Supplementary Figure S3**). Thus, any effects of MT-CM on INS-1 cells should be ascribed to factors generated by MT and not the other components of the media. Moving forward, the controls for II-MT-CM or MI-MT-CM, were the 3:1 mix containing either αMEM + II not conditioned by MT or αMEM + MI not conditioned by MT, respectively.

Effects of Stress-Induced Secreted Factors on INS-1 Cells

Insulin Secretion

CM derived from either ND- or T2D-MT treated under the II condition had no effect on any of the markers of INS-1

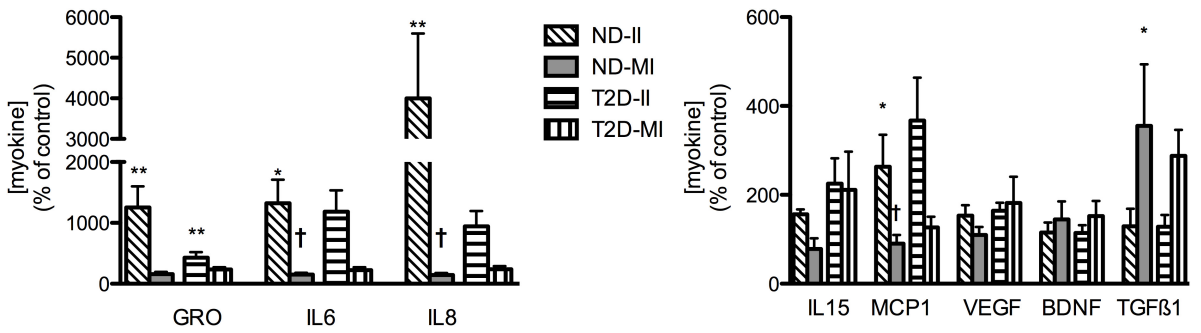

FIGURE 6 | Regulation of myokine secretion by treatment with II or MI. Results presented as a percentage of the matched control (no addition) CM from MT from the same individual, Ave + SEM. The numbers of individual sets of MT evaluated (in duplicate) for each factor are given in the order ND-II/ND-MI/T2D-II/T2D-MI): GRO (11/12/10/12), IL6 (12/12/12/13), IL8 (11/12/12/15), IL15 (5/5/5/5/), MCP1 (6/6/7/7), VEGF (8/8/4/4/), BDNF (4/4/5/5), TGFβ1 (3/3/5/5). *$p < 0.05$ vs. matched control, **$p < 0.01$ vs. matched control, †$p < 0.05$ vs. matched response to II in MT from the same subject.

cell viability (**Figures 7A,B** and **Supplementary Figure S3C**). Total insulin content was also unaltered (**Figure 7C**). However, after treatment with II-ND-CM, GSIS was doubled (100 ± 25% of control), compared to the relevant non-conditioned media control (**Figure 7D**): CM from T2D MT (II-T2D CM) showed no such effect. This effect of II-ND-CM was limited to GSIS, as both IS in the presence of low [glucose] (**Figure 7**) and IS_{max} (**Figure 7E**) were unaltered.

CM from MI-treated ND- and T2D-MT also had no effect on INS-1 cell viability or total insulin content (**Figures 7A,B** and **Supplementary Figure S3C**). In contrast to the response to the II condition, MI-ND-CM had no effect on either GSIS or IS_{max} (**Figures 7E,F**). However, exposure to the CM from T2D-MT (MI-T2D-CM) reduced GSIS, to 65 ± 5% of control ($p < 0.05$) (**Figure 7D**). Again, IS in the presence of low [glucose] (**Figure 7D**) and IS_{max} (**Figure 7F**) were unperturbed.

IkBα content, was not altered by either II or MI/ND-CM or II or MI/T2D-CM (**Figures 7G,H**). Similarly, the various CM had only modest, or no, effects on inflammatory signaling (**Figure 7H**): only an effect of MI/T2D-CM to reduce p38 protein (by 10%) attained statistical significance.

Infectious and Metabolic Inflammation Regulation of Insulin Secretion

In studying further the nature of regulation of β-cell function by MT-secreted factors, we focused on the two conditions where GSIS was altered; treatment with II-ND-CM and MI-T2D-CM.

As one step in determining the potential nature of the active factor(s) responsible for regulation of GSIS, INS-1 cells were cultured for 24 h with the indicated MT-CM that had either been left unprocessed or boiled for 10 min before treating cells. Boiling fully abrogated stimulation of GSIS by II-ND-CM or the suppression by MI-T2D-CM (**Figure 8A**), suggesting that the factor(s) responsible for the effects on GSIS are protein in nature and therefore, by definition, can be termed myokines.

The signaling pathways involved in myokine regulation of β-cell function were investigated by simultaneous treatment of INS-1 cells with specific MT-CM and inhibitors of PI3K (wortmannin), PKC (Ro 31-8220) and p38 MAPK (SB 203590) for 24 h. All three of the inhibitors were able to fully reverse II-ND-CM stimulation of GSIS (**Figure 8B**). Indeed, each inhibitor was also able to suppress GSIS below control. Conversely, each of the inhibitors restored GSIS after MI-T2D-CM treatment (**Figure 8B**). These results suggest that multiple pathways, and potentially multiple factors, participate in MT regulation of GSIS.

DISCUSSION

The defining characteristics of T2D are insulin resistance in peripheral tissues and, ultimately, β-cell failure (DeFronzo and Tripathy, 2009). The importance of insulin resistance in SkM itself is well known (reviewed in DeFronzo and Tripathy, 2009). More recently, attention has been paid to a role for SkM in regulating β-cell mass and function (Shirakawa et al., 2017). Such regulation could be mediated by myokines, protein factors secreted from SkM (Barlow and Solomon, 2018). For example, exercise is known to improve/protect β-cell viability (Narendran et al., 2017; Paula et al., 2018) at least in part through IL6 released from SkM, either acting directly on β-cells (Christensen et al., 2015; Paula et al., 2015), or by enhancing GLP-1 secretion from L cells and alpha cells (Ellingsgaard et al., 2011). Yet, others have found no effects of physiologic levels of IL6 on insulin secretion (Barlow et al., 2018). Other exercise-induced factors that have been suggested to improve β-cell mass and/or function include irisin (Natalicchio et al., 2017), RANTES (Mizgier et al., 2017) and Fractalkine (CX3CL1) (Barlow and Solomon, 2018).

The muscle secretome could also exert negative effects on β-cell mass and function. For example, CM from myotubes from healthy subjects where an insulin resistant state was induced by treatment with TNFα proved capable of reducing both β-cell mass and GSIS (Bouzakri et al., 2011), while IL13 protected islets against cytokine-induced apoptosis, with no effects on GSIS (Rutti et al., 2015). Conversely, while feeding of a high fat diet (20% palmitate) made male C57BL/6 mice insulin resistant, exosome-like vesicles isolated from muscle of these animals were able to induce proliferation of MIN6B1 cells and isolated islets (Jalabert et al., 2016), possibly to compensate for the insulin

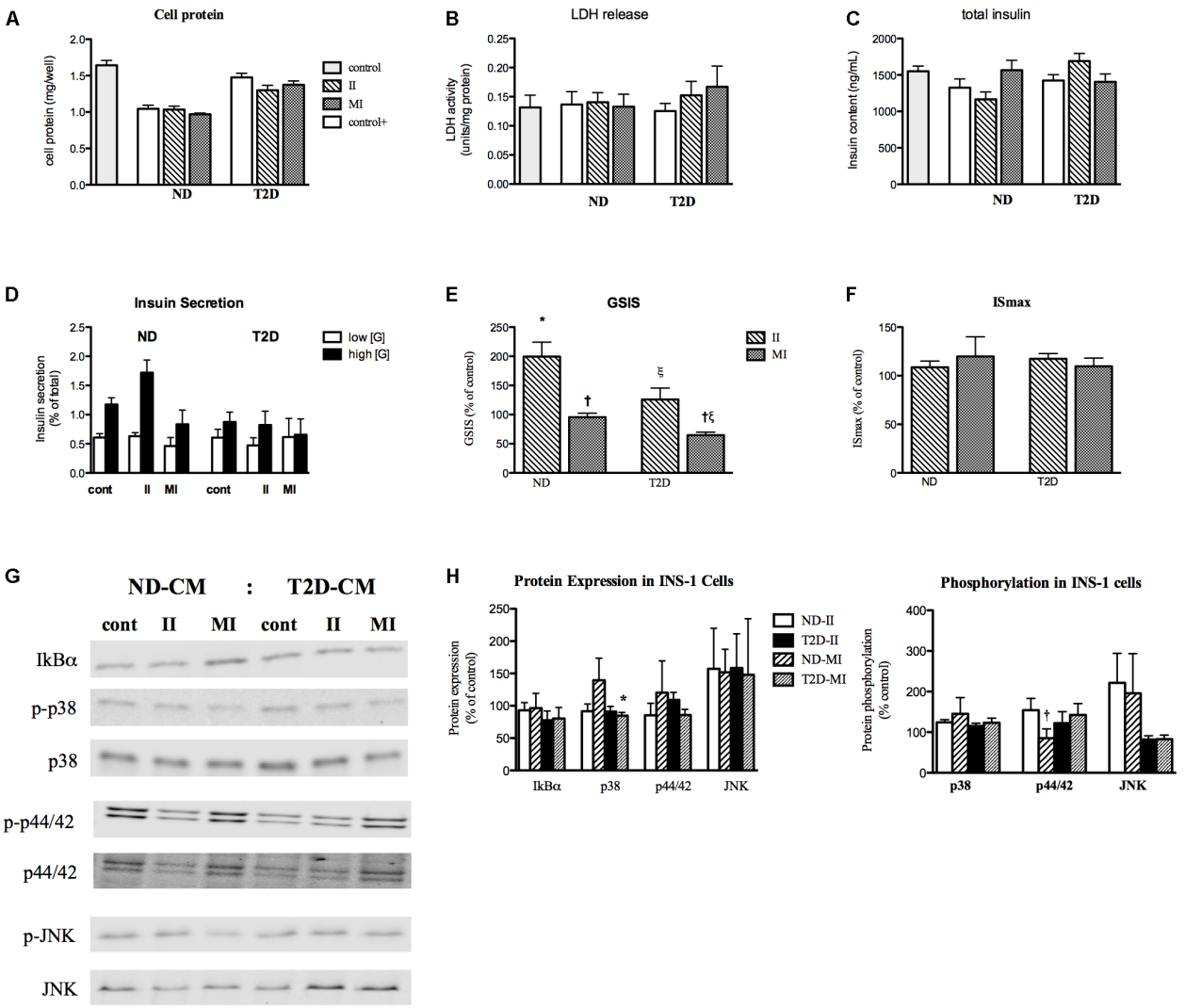

FIGURE 7 | Effects of CM from ND- and T2D-MT challenged with II and MI conditions on INS-1 cell function. INS-1 cells treated with the indicated CM for 24 h before harvest or assay. **(A)** Total cell protein (n = 12–24). **(B)** LDH release (n = 12–24). **(C)** Total insulin content (n = 12–24). **(D)** Insulin secretion (n = 10–14). **(E)** GSIS (n = 10–14). **(F)** IS_{max} (n = 8–14). **(G)** Representative western blots for IkBα, total and phosphorylated p38, p44/42, and JNK. **(H)** Quantization of western blots (n = 4–8). Results presented as absolute value or as a percentage of the appropriate control, II or MI non-conditioned media. Ave + SEM. Panels **(A–C)**; Control = RPMI: a-MEM (3:1) w/o treatment conditioned by MT from the same individual, control+ = RPMI: a-MEM (3:1) + II or MI not conditioned by MT. Panels **(D,E,G)**, control = RPMI: a-MEM (3:1) w/o treatment conditioned by MT from the same individual. *$p < 0.05$ vs. control, †$p < 0.05$ vs. II.

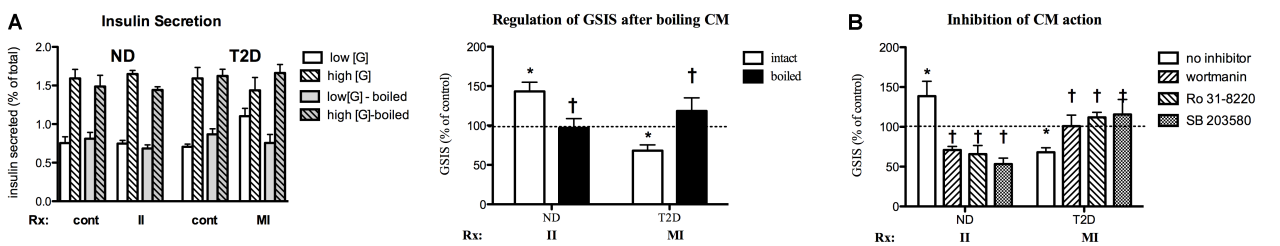

FIGURE 8 | Characterization of MT-CM regulation of GSIS. **(A)** Cells treated for 24 with intact MT-CM or MT-CM boiled before exposure: Left panel – insulin secretion, Right panel – GSIS (n = 10). **(B)** Inhibition. Cells treated with the indicated CM in the absence or presence of SB203580 (100 nM, n = 6 for ND/5 for T2D), Ro 31-8220 (50 nM, n = 6/5), or wortmannin (100 nM, n = 6/8) before GSIS determined. Control = RPMI: αMEM (3:1) w/o treatment conditioned by MT from the same individual. *$p < 0.05$ vs. matched control, †$p < 0.05$ vs. intact media **(A)** or no inhibitor **(B)**.

resistant state. These authors implicated miR-16 in this action (Jalabert et al., 2016).

To date, investigations of SkM effects on β-cell viability and function have focused on tissue or cells from healthy animals or humans, manipulated by exercise, diet, or other treatments to induce insulin resistance, but not on the impact of T2D itself. Since we, and others have reported that altered secretion at the protein (Brandt et al., 2012; Ciaraldi et al., 2016), miRNA (Massart et al., 2016, 2017), and metabolite levels (Rai and Demontis, 2016), is an intrinsic property of muscle in T2D (reviewed in Eckardt et al., 2014; Garneau and Aguer, 2019), we sought to determine if β-cell/muscle communication was altered in T2D. To that end, we employed the human SkM cell system (Henry et al., 1995). Multiple investigators have shown that human SkM satellite cells, when cultured and differentiated to myotubes display many of the properties of intact muscle, including T2D-related differences in glucose and fat metabolism (reviewed in Aas et al., 2013; Gaster, 2018), protein expression (Al-Khalili et al., 2014) and, most important to the current goals, secretory profile (Ciaraldi et al., 2016; Massart et al., 2016). Media conditioned by culture with myotubes, was taken as a surrogate for secretion into the extracellular space, and ultimately into the circulation.

When cultured under standard conditions, with glucose and insulin levels similar to those present in the circulation of ND subjects, CM from neither ND nor T2D-MT had any effects, over 24 h, on INS-1 cell viability and function (**Supplementary Figure S1**), or on markers of inflammation. Thus, the T2D-related intrinsic differences in secretion of myokines and other factors we, and others have reported (Ciaraldi et al., 2016; Massart et al., 2016), alone would not appear to contribute to changes in β-cell mass or regulated insulin secretion over this time frame. Unlike our finding of no effect of ND-MT-CM on GSIS in INS-1 cells, Bouzakri et al. (2011) reported that media conditioned by MT from healthy subjects increased GSIS in primary rat β-cells. Multiple differences in experimental conditions, including the target cell itself might account for this difference. It is critical to note that the control environment in which the MT were cultured and CM generated represents, at least for T2D-MT, an artificial situation. For that reason, we elected to challenge myotubes under conditions that more closely modeled the environment seen *in vivo* in T2D individuals. To mimic the chronic, low-grade systemic inflammation present in T2D (Olefsky and Glass, 2010), we chose treatment with LPS (II), a widely employed intervention (reviewed in Boutagy et al., 2016). While LPS treatment stimulated secretion of a number of pro-inflammatory myokines (**Figure 6**) from both ND and T2D-MT, this was accompanied by only modest, if any, changes in cellular indicators of inflammation or inflammatory signaling in the myotubes. Insulin signaling through Akt phosphorylation was also unaltered by the II condition.

Meanwhile, as might be expected, the hyperglycemic/hyperinsulinemic/hyperlipidemic T2D-like conditions induced insulin resistance at the level of Akt phosphorylation in ND-MT, but did not exacerbate the already impaired Akt response in T2D-MT; it may be that even more severe (non-physiologic) conditions would be needed to induce even more severe insulin resistance for this response. ND- and T2D-MT differed in a number of other ways in their responses to the MI environment. Stimulation of myokine secretion, while modest compared to the II response, was seen for a number of factors from T2D-MT, unlike with ND-MT, where only TGFβ1 secretion was altered significantly. These results reveal another aspect of the T2D phenotype in muscle that is retained in MT, an increased sensitivity to a hyperglycemic/hyperinsulinemic/hyperlipidemic environment.

Given the different conditions under which human myotubes and INS-1 cells, the established β-cell model employed for these studies, are cultured, considerable effort had to be put into determining how the MT media might influence INS-1 cells, even in the absence of contributions from MTs. Fortunately, only minor modifications in serum-free α-MEM were needed to maintain INS-1 cell viability and function (**Supplementary Figure S1**). Furthermore, the II and MI conditions used to treat MT to generate CM had only modest direct effects on the outcomes of interest in INS-1 cells, which were accounted for by inclusion of the appropriate controls. Thus, any effects of II- or MI-MT-CM on INS-1 cell mass and/or function could be ascribed to contributions from the ND and T2D myotubes.

The major findings of the current studies are those presented in **Figures 6**, **7**; that MT from ND and T2D subjects respond differently to the challenges represented by infectious and metabolic inflammation with regard to both their secretome, and the effects of those secretions on β-cell function. Furthermore, these differential effects are highly specific for glucose regulated insulin secretion, as neither markers of β-cell mass, insulin production, nor maximal insulin secretion, were impacted. While ND-MT are able to produce and secrete factor(s) that augment (II) or possibly protect (MI) GSIS, T2D-MT lacked these capacities.

Further studies provided additional information about the nature of the mechanisms by which factors secreted from ND- and T2D-MT influence GSIS. We learned that both the beneficial (II-ND-CM) and negative (MI-T2D-CM) effects are mediated by protein factors (**Figure 8A**) and therefore could be considered to be myokines. Another common feature of the two responses is that multiple signaling pathways; PI3-K, PKC and p38 MAPK, are involved (**Figure 8B**).

In trying to identify the specific factors present in CM responsible for regulation of GSIS one would look for those that are uniquely altered in II-ND-CM and MI-T2D-CM. Such factors would need to meet the following criteria: (1) differentially regulated in ND-CM under II vs. control or MI conditions, (2) differentially regulated in T2D-CM under MI vs. control or II condition, (3) changed in ND- but not T2D-CM under the II condition, (4) changed in T2D- but not ND-CM under MI condition. Of the proteins measured (**Figure 6**), no individual protein met all of the necessary criteria. The implication that multiple signaling pathways mediate these specific instances of modulation of regulated insulin secretion (**Figure 8B**) suggests that multiple factors might be involved, which was not considered in our initial analysis of the data.

A number of muscle-derived factors have been shown to modulate GSIS: irisin (Natalicchio et al., 2017), IL6 (Ellingsgaard et al., 2011; Christensen et al., 2015), fractalkine (Barlow and Solomon, 2018), angiogenin, and osteoprotegerin (Rutti et al., 2018) and Wnt3a (Kozinski et al., 2016); RANTES improved basal insulin secretion, but was without effect on GSIS (Mizgier et al., 2017). The involvement of any of these factors in the responses described in the current report might be questioned, as many of these same factors also protected the β-cell or islet systems studied from apoptosis, while we found no such effects of specific MT-CM on caspase 3 cleavage, an accepted marker of apoptosis. However, comparisons between studies are complicated by the use of different challenges for muscle cells; palmitate alone (Natalicchio et al., 2017), insulin alone (Mizgier et al., 2017), or selected cytokines (Christensen et al., 2015; Rutti et al., 2015, 2018). Also, none of these studies evaluated MT from individuals with T2D. Obviously additional studies, possibly proteomic analysis of specific CM, are needed to identify the factor(s) responsible for the regulation of GSIS reported here.

While Kozinski et al. (2016) reported that reciprocal changes in Wnt3a and Wnt4 secretion from adipose tissue and SkM tissue and C2C12 cells could influence islet proliferation and GSIS, we were unable to detect either protein in MT-CM, even after concentration. Several factors could contribute to such a discrepancy in the results. In intact SkM, Wnts are found in axons and satellite cells, where they play a role in differentiation (Cisternas et al., 2014). It is possible that Wnt expression could be lost with terminal differentiation of human myotubes, while C2C12 cells may retain aspects of the myoblast phenotype. However, that does not rule out a role for the Wnt system in mediating β-cell adaptations to specific stressors, merely that, in humans, adipose tissue and not muscle may represent the primary source of Wnt signaling proteins.

The relationship between chronic low-grade inflammation in adipose tissue and insulin resistance in obesity and T2D is well established (reviewed in DeFronzo and Abdul-Ghani, 2011); the situation is less clear with regard to SkM, as there are reports of both normal (Tam et al., 2012; Amazou et al., 2016; Perry et al., 2016) and elevated (Fink et al., 2013; Patsouris et al., 2014; Brown et al., 2015) markers of inflammation in SkM from obese insulin-resistant and T2D individuals. Under the normoglycemic/insulinemic/low lipid, control culture conditions employed in this study we found no differences between ND and T2D MT for multiple markers of inflammation, even as T2D-MT displayed a tendency (this report) to secrete elevated levels of several pro-inflammatory cyto- and chemokines (**Figure 2**). These results suggest that inflammation in SkM may not be an effect autonomous to myotubes, but due to the recruitment of pro-inflammatory cells into SkM.

Conversely, it has been reported that MT from T2D subjects display elevated phosphorylation of p38 MAPK (Brown et al., 2015). Yet, in that instance the indication of inflammation was uncoupled from insulin resistance, as glucose uptake was unaltered (Brown et al., 2015). A similar uncoupling of SkM inflammation and insulin resistance has been seen in obese subjects (Amazou et al., 2016), after SkM-specific over-expression of MCP-1 (Evers-van Gogh et al., 2016), and with palmitate treatment of L6 myotubes (Sinha et al., 2004). While we found that LPS treatment of MT resulted in increased secretion of multiple pro-inflammatory factors, the finding of only modest changes in markers of inflammation is additional support for the importance of non-MT cells to the establishment of inflammation in SkM. Most interesting are the responses of MT to the diabetic-like, MI conditions, where in ND-MT there was minimal or no impact on either secretion of the myokines measured or markers of inflammation, which would be consistent with the lack of effects of MI-ND-CM on insulin secretion, while the ability of MI-T2D-CM to suppress GSIS was accompanied by modest changes in p38 and p44/42 MAPK protein expression and phosphorylation. The differing responses to the II and MI treatments reveal additional aspects of the T2D phenotype that are intrinsic to SkM, as they are retained in culture.

Several limitations in the current studies require mention. One is the nature of the human SkM cell system. While, as mentioned earlier, these cells reflect many of the properties of their donors, they also represent denervated muscle. Denervation in itself can induce insulin resistance (Turinsky and DAmrau-Abney, 1998), while removing the contributions of central, neural, control of metabolism, which could also be a site of difference between healthy individuals and those with T2D (Porte et al., 2005). Furthermore, while inflammatory cytokines have been shown to inhibit myoblast differentiation, this effect was not present on myotubes (Langen et al., 2001); we avoided this effect by treating fully differentiated myotubes. An advantage of the human MT system is that it permits study of the cell-autonomous effects of MT on β-cell mass/function, yet it neglects the contributions of other cell types, such as pro- and anti-inflammatory cells infiltrating SkM (see above). Another difference from whole body physiology is the static nature of the current studies, both for generation of CM and treatment of INS-1 cells. In addition, we utilized confluent INS-1 cells, which while that would allow evaluation of myokine effects on cell viability, including apoptosis, it precludes following effects on proliferation, limiting comparisons to some of the other reports on myokine effects on β-cells.

In summary, we report here, in agreement with the work of others, that factors secreted from SkM can influence regulated insulin secretion. Novel information includes the observation that MT from individuals with T2D differ from MT from ND subjects with regard to the factors they secrete in response to conditions modeling aspects of the environment present in T2D. While ND-MT are able to protect or augment GSIS when faced with these challenges, T2D-MT lack these compensatory responses. Rather, under T2D-like conditions (MI), T2D-MT act in a manner to impair GSIS, which, together with the impaired metabolism and insulin resistance intrinsic to T2D SkM, would contribute in multiple ways to the metabolic dysfunction characteristic of T2D. Under the conditions investigated in this report, muscle secretome regulation of GSIS is mediated by currently unknown protein factors that act through both pro-inflammatory (p38 MAPK) and other (PI3-K and PKC) signaling pathways.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The experimental protocol was approved by the Human Research Protection Programs of the Veterans Affairs San Diego Healthcare System and the University of California, San Diego. Informed written consent was obtained from all subjects after explanation of the protocol.

AUTHOR CONTRIBUTIONS

AR and TC conceived and designed the study, performed the experiments, researched the data, and wrote the manuscript. RH contributed to study design, reviewed and edited the manuscript. TC is the guarantor of this work and, as such, had full access to all of the data in the study and takes full responsibility for the accuracy of the data analysis.

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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.2019.01608/full#supplementary-material

FIGURE S1 | Effects of culture media on INS-1 cell viability and function. INS-1 cells treated with the indicated media, RPMI1640 or RPMI+ the indicated proportion of αMEM, for 24 h before harvest or assay. **(A)** Total cell protein (n = 12). **(B)** LDH release (n = 9). **(C)** Representative western blot for total and cleaved caspase 3: 1 – RPMI1640, 2 – αMEM, 3–5 – T2D-MT-CM, 6–8 – ND-MT-CM. **(D)** Total, secreted and cell-associated, insulin content (n = 12). **(E)** GSIS (n = 10). **(F)** IS_{max} (n = 5). For panels **(D–F)**, αMEM = 3:1 RPMI + αMEM. *$p < 0.05$ vs. paired control.

FIGURE S2 | Effects of II and MI conditions on MT viability. MT treated for 48 h (cell harvesting). **(A)** Total cell protein (n = 12–14). **(B)** Representative western blot for total and cleaved caspase 3: cont – Jurkat cell extract treated + cytochrome C, αMEM = 3:1 RPMI + αMEM not conditioned, +II = 3:1 RPMI + (αMEM not conditioned + LPS), + MI = 3:1 RPMI + (αMEM not conditioned + MI mix).

FIGURE S3 | Effects of II and MI media on INS-1 cell viability and function. INS-1 cells treated with the indicated media for 24 h before harvest or assay. **(A)** Total cell protein (n = 10–12). **(B)** LDH release (n = 10–12). **(C)** Representative western blots for total and cleaved caspase 3: I – non-conditioned αMEM, 1 – control, 2 – +II, 3 – +MI; II – ND-MT-CM, III – T2D-MT-CM; cont – Jurkat cell extract treated + cytochrome C. **(C)** Total, secreted and cell-associated, insulin content (n = 10–11). **(D)** Insulin secretion (n = 7–10). **(E)** GSIS (n = 7–10). **(F)** IS_{max} (n = 8–12). *$p < 0.05$ vs. paired control.

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Exercise-Released Myokines in the Control of Energy Metabolism

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Physical activity reduces cardiometabolic risk, while physical inactivity increases chronic diseases risk. This led to the idea that exercise-induced muscle contraction contributes to metabolic regulation and health. It is now well established that skeletal muscle, through the release of endocrine factors, i.e., so-called myokines, crosstalk with metabolic organs such as adipose tissue, liver and pancreas. Recent advances suggested that a number of myokines are able to modulate adipose tissue metabolism and thermogenic activity, liver endogenous glucose production and β-cell insulin secretion. This novel paradigm offers a compelling hypothesis and molecular basis to explain the link between physical inactivity and chronic diseases. Herein, we review major findings and recent advances linking exercise, myokines secretion and inter-organ crosstalk. Identifying the molecular mediators linking physical activity to metabolic health could open the path toward novel therapeutic targets in metabolic diseases.

Keywords: skeletal muscle, exercise, exerkine, crosstalk, metabolism

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INTRODUCTION

Physical activity reduces cardiometabolic risk, whereas sedentary behaviors favor the development of chronic diseases (Janiszewski and Ross, 2009; Booth et al., 2017; Powell et al., 2018). These observations led to the concept that exercise, by stimulating muscle contraction, participates in the regulation of energy homeostasis and organ function. It is now well known that skeletal muscle, through the secretion of endocrine factors, is able to communicate with several key metabolic organs involved in the control of energy metabolism (Pedersen and Febbraio, 2012). Recent advances emphasized the role of secreted proteins, i.e., myokines, which are able to regulate lipid mobilization from adipose tissue, liver endogenous glucose production, insulin secretion by beta pancreatic cells or to activate thermogenesis. In this review, we will describe the main metabolic effects of exercise-released myokines on main metabolic organs, i.e., skeletal muscle, adipose tissue, liver and pancreas. We will also discuss how this new paradigm offers a molecular basis to explain the link between sedentary behaviors and metabolic diseases.

SKELETAL MUSCLE ADAPTATIONS TO EXERCISE

Physical inactivity is a major risk factor for obesity, type 2 diabetes, cardiovascular diseases, certain cancers, osteoporosis and early mortality (LaMonte et al., 2005; LaMonte and Blair, 2006). Aerobic capacity, or cardiorespiratory endurance, is a major predictive factor of cardiovascular mortality

(Lee et al., 2010). Physical exercise is well-known to stimulate muscle oxidative capacity and lipid oxidation. Cross-sectional studies have demonstrated increased lipid oxidation in endurance-trained individuals compared to sedentary controls during an exercise bout with the same relative and absolute workload (Nordby et al., 2006). Longitudinal studies have shown that lipid oxidation is improved during an acute exercise bout performed after an endurance-training program for the same relative and absolute workload in either individuals with normal weight (de Glisezinski et al., 2003), overweight (Johnson et al., 2010) or obesity (Tarnopolsky et al., 2007). Even if several biochemical adaptations have been reported (Hawley et al., 2014), the greater mitochondrial oxidative capacity seems to be the main determinant. The increase in lipid oxidation during exercise is accompanied by an increase in the number of mitochondria and in mitochondrial enzymes activity in response to exercise training (Menshikova et al., 2005). Several studies have also demonstrated an increase in mitochondrial density through electron microscopy and in the expression of mitochondrial respiratory chain complexes (Menshikova et al., 2005; Engeli et al., 2012).

These molecular adaptations to training seems to be largely mediated by transcription factors such as peroxysome proliferator-activated receptor (PPAR)-gamma coactivator-1α (PGC-1α) and PPARβ, and metabolic sensors such as 5′-AMP activated kinase (AMPK). Thus, a skeletal muscle-specific overexpression of PGC-1α and PPARβ markedly improves oxidative capacity and endurance in mice (Lin et al., 2002; Wang et al., 2004). These animal models also display an improvement in insulin sensitivity and glucose tolerance. Several human studies also confirm the association between increased muscle oxidative capacity and insulin sensitivity in response to endurance or resistance training (Bruce et al., 2006; Toledo et al., 2007). However, most of the cellular and molecular mechanisms linking physical exercise to health benefits remain poorly understood. Identification of these mechanisms may lead to the discovery of new therapeutic targets in metabolic diseases but also allows the development of personalized exercise medicine (Neufer et al., 2015). Considering the benefits of physical exercise on many organs and in multiple chronic diseases, skeletal muscle has been longstanding considered as able to release humoral factors into the bloodstream. We will here describe the main myokines induced by physical exercise, i.e., exerkines, and involved in the regulation of energy metabolism.

MYOKINES: MESSENGER OF SKELETAL MUSCLES TO REMOTE METABOLIC ORGANS

By analogy with endocrine glands and adipose tissue, myokines are peptides, which are expressed, produced and secreted by muscle fibers. These myokines display autocrine/paracrine and endocrine effects, and could mediate the beneficial effects of exercise on other key organs involved in the regulation of energy homeostasis (**Figure 1**). The hypothesis that skeletal muscle produces secreted factors during exercise has been suggested when it was observed that muscle contraction induces physiological and metabolic adaptations in other organs, which are not mediated by the nervous system. Thus, an electric stimulation in skeletal muscle without nervous afference or efference in paraplegic patients recapitulates several of the physiological adaptations observed in valid individuals (Mohr et al., 1997).

By using different techniques, recent studies suggested that skeletal muscle may release several hundreds of proteins, although only a few have been characterized and their biological function demonstrated (Catoire et al., 2014; Hartwig et al., 2014; Deshmukh, 2016). Pourteymour et al. (2017) recently identified by RNA sequencing novel candidate myokines, and among those characterized colony-stimulating factor-1 (CSF1) as a novel exercise-regulated myokine. The biological role of CSF1 in the context of exercise still remains to be determined. Collectively, this indicates that skeletal muscle is therefore able to communicate with remote organs, through humoral factors secreted into the bloodstream during exercise.

MYOKINES WITH AN AUTOCINE/PARACRINE ACTION IN SKELETAL MUSCLE

Among these factors, we find the so-called myostatin, also known as *growth/differentiation factor 8*, whose gene invalidation leads to an excessive increase in muscle mass (McPherron et al., 1997). Myostatin seems to act localy by inhibiting muscle hypertrophy, but is also secreted in the bloodstream to act remotely on adipose tissue (McPherron and Lee, 2002). Other factors have been identified such as brain-derived neurotrophic factor (BDNF) (Rasmussen et al., 2009), musclin also called osteocrin (Subbotina et al., 2015), and interleukin-15 (IL-15) (Quinn et al., 2013) to control muscle hypertrophy and oxidative capacity in an autocrine fashion. At the muscle level, IL-6 seems to stimulate intramuscular triacylglycerols lipolysis and fatty acids oxidation in an autocrine manner (Wolsk et al., 2010). A human study has also demonstrated that IL-6 injection, at a dose reproducing physiological variations as to those observed during exercise, stimulates GLUT4 translocation to the plasma membrane and improves insulin sensitivity in skeletal muscle (Carey et al., 2006). This mechanism could involve the activation of AMPK. Many other proteins secreted by skeletal muscle with an autocrine effect have been identified, such as SPARC, fibroblast growth factor-21 (FGF-21), decorin, myonectin and irisin. Extensive reviews on this topic have been published elsewhere (Lee and Jun, 2019; Piccirillo, 2019).

More recently, we identified apelin as a novel myokine in human skeletal muscle. We showed that apelin gene expression is up-regulated in response to 8-week aerobic exercise training in obese male subjects (Besse-Patin et al., 2014). Apelin gene expression is up-regulated in human primary skeletal muscle cells in response to exercise-mimetic treatment including Ca^{2+} and cAMP releasing agents (Besse-Patin et al., 2014). It was further demonstrated that apelin is acutely released upon muscle contraction during exercise to act in an autocrine fashion

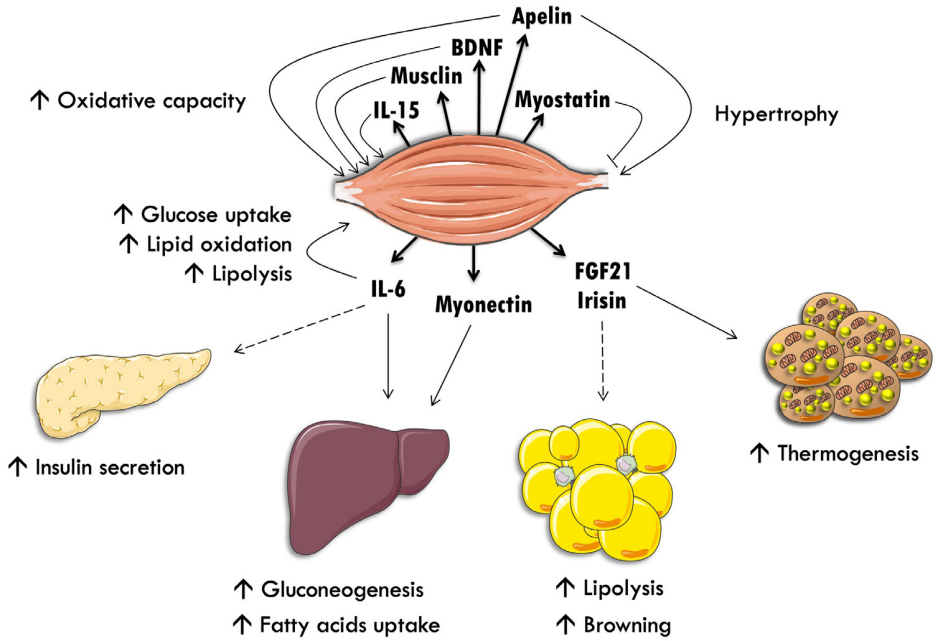

FIGURE 1 | Skeletal muscle as an endocrine organ. Interleukin-15 (IL-15), brain-derived neurotrophic factor (BDNF) and interleukin-6 (IL-6) stimulate lipid oxidation and oxidative metabolism in an autocrine fashion. IL-6 also stimulates intramyocellular triacylglycerol lipolysis and glucose uptake. IL-6 stimulates pancreatic insulin secretion and exercise-induced hepatic neoglucogenesis through an endocrine communication. Myostatin inhibits muscle hypertrophy. Finally, fibroblast growth factor-21 (FGF-21) stimulates white adipose tissue lipolysis and brown adipose tissue thermogenesis.

to activate skeletal muscle hypertrophy. A progressive decline in muscle apelin expression and secretion with aging may contribute to the degenerative loss of muscle mass, quality and strength, i.e., sarcopenia (Vinel et al., 2018).

In a recent study, Furrer et al. (2017) demonstrated that B-type natriuretic peptide (BNP), which was previously thought to be mainly expressed in the heart (Moro and Lafontan, 2013), is also a PGC1α-dependent myokine released by contracting skeletal muscle to modulate macrophage function in a paracrine fashion. A tightly controlled crosstalk between muscle fibers and immune cells is thus a key event in training adaptation and for muscle regeneration and repair. This work resonates with the study of Subbotina et al. (2015) indicating that proteins belonging to the natriuretic peptide family may play an important physiological role in controlling muscle metabolism and function.

MYOKINES WITH AN ENDOCRINE ACTION ON METABOLIC ORGANS

White and Brown Adipose Tissues

Interleukin-6 was the first myokine to be discovered by Bente Pedersen and colleagues (Pedersen et al., 2003). Circulating IL-6 levels markedly increase in response to an acute exercise bout, up to 100-fold increase above resting level. This increase seems to be independent of muscle fiber damages and is tightly correlated with exercise intensity, duration and muscle mass involved in the mechanic workload (Suzuki et al., 2002). Several studies have demonstrated an increase in IL-6 expression and secretion in skeletal muscle in response to contraction, particularly when muscle glycogen content is depleted (Keller et al., 2001; Steensberg et al., 2001; Febbraio et al., 2003). This observation led to the suggestion that IL-6 is secreted by skeletal muscle in response to exercise and acts as a metabolic sensor. Thus, IL-6 could stimulate adipose tissue lipolysis and lipid mobilization during exercise to provide energy for muscle contraction and spare glucose (van Hall et al., 2003). An elegant clinical study has recently demonstrated that IL-6 is required to reduce visceral adipose tissue mass in response to exercise training in humans (Wedell-Neergaard et al., 2019). However, the role of IL-6 on adipose tissue lipolysis is still a matter of debate, as it was demonstrated that an acute elevation of IL-6 at a normophysiological level increased the rate of whole-body lipolysis due to an increase in muscle fatty acids release, whereas adipose tissue lipolysis remained unaffected (Wolsk et al., 2010). In addition, IL-6 does not display any acute effect on adipocyte lipolysis *in vitro*, as 2 days of treatment are required to induce glycerol release in culture adipocytes (Trujillo et al., 2004). Altogether, these data are therefore challenging the role of IL-6 in acute stimulation of adipose tissue lipolysis during exercise.

Fibroblast growth factor-21 is a new member of the fibroblast growth factor (FGF) family, which has been discovered in the early 2000s. Even if it has been primarily identified in the liver, FGF-21 is expressed in mouse and human skeletal muscle and seems to be regulated by insulin (Hojman et al., 2009). Circulating FGF-21 levels increases in response to a 2-week endurance training program (Cuevas-Ramos et al., 2012).

FGF-21 displays anti-diabetic effects in mice through the stimulation of glucose transport in adipose tissue (Kharitonenkov et al., 2005) and stimulates lipolysis and thermogenesis in BAT (brown adipose tissue) (Inagaki et al., 2007; Hondares et al., 2010). FGF-21 has been described to regulate the expression of PGC-1α and favor the thermogenic program and browning in white adipose tissue (Fisher et al., 2012), as recently reviewed (Cuevas-Ramos et al., 2019). FGF-21 exhibits the characteristics of a metabolic myokine inducing an increase in energy expenditure and an improvement of insulin sensitivity in response to chronic exercise. It is important to note that some authors recently caught attention on potential detrimental effects of FGF-21 at high circulating plasma concentrations (Tezze et al., 2019). FGF-21 has been proposed as a biomarker of mitochondrial dysfunction in skeletal muscle and used as a predictor of disease progression. For instance, high circulating levels of FGF-21 are associated with high rates of mortality in end-stage chronic kidney diseases (Kohara et al., 2017).

Irisin is a myokine, which has been recently identified by the group of Bruce Spiegelman (Bostrom et al., 2012). As previously discussed, PGC-1α is a transcriptional co-activator induced by physical exercise, which activates mitochondrial biogenesis and muscle oxidative capacity. The authors hypothesized that PGC-1α stimulates the secretion of specific myokines, which are able to modulate the function of other organs. They identified irisin, which is the product of proteolytic cleavage of the membrane protein fibronectin type-III domain-containing protein 5 (FNDC5). FNDC5 is largely overexpressed in skeletal muscle of MCK-PGC-1α mice. Interestingly, browning of subcutaneous adipose tissue occurs in MCK-PGC-1α mice and a sharp increase in thermogenic genes such as uncoupling protein 1 (UCP1) and cell death-inducing DFFA-like effector A (CIDEA) is observed. The authors also showed that the treatment of differenciated primary adipocytes with recombinant irisin or with conditioned media from MCK-PGC-1α mice primary myocytes induces the expression of UCP1 and CIDEA *in vitro*. Irisin also stimulates the expression of PGC-1α and PPARα as well as basal and uncoupled respiration *in vitro* in murine subcutaneous adipocytes primary cell culture. The molecular mechanism by which irisin induces UCP1 could involve a partnership between PGC-1α and PPARα. Finally, a short 10-day treatment of wild-type mice with irisin induces an increase in UCP1 expression and the browning of subcutaneous adipose tissue. This treatment seems sufficient to increase total energy expenditure, slightly decrease fat mass and improve insulin sensitivity. These data seem to be physiologically relevant as irisin muscle expression and plasma level are increased in humans in response to a 10-weeks endurance training program or in older individuals (Timmons et al., 2012). However, the authors of this latter study did not observe significant variations in muscle irisin expression in younger subjects or in response to a resistance training program. Another longitudinal study also confirms the rise in circulating irisin level in response to an endurance training program in young healthy subjects (Huh et al., 2012). Altogether, irisin seems to be a metabolic myokine able to stimulate white adipose tissue thermogenesis, and thus contributes to energy balance control in response to physical activity in mice. This polypeptide may have a potential therapeutic interest in the management of obesity and its metabolic complications. However, the relevance of irisin in humans remains highly controversial, as some research groups have pointed out that the commercially available antibodies used to detect irisin might be unspecific (Albrecht et al., 2015). In addition, Jürgen Eckel's group demonstrated that the translation of mice studies to humans might be compromised due to the presence of a stop codon in the gene encoding the precursor of irisin in humans (Raschke et al., 2013). Finally, other studies conducted in different groups of subjects (i.e., young versus old, lean versus overweight) and assessing different exercise modalities did not report a consistent rise of muscle irisin mRNA or circulating levels with exercise training, except for highly active elderly subjects (Timmons et al., 2012; Norheim et al., 2014).

A study from Lee et al. (2014) has shown that both irisin and FGF21 are able to increase mitochondrial respiration and induce thermogenesis *in vitro* when applied on human brown adipocytes from neck fat biopsies. The authors further demonstrated a fat depot-specific effect of FGF21 and irisin (or a combination of both). The beiging effect was greater in neck adipocytes compared to subcutaneous white adipocytes, and no effect was observed in visceral adipocytes. These data are intriguing when compared to the now well-accepted higher beiging ability of subcutaneous adipose tissue compared to visceral adipose tissue in humans (Zuriaga et al., 2017). Because irisin and FGF21 are released by muscle during exercise, one could hypothesize that exercise enhances WAT beiging. If such an effect has been observed in mice (Stanford et al., 2015), it is not the case in humans (Norheim et al., 2014). The effect of exerkines on thermogenesis in humans remains therefore an open question and further studies need to characterize the impact of exerkines on both WAT beiging and BAT activation in humans (Lehnig and Stanford, 2018).

Myonectin, also known as C1q tumor necrosis factor α-related protein isoform 15 (CTRP15), was identified as a nutrient-sensitive myokine by Seldin et al. (2012). It has been demonstrated that myonectin is an exercise training-responsive myokine. Myonectin plasma level is increased in response to an 8-week aerobic exercise-training program in humans (Pourranjbar et al., 2018) and muscle myonectin gene and protein expression are both induced after treadmill endurance exercise in mice (Rabinovich-Nikitin and Kirshenbaum, 2018). Myonectin has been shown to target adipose tissue, but its metabolic role in response to muscle contraction has not been clearly established yet. Myonectin deletion impairs lipid handling resulting in an increased adipose tissue mass in diet-induced obese mice. However, these changes are independent of modifications in adipose tissue lipolysis in myonectin knockout mice, and are due to increased lipid uptake into adipocytes in postprandial conditions (Little et al., 2019). Furthermore, myonectin does not seem to be required for the physiological responses to exercise in mice (Little et al., 2019). Therefore, its role in the context of exercise warrants further investigations.

Collectively, adipose tissues appears to be important targets of myokines controlling energy fluxes and fuel availability to the contracting muscle. Some of these myokines can also

improve adipocyte oxidative capacity and glucose uptake, a physiological adaptation contributing to endurance exercise-mediated metabolic health improvement. These myokines also seem to activate thermogenesis through a direct action on BAT. However, caution should be taken when interpretating the relevance of thermogenesis in mice, since some studies are not performed at thermoneutrality but instead at room temperature (i.e., almost 21°C). The thermal stress induced by temperature below thermoneutrality increases metabolic rate beyond what is observed in humans in the resting state. Therefore caution should be taken when translating phenotypes in mice to humans.

Pancreas

We recently observed a significant increase in glucose-stimulated insulin secretion from isolated mice islets in response to conditioned media from resting non-treated human primary myotubes. This indicates that skeletal muscle cells produces basal rates of secreted factors capable of modulating insulin secretion (Mizgier et al., 2017).

Previous studies suggested a potential crosstalk between skeletal muscle and pancreatic β-cell through IL-6 (Handschin et al., 2007; Ellingsgaard et al., 2011). In the first study, authors observed an elevated IL-6 secretion rate from muscle-specific PGC-1α knockout mice. They further showed that this translated into higher plasma IL-6 levels disrupting insulin secretion by pancreatic β-cell. In contrast, the study by Ellingsgaard et al. (2011) suggested that IL-6 released by contracting muscle can induce glucagon-like peptide 1 secretion by intestinal L cells and pancreatic α-cells, which consequently promotes insulin secretion. The discrepancy between the two studies may lie in the various physiological contexts behind the studied mouse models. In a context of systemic inflammation and lipotoxicity, chronic elevation of IL-6 could have detrimental effects on insulin secretion by altering the positive regulation of β-cell by other hormones. Physiologically, insulin secretion is expected to decrease during acute exercise to relieve a break on lipolysis and hepatic glucose production. Controversies regarding the role of IL-6 as a myokine on insulin secretion therefore still exist and further research is needed.

In a recent study, it was shown that the PGC1α-dependent myokine irisin (FNDC5) behaves as a novel pancreatic β-cell secretagogue (Natalicchio et al., 2017). Interestingly, irisin enhanced insulin biosynthesis and glucose-stimulated insulin secretion in human and rat pancreatic β-cells. More interestingly, human skeletal muscle cells cultured under lipotoxic conditions increased irisin secretion. These conditioned media containing irisin were able to reduce β-cell apoptosis under lipotoxic stress induced by palmitate treatment. Altogether, these data show that irisin promotes β-cell survival and contributes to a crosstalk between skeletal muscle and β-cell under lipotoxic stress.

In conclusion, these studies highlight the existence of a crosstalk between skeletal muscle and pancreatic β-cell, which may be relevant in physiological conditions and diseases state.

Liver

Pedersen and Febbraio nicely showed that recombinant human IL6 infusion in lean healthy subjects during moderate intensity exercise increases hepatic glucose production to the same extent as those observed during high intensity vigourous exercise (Febbraio et al., 2004). This demonstrates that IL-6 has the ability to enhance hepatic glucose production when released by contracting skeletal muscle during exercise. Seldin et al. (2012) showed that myonectin improves systemic lipid metabolism by increasing liver fatty acid uptake through up-regulation of fatty acid transporter genes such as cluster of differentiation 36 (CD36), fatty acid transport protein (FATP) and fatty acid binding protein (FABP) (Seldin et al., 2012). In summary, only few exerkines have been shown to modulate liver functions. Future studies will likely identify novel exercise-regulated myokines capable to crosstalk with the liver.

Brain

The brain plays a central role in the integration of whole-body afferent signals (nervous, hormonal, and nutritional), including muscle signals in the context of exercise, and sends downstream efferent messages to peripherical metabolic organs. A number of myokines including cathepsin B, irisin, BDNF and FGF-21 have been suggested to play a role in the muscle-brain crosstalk (Delezie and Handschin, 2018). They do not seem to induce efferent signals to peripherical metabolic organs but rather to mediate exercise-induced improvement of brain functions such as cognition, memory, neuroplasticity, motor coordination, sleep, and mood. Some of these effects are mediated through neuroprotection and reduction in brain inflammation. This goes beyond the scope of this review and has been detailed elsewhere (Moon et al., 2016; Delezie and Handschin, 2018; Kim et al., 2019). FGF-21 seems to be able to cross the blood-brain barrier and to modulate sympathetic nerve output to brown adipose tissue in rodents (Owen et al., 2014). Further studies are required to better understand the muscle-brain crosstalk and brain-mediated effects of these myokines on remote metabolic organs, particularly in humans.

IMPAIRED MYOKINE SECRETION AS A TRIGGER OF ADVERSE HEALTH EFFECTS LINKED TO SEDENTARY BEHAVIORS?

It is well established that physical inactivity (<150 min/week of moderate-to-vigorous physical activity) and sedentary behaviors like sitting increase the risk of type 2 diabetes, a number of cancers, cardiovascular diseases and other chronic diseases. Because myokines allow muscle contractions to modulate other tissues through a pathway independent of the nervous system (Kjaer et al., 1996), the adverse health effects of physical inactivity and sedentary behaviors may result from the lack of muscle contraction. Given the anti-inflammatory effects of the myokines secreted in response to exercise, physical inactivity may lead to inflammation and inability to store fat in subcutaneous adipose tissue. Muscle unloading during bed-rest studies has been associated in healthy adults with a shift toward pro-inflammatory conditions (Rudwill et al., 2013;

Mutin-Carnino et al., 2014) and an increase in visceral adipose tissue (Belavy et al., 2014). While concomitant exercise can prevent the increase in pro-inflammatory markers (Rudwill et al., 2013; Mutin-Carnino et al., 2014), no effect of resistive exercise was reported on body fat redistribution (Belavy et al., 2014). Similarly, resistive exercise training did not prevent the development of insulin resistance, hypertriglyceridemia and alterations in nutrient oxidation by bed-rest-induced physical inactivity (Bergouignan et al., 2006). These metabolic alterations were, however, mitigated when aerobic exercise was performed in addition to resistive exercise suggesting either the existence of a dose-response relationship between the number of muscle contractions and the health benefits, or that other factors such as activity or total energy expenditure have effects on metabolism in addition to or in synergy with muscle contractions. Further studies are warranted to better understand the respective contribution between lack of muscle contraction and low energy expenditure in the development of metabolic diseases associated with physical inactivity.

WHERE DO WE GO FROM HERE?

One of the major challenges in the next few years will be to address whether the findings observed in animal and cell models can be translated to human physiology. In addition, many novel myokines still remain to be identified and the currently described set of myokines probably only reflects the tip of the iceberg. Given the beneficial effects of exercise on adipose tissue remodeling, we believe research efforts should be placed on the identification of novel exercise-induced myokines that may control adipose tissue metabolism and function. As physical activity is known to influence appetite and energy intake (Beaulieu et al., 2016; Thivel et al., 2016), understanding the role of the muscle secretome on the central control of appetite and energy intake is another important field of research to pursue. Finally, it is still unclear whether all myokines are required to confer the beneficial adaptations to exercise. Future studies should better characterize the respective role of each myokine and assess their potential additive or synergistic contribution to whole-body phenotype.

CONCLUSION

Skeletal muscle behaves as an endocrine organ through the secretion of myokines and modulates the function of key metabolic organs in response to exercise. This original crosstalk between skeletal muscle and metabolic organs provides a conceptual basis to understand how exercise lowers the risk to develop several chronic diseases and increases health- and life-span. Highlighting the potential therapeutic role of novel metabolic myokines emphasizes the importance of inter-organ crosstalk mediated by exercise. It now seems well established that some exerkines mediate the beneficial physiological adaptations of exercise in the control of energy homeostasis and insulin sensitivity. It is therefore reasonable to think that physical inactivity and conditions altering muscle integrity (neuromuscular diseases, aging...) impair the secretion of various metabolic myokines, favoring in the long term the development of chronic diseases such as obesity, diabetes and cardiovascular diseases.

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

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

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Plasma Myokine Concentrations After Acute Exercise in Non-obese and Obese Sedentary Women

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Exercise and physical activity levels influence myokine release from skeletal muscle and contribute to circulating concentrations. Indeed, many myokines, including interleukin (IL)-6, IL-15, secreted protein acidic rich in cysteine (SPARC), and fibroblast growth factor (FGF) 21 are higher in the circulation after an exercise bout. Since these peptides modulate muscle metabolism and can also be targeted toward other tissues to induce adaptations to energy demand, they are of great interest regarding metabolic diseases. Therefore, we set out to compare, in six women with obesity (BMI $\geq$30 kg/m^2) and five healthy women (BMI 22–29.9 kg/m^2), the effect of an acute bout of moderate-intensity, continuous cycling exercise (60 min, 60% VO_2peak) on the release of myokines (IL-6, IL-8, IL-10, IL-13, IL-15, SPARC, and FGF21) in plasma for a 24-h time course. We found that plasma IL-8 and SPARC levels were reduced in the group of women with obesity, whereas plasma IL-13 concentrations were elevated in comparison to non-obese women both before and after the exercise bout. We also found that plasma FGF21 concentration during the 24 h following the bout of exercise was regulated differently in the non-obese in comparison to obese women. Plasma concentrations of FGF21, IL-6, IL-8, IL-15, and IL-18 were regulated by acute exercise. Our results confirm the results of others concerning exercise regulation of circulating myokines while providing insight into the time course of myokine release in circulation after an acute exercise bout and the differences in circulating myokines after exercise in women with or without obesity.

Keywords: myokines, exercise, interleukins, FGF21, secreted protein acidic rich in cysteine, training, obesity, skeletal muscle

INTRODUCTION

Since the beginning of the 2000s, skeletal muscle has gained notoriety as an important source of secreted factors such as peptides, RNAs, metabolites, and extracellular vesicles that can contain any of these molecules. These factors are significant regulators of whole-body energy metabolism. The peptides secreted by the skeletal muscle are called myokines, as many of these peptides are also cytokines [e.g., the interleukins (IL)] (Pedersen et al., 2003). Myokines have been studied not only

for their effect on skeletal muscle metabolism but also as endocrine effectors of energy metabolism adaptions (Nielsen and Pedersen, 2007; Garneau and Aguer, 2019).

During exercise and/or muscle contraction, the levels of some myokines in the muscle are greatly increased, and circulating levels of these peptides are also modulated by exercise. For example, after an acute bout of moderate-intensity cycling on a stationary bicycle (70% VO_2max), IL-6 and fibroblast growth factor (FGF) 21 expression was increased in skeletal muscle, while serum levels of IL-6, IL-15, and FGF21 were significantly increased (Sabaratnam et al., 2018). The effect of exercise on the secretion of myokines from skeletal muscle locally cannot necessarily be translated to the release of these peptides in the circulation, as reviewed in Garneau and Aguer (2019). Indeed, some myokines are believed to act directly on skeletal muscle to improve its energy metabolism during contraction. For example, IL-6 increases muscle fatty acid oxidation and glucose uptake in L6 myotubes and human primary muscle cells from *rectus abdominis* muscle (Al-Khalili et al., 2006; Carey et al., 2006), while IL-13 treatment directly stimulates glucose uptake and oxidation in human primary myotubes (Jiang et al., 2013). On the contrary, the mechanism of action of myokines can also require their release in the circulation to be delivered to their target tissue(s). Examples of such mechanisms include the stimulation of fatty acid oxidation and glucose uptake into white adipose tissue by IL-6 during exercise recovery (Knudsen et al., 2017) and the positive effect of IL-13 on both rat and human pancreatic β-cell survival (Rutti et al., 2016). This underscores the different mechanisms of action of myokines depending on their release locally in skeletal muscle [muscle protein and messenger RNA (mRNA)] and in the circulation (plasma or serum protein). The metabolic functions of myokines draw a link between exercise adaptations and amelioration of muscle and whole-body metabolisms, which is of interest in the context of obesity, as it is an important risk factor for the development of type 2 diabetes (T2D) (Muoio and Newgard, 2008).

In patients affected with certain chronic non-communicable diseases (e.g., sarcopenia, arthritis, obesity, and T2D), circulating myokine levels are altered. These diseases are generally associated with systemic, low-grade inflammation (Gregor and Hotamisligil, 2011). For example, IL-6 and FGF21 were found to be elevated in patients with obesity and correlated with whole-body adiposity (Vozarova et al., 2001; Zhang et al., 2008); circulating IL-8 and secreted protein acidic rich in cysteine (SPARC) correlated positively with BMI (Kim et al., 2006; Wu et al., 2011); while plasma IL-15 not only correlated negatively with trunk fat mass and body-fat percentage (Nielsen et al., 2008) but have also been shown to be elevated in the setting of obesity (Perez-Lopez et al., 2018). It is well-established that acute and chronic exercise can modulate circulating myokine levels. However, it is currently unclear if the presence of obesity (BMI >30 kg/m^2) can impact the myokine response in sedentary subjects. In this regard, our study design allows us to observe the potential effects of obesity on the variations in circulating myokines following an acute bout of exercise.

Here, we evaluate the dynamic and time-dependent changes in plasma myokine concentrations [pre-, immediately after an acute bout of moderate-intensity cycling exercise (0 h), 1, 2, 3, 4, 12, and 24 h postexercise] in sedentary women with and without obesity.

MATERIALS AND METHODS

Participants and Study Design

A total of 11 women, 18–40 years of age, were included in this study; six women were classified as obese (BMI >30 kg/m^2), and five were classified as non-obese (BMI = 22–29.9 kg/m^2). Other inclusion and exclusion criteria were as follows: weight stable (<3 kg variations in the last 8 weeks); not involved in any exercise program; willing to stop caffeine and alcohol consumption 48 h before blood draw; no history of T2D; glycated hemoglobin (HbA1c) ≤ 6.5% (measured in the obese group only); favorable anatomy for continuous venous blood sampling; no presence of clinically significant abnormalities on ECG; no significant renal, cardiac, liver, lung, or neurological disease (controlled hypertension acceptable); no use of drugs known to affect metabolism or body weight (e.g., orlistat, sibutramine, ephedrine, phenylpropanolamine, corticosterone); current treatment with blood thinners or antiplatelet medications that cannot be stopped safely; no new onset (<3 months) of oral contraceptives or hormone replacement therapy; not current smokers (>3 months); not currently pregnant or having nursed a child within the last 12 months; no gait problems; no increased liver function tests (aspartate aminotransferase/alanine aminotransferase/gamma-glutamyl transferase or alkaline phosphatase >2.5 times the upper limit of normal); no in-body metal objects that would interfere with body composition measurements; no New York Heart Association any class heart failure; no history of deep vein thrombosis or pulmonary embolism; no significant varicose veins; no abnormal blood count or blood donations in the last 2 months; no major surgery of the abdomen, pelvis, or lower extremities in the last months; no bariatric surgery or liposuction within the previous 3 years; no cancer; no rheumatoid disease; no bypass graft in limb; no known genetic factor (e.g., Factor V Leiden) or hypercoagulable state; no diagnosed peripheral arterial or vascular disease or intermittent claudication; no peripheral neuropathy; no claustrophobia; no major depression; no presence of an eating disorder or eating attitudes/behaviors that could interfere with the study; and no nocturnal urination and/or sleep apnea.

Two days before performing the exercise bout, participants underwent a diet stabilization period and were given standard diet composition meals (35% fat, 15% protein, and 50% carbohydrates; prepared and prepacked at the exercise facility). The day before the exercise test, participants came fasted at the facility, underwent all baseline measures, then ate the standardized diet for breakfast and lunch. At night, they consumed a high-carbohydrate dinner and snack (70–75% carbohydrate, 12–15% fat, 10% protein), then performed the cycling exercise bout at around 7 AM the following morning. Plasma samples from these participants were obtained immediately before (pre-), immediately after (0 h), and 1, 2, 3, 4, 12, and 24 h into the recovery period after a fasted acute

bout of moderate-intensity continuous exercise. The exercise bout consisted of 60 min of cycling on an upright stationary bicycle (Vision Fitness U40, Wisconsin, United States) at 60% VO_2peak. Participants remained fasted for the first 4 h of recovery with limited liquid for consumption (600 ml) and were fed meals of standard diet composition (35% fat, 15% protein, and 50% carbohydrates) after the "4 h" blood draw, with limited total liquid consumption of 1,000 ml. Blood samples were collected with a venous catheter in standard anticoagulant (ethylenediaminetetraacetic acid) tubes, centrifuged at 2,000 × *g* for 10 min, aliquoted, and stored at −80°C until analyses.

Body Composition, Blood Analyses, and VO_2max Determination

Whole-body fat and lean mass were assessed through dual energy X-ray absorptiometry scans (GE Lunar iDXA whole-body scanner, GE Healthcare, United States). Glucose, insulin, and HbA1c were measured in fasted blood samples in the clinical chemistry laboratory at either Florida Hospital or onsite at the Translational Research Institute for Metabolism and Diabetes, as previously described (Stephens et al., 2018) to assess the eligibility of the participants. An HbA1c ≥ 6.5% would have resulted in the exclusion of the participant from the study. Glucose and insulin levels were used to determine homeostatic model assessment of insulin resistance (HOMA-IR) values {HOMA = [(fasting insulin, IU/ml) × (fasting glucose, mmol/L)]/22.5}, which attest of the sensitivity to insulin, therefore of the metabolic health of the participants (Antuna-Puente et al., 2011). All tests were performed during the follicular phase of the participants' menstrual cycle to avoid the confounding effects of the hormonal surges during the other phases on circulating cytokine levels. Aerobic fitness was assessed through maximal oxygen consumption (VO_2max) incremental test on a cycle ergometer as in Costford et al. (2010), and VO_2max was reached if the respiratory exchange ratio increased to 1.10 or higher and the participant's heart rate increased to within 10 beats of the age-predicted maximum [208 - (0.7×age)] or the rate of perceived exertion was 17 or over on a scale of 6–20.

Myokine Quantification

Secreted protein acidic rich in cysteine (also known as osteonectin) and FGF21 were quantified by single plex assays from MesoScale Discovery (R-plex; MSD, Maryland, United States), whereas IL-6, IL-8, IL-10, IL-13, IL-15, and IL-18 were quantified by multiplex assay (U-plex; MSD) in plasma obtained from venous blood samples taken from the arm of the participants. Intra-assay coefficients of variations for the standards for SPARC, FGF21, and the multiplex were 10.00, 8.02, and 8.28%, respectively. Samples were measured only once; therefore, their intra- and interassay coefficients of variations cannot be calculated. All antibodies, with the exception of SPARC, were validated for target specificity. Information can be found on the datasheet of the different U-plex antibody sets[1] in the **Supplementary Table S2**.

Statistical Analyses

Data are presented as means ± standard deviation (SD). Anthropometric measures were analyzed by unpaired *t*-test or Welch's *t*-test (depending on the standard error values), while all myokine quantifications were analyzed by two-way ANOVA with repeated measures or mixed model analysis (when samples from certain time points were missing) along with Dunnett's multiple comparison, as *post hoc* tests and area under the curve (AUC) of all time points were assessed and were analyzed by unpaired *t*-test or Welch's *t*-test. All statistical analyses were performed with Prism 8 software (San Diego, CA, United States). A $p \leq 0.05$ was considered significant.

RESULTS

Characteristics of the Participants

The characteristics of the participants are presented in **Table 1**. No statistical differences were found between the two groups that performed the acute bout regarding age, blood glucose, insulin, insulin sensitivity (HOMA-IR), and VO_2max, but the BMI ($p < 0.05$), the fat mass ($p < 0.05$), and the total lean mass ($p < 0.01$) of the women from the obese group were significantly higher than the non-obese group. The HOMA-IR values in the two groups of non-obese or obese sedentary individuals suggest that these participants were insulin resistant.

Time Course of Plasma Myokine Concentrations in Response to Acute Exercise

In response to an acute cycling bout, FGF21, IL-6, IL-8, IL-15, and IL-18 concentrations were significantly altered in plasma of both obese and non-obese women subjects over 24 h after the exercise (**Figure 1**). For IL-6 and IL-18, these myokines increased transiently after the exercise bout and returned to pre-exercise values in the circulation within 24 h into the recovery period in both subject groups. On the contrary, IL-8 levels in plasma

[1]https://www.mesoscale.com/

TABLE 1 | Participant characteristics of women having completed a single, acute bout of moderate intensity continuous exercise.

Group	*n*	Age (years)	BMI (kg/m^2)	Fat mass (kg)	Lean mass (kg)	Glucose (mmol/L)	Insulin (μIU/ml)	HOMA-IR	HbA1c (%)	VO_2max (ml/min)
Non-obese	5	28.80 ± 8.41	27.38 ± 1.40	27.69 ± 4.95	38.16 ± 4.54	5.01 ± 0.43	14.04 ± 7.80	3.21 ± 1.92	–	1,606 ± 305
Obese	6	30.00 ± 6.93	35.43 ± 5.44†	45.93 ± 12.43†	50.22 ± 2.84††	5.02 ± 0.32	14.48 ± 4.96	3.28 ± 1.30	5.73 ± 0.33	1,817 ± 282

Data are means ±SD. $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ in comparison to non-obese group.

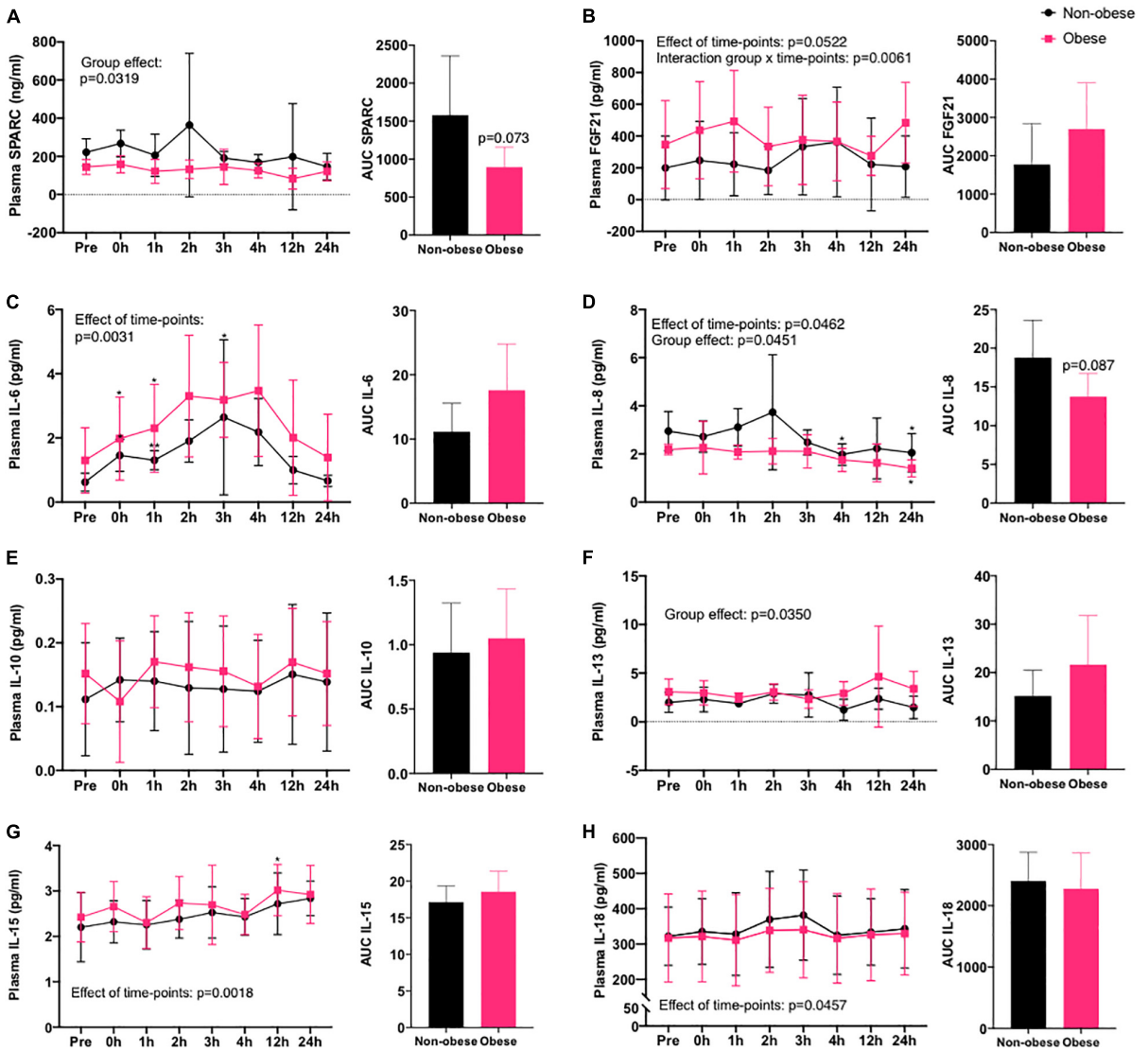

FIGURE 1 | Myokines in circulation after an acute bout of exercise in women with or without obesity. Time course of concentrations of myokines in plasma of non-obese and obese women following 60 min of cycling at 60% VO_2peak at eight different time points (pre-, immediately after (0 h), 1, 2, 3, 4, 12, and 24 h into recovery) and AUC of all time points. **(A)** SPARC, **(B)** FGF21, **(C)** IL-6, **(D)** IL-8, **(E)** IL-10, **(F)** IL-13, **(G)** IL-15, and **(H)** IL-18. Non-obese group, $n = 5$ (black circles) and obese group, $n = 6$ (pink squares). $^*p < 0.05$, $^{**}p < 0.01$ in comparison to pre-exercise (pre), effect of time points relates to variations after the exercise bout and group effect relates to BMI classifications.

decreased gradually over the 24 h following the exercise bout, reaching significance at the 4 and 24 h time point, while IL-15 levels increased continuously to reach levels higher than basal after 12 h into recovery. No significant effect of the exercise bout was detected on plasma levels of SPARC, IL-10, and IL-13 over the 24 h of blood collection. IL-6 was the only myokine significantly increased in plasma immediately after the exercise bout, and the absolute increase relative to baseline was similar in both groups. At all time points (basal levels and levels in response to the exercise bout), SPARC and IL-8 levels in plasma were lower in the obese group, while IL-13 levels were higher in obese women in comparison to non-obese subjects. Quantification of the AUC indicated a trend toward decreased overall IL-8 ($p = 0.087$) and SPARC ($p = 0.073$) in the obese group. Finally, a significant interaction between obesity status and the variation in FGF21 levels following the exercise bout was detected, suggesting the rise in levels of this myokine in plasma after acute exercise was earlier in the participants with obesity, and they remained elevated after 24 h rather than returning to basal as in the non-obese group.

DISCUSSION

This study focused on the comparison in plasma myokine levels in women with obesity vs. women without obesity after a bout of moderate-intensity continuous cycling. Plasma myokines were

assessed over a 24-h time course following the acute exercise to account for the different timing of secretion of individual peptides. Most of the myokines measured were regulated by acute exercise (FGF21, IL-6, IL-8, IL-15, and IL-18), and some were released at different levels in plasma over the 24 h in the group of women with obesity in comparison to the non-obese group (SPARC, IL-8, and IL-13). The regulation of plasma FGF21 following acute exercise was different in the non-obese group in comparison to the obese group.

IL-6

Regarding IL-6, the magnitude and the time course of the increase in plasma IL-6 following the exercise bout was similar to a previous study in which normal-weight and obese subjects had completed 30 min of moderate-intensity aerobic exercise (Slusher et al., 2015). In muscle biopsies from healthy individuals taken at different time points following a running bout, IL-6 mRNA expression was found to be induced immediately by exercise (Louis et al., 2007). These results align with the variations in circulating IL-6 levels that we observed over the 24 h following the acute exercise bout completed by our sedentary participants. As reviewed by Lombardi et al., acute increases in IL-6 secretion are better detected in plasma than in serum, further highlighting the relevance of our results (Lombardi et al., 2017).

IL-8

No increase in plasma IL-8 was detected after the cycling bout, although others found increased muscle IL-8 expression after a running exercise (Louis et al., 2007). This suggests that the increased expression of IL-8 during muscle contraction might result in the release of the protein within the muscle *interstitium*. These results support the hypothesis of Nielsen and colleagues regarding the potential autocrine role of IL-8 in muscle metabolic adaptations to physical activity (Nielsen and Pedersen, 2007). On the other hand, IL-8 levels are higher in serum than in plasma (Lombardi et al., 2017). Perhaps, differences in circulating IL-8 levels after the exercise bout would have been more significant in serum than in plasma, as this myokine seems to be released primarily in that fraction of the blood. Circulating resting and exercise-induced IL-8 was reduced in participants from the obese group, which could potentially be explained by their body composition, as women in the non-obese group had significantly less total lean and fat mass. Since myokines were measured solely in the circulation, we cannot hypothesize on the effect of either tissue to explain this difference between the study groups.

IL-10

To our knowledge, no studies have been published on the effect of exercise on circulating levels of IL-10 in metabolically healthy subjects. On the other hand, in support of our findings, others found no effect of acute exercise on plasma IL-10 levels in patients with T2D (Korb et al., 2018). Since this myokine has been shown to have a positive effect on skeletal muscle metabolism (Hong et al., 2009; Dagdeviren et al., 2016), perhaps its mechanism of action in response to exercise is autocrine and unrelated to circulating concentrations. This hypothesis would support our findings, as well as those of others, regarding the lack of response to an acute bout of exercise in circulating IL-10. A more molecular analysis of the signaling mechanisms in muscle *per se* would be required to elucidate the effects of exercise on muscle IL-10 secretion and/or expression.

IL-13

Concerning IL-13, plasma levels were higher in the obese in comparison to the non-obese group at rest and in response to the acute bout of exercise. Others have shown that serum and muscle IL-13 is reduced in non-obese patients with T2D, a common comorbidity of obesity (Jiang et al., 2013). Further investigations would be required to assess how obesity and/or T2D affect circulating IL-13. Our results for plasma IL-13 in response to acute exercise showed great variability, preventing us to draw any clear conclusion. In many samples, IL-13 was undetected, which affected the power to detect any significant differences between groups or in response to exercise.

IL-15

Our results demonstrated that IL-15 circulating levels were not immediately increased after the bout of cycling, although this myokine was previously found to be higher in plasma after an acute bout of cycling exercise (Sabaratnam et al., 2018). In their study, plasma IL-15 levels returned to lower levels than baseline 3 h into recovery, while we show a steady increase as far out as 12 h. These discrepancies could be explained by the fact that our participants were women, while theirs were men. On another note, an acute running bout in healthy participants induced a gradual increase in muscle IL-15 mRNA expression over the 24 h following exercise, aligning with our results (Louis et al., 2007). Furthermore, IL-15 can be found in circulation in its free form or complexed to a soluble form of the alpha subunit of the IL-15 receptor (sIL-15Ralpha), which may alter IL-15 activity depending on the target cells as discussed in Nadeau and Aguer (2019). The kit used in the study mentioned previously shows ∼21% cross-reactivity with the complexed form of IL-15 according to the manufacturer (Human IL-15 Quantikine ELISA Kit, R&D Systems Inc., MN, United States), while the one we used (MSD) showed average reactivity at physiologically relevant doses (1.1–17.2 pg/ml) of ∼38% (**Supplementary Figure S1**). Since the half-life of free IL-15 is relatively short (∼30 min) and increases when IL-15 is complexed to sIL-15Ralpha (20–25 h), it is likely that the increase we observed after acute exercise lasting into the recovery period was due to the recognition of the complex, as discussed in Nadeau and Aguer (2019). Further studies would be required to better understand the mechanisms of free IL-15 and IL-15/sIL-15Ralpha complex secretion in response to exercise. Moreover, resting plasma IL-15 levels were found to be lower in physically active individuals in comparison to sedentary subjects and even higher in sedentary obese patients (Perez-Lopez et al., 2018). Therefore, we anticipated that the group of women with obesity would show higher plasma IL-15, but we found no significant differences between the two groups. Since Perez-Lopez and colleagues used the kit mentioned previously to detect IL-15, we again speculate that the differences in our results stem from the detection of the complexed form of IL-15 in our assay. Finally, the matrix does not affect IL-15 recovery when

measuring this myokine in the circulation, which suggests that the results would have been similar if serum samples had been analyzed (Lombardi et al., 2017).

SPARC

Circulating SPARC levels were unaffected immediately after an acute exercise bout, although others found an increase in serum SPARC in rats after acute exercise that mirrored increased muscle protein content (Matsuo et al., 2017). The same group also found a transient increase in serum SPARC in humans after a bout of high-intensity interval exercise or moderate-intensity continuous exercise. Data relating to preanalytical parameters for the quantification of SPARC in the circulation are lacking; it is possible, however, that variations in SPARC levels after exercise are better detected in serum than plasma. Nevertheless, others showed that a single 20-s "all-out" cycling sprint induced a significant rise in serum SPARC but that correcting circulating SPARC levels with the exercise-induced changes in plasma volume negated this effect (Songsorn et al., 2017). This finding highlights the importance of measuring hematocrit and hemoglobin levels pre- and postexercise to verify that the potential exercise-induced alterations in circulating protein concentrations are not due to the changes in plasma volume after exercise (Alis et al., 2015; Dill and Costill, 1974).

FGF21

FGF21 is primarily expressed and produced by the liver (Nishimura et al., 2000), but its expression is also induced in skeletal muscle during exercise, and serum FGF21 increases following an aerobic exercise bout (Tanimura et al., 2016). We observed no significant increase in plasma FGF21 after the cycling bout in either groups, which confirms the findings of others regarding changes in plasma or serum FGF21 after exercise in patients with obesity, but contradicts the results obtained in healthy participants (Slusher et al., 2015; Sabaratnam et al., 2018). In normal weight (BMI = 18.5–24.9 kg/m^2) men and women, FGF21 was found to be acutely increased in plasma after exercise; however, since our sedentary group had an average BMI classified as overweight (25–29.9 kg/m^2), perhaps the response in FGF21 secretion was altered similarly as in patients with obesity. Of note, the average circulating FGF21 levels detected in our groups were slightly elevated compared to values from previous studies. Hansen et al. (2015) compared the effect of exercise on FGF21 release from the liver in comparison to leg muscle and found no significant release of this protein in the circulation from the skeletal muscle, whereas the splanchnic bed secreted up to fourfold more FGF21 after acute exercise. These findings confirm the hypothesis that skeletal muscle is not the main source of circulating FGF21, even during exercise.

Short-Comings and Limitations

Our study design did not allow for the measurement of myokine secretion directly from skeletal muscle, as similar peptides can be secreted by other tissues (e.g., adipose tissue, liver, brain, etc.) (Garneau and Aguer, 2019; Weigert et al., 2019). Therefore, we cannot say for certain that all of the measured peptides originated from skeletal muscle in response to exercise. However, we found no correlations between any of the myokines and fat mass or BMI (data not shown), suggesting that adipose tissue is likely not responsible for any of the observed effects of acute exercise on variations in circulating myokines. Nevertheless, it is possible that other organs, such as the liver, might have contributed to the changes in circulating peptides levels following the cycling bout, as discussed regarding FGF21. A solution to this problem is the use of skeletal muscle biopsies to quantify myokine secretion directly from the muscle (Garneau and Aguer, 2019). However, myokines measured in skeletal muscle cannot be assumed to originate solely from muscle secretion, as the peptides could be secreted by other tissues to act on skeletal muscle during exercise. In addition, increased levels of a peptide in muscle after exercise does not necessarily translate into its release in the circulation.

Although some of the myokines measured are more stable and/or better detected in plasma, others are ideally quantified in serum. Therefore, changes in myokine secretion in the circulation might go undetected by analyses performed solely in plasma samples. Another limitation of our analyses consists in the single measurement of every sample, preventing us to calculate intra- or interassay coefficients of variation for all myokines. In addition, exercise modalities might be great contributors to the outcome on muscle signaling. The combination of aerobic and resistance exercises confers more significant changes in glucose homeostasis and muscle substrate metabolism in patients with T2D and is therefore of great interest regarding muscle signaling adaptations to exercise (Church et al., 2010; Sparks et al., 2013). Similarly, interval exercise of elevated intensity such as Tabata exercises or sprint intervals has been shown to stimulate myokine release acutely in the circulation (Harnish and Sabo, 2016). Consequently, the plasma concentrations of myokines in the 24 h following an exercise bout might evolve differently as a function of the exercise modality performed.

Another confounding effect in our study design that could have affected the measurement of certain myokines that are also cytokines (the interleukins) is the use of a venous catheter for blood collection. This method was chosen, as it is less invasive and stressful than repetitive venipuncture for subjects undergoing eight blood draws over 24 h. However, others have shown that this method of blood sampling could induce local inflammation and affect levels of circulating cytokines to a greater extent than venipuncture (Dixon et al., 2009). In addition, it has been shown that differences in plasma volume can affect the analysis of exercise-induced circulating myokine variations (Songsorn et al., 2017). Since we did not have values for hematocrit and hemoglobin for the time points following the cycling bout, we were unable to measure plasma volume variations as a function of time during the recovery period and therefore could not correct circulating myokine levels to variations in blood composition (Dill and Costill, 1974). As we did not detect an increase in all the myokines measured in plasma following acute exercise, it is likely, nonetheless, that differences in plasma volume did

not cause erroneous conclusions regarding exercise-induced myokines. Moreover, interindividual variability is a major source of concern in observing the muscle signaling adaptations to acute or chronic exercise (He et al., 2018), and this could explain the impossibility to find an effect of acute exercise for certain peptides that we measured in plasma. Likewise, since our sample size was very small, some significant effects of exercise might have been undetected. This factor is the greatest limitation of our study. Regardless, we were able to find interesting effects of both acute exercise and obesity status on circulating target myokines among our study groups. Another limitation of our study is the fact that only women were represented, but this feature can also be considered positive, as women are generally underrepresented in the literature regarding exercise metabolism and muscle signaling.

Conclusion

Our findings relating to the time course of plasma myokine levels following acute exercise depending on BMI classification (obese or non-obese) shed light on the mechanisms of endocrine signaling after physical activity. We found a regulation of IL-6, IL-8, IL-15, IL-18, and FGF21 in plasma by exercise, while obesity status increased IL-13 and decreased IL-8 and SPARC in the circulation. The next step would be to assess the effects of acute exercise on muscle signaling directly in these two groups, for example, by measuring myokine secretion from muscle biopsies harvested before and after the exercise bout. In this sense, it would be possible to compare signaling adaptations in participants with or without obesity in the circulation with the ones observed locally in skeletal muscle and potential correlations or distinctions between myokine regulation at both levels could be made as a function of body composition (i.e., BMI).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at AdventHealth Orlando. The Institutional Review Board at AdventHealth Orlando approved this research for ethical standards, scientific merit, and regulatory compliance. The Office of Research Administration provided support and oversight to ensure the integrity of this research at AdventHealth Orlando. ClinicalTrials.org Identifier: NCT01911091. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LS and SS provided laboratories of samples. CA and LG performed sample analyses, data collection, and analyses at EM's laboratory facilities. All authors contributed to the review 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.2020.00018/full#supplementary-material

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Dipeptidyl Peptidase IV as a Muscle Myokine

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Dipeptidyl peptidase IV (DPP-IV) is a unique serine protease that exists in a membrane bound state and in a soluble state in most tissues in the body. DPP-IV has multiple targets including cytokines, neuropeptides, and incretin hormones, and plays an important role in health and disease. Recent work suggests that skeletal muscle releases DPP-IV as a myokine and participates in control of muscle blood flow. However, few of the functions of DPP-IV as a myokine have been investigated to date and there is a poor understanding about what causes DPP-IV to be released from muscle.

Keywords: peptidase, muscle, secretome, exercise, whey protein, metalloproteases

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INTRODUCTION

Dipeptidyl Peptidase IV (DPP-IV) is a serine protease that cleaves a variety of proteins that contain the X-Pro and X-Ala dipeptides including incretin hormones, many neuropeptides, and some cytokines (Mulvihill and Drucker, 2014). DPP-IV is present in a membrane bound form in most cells the body including the gut, kidneys, on the surface of T-cells, on the endothelial layer of arteries and arterioles and on skeletal muscle. The function of membrane bound DPP-IV in T-cells is well characterized (Boonacker and Van Noorden, 2003), but its action in other tissues is less well understood.

Dipeptidyl Peptidase IV can also exist as an active enzyme in a soluble form in the interstitial space and in the blood. The blood contains a concentration of soluble DPP-IV in the plasma at activities ranging from 12.5 to 42 U/L in the general population (Durinx et al., 2001) and from 13.6 to 73 U/L in a healthy younger group (Neidert et al., 2016c). The origin of blood DPP-IV is believed to be all of the sources of DPP-IV including the gut, kidneys, muscle, T-cells and fat (Mentlein, 1999). The most well understood purpose of DPP-IV in the plasma is truncating incretin hormones such as GLP-1 into a non-usable form and thus, altering insulin release (Batterham and Bloom, 2003). Inhibiting DPP-IV is a pharmacological treatment for diabetes and results in significant improvement in bioactive GLP-1 and insulin release (Lambeir et al., 2003). Additional benefits of DPP-IV inhibition are a reduction in TNF-alpha (Akarte et al., 2012) and a reduction in cytokines like IL-2, IL-6, and IL-10 in some studies (Klemann et al., 2016). DPP-IV inhibition also improves exercise capacity and mitochondrial function in mice with heart failure (Takada et al., 2016) and improves liver function in diabetic mice (Tanimura et al., 2019). On the negative side, DPP-IV inhibitors can cause severe joint/muscle pain and neuropeptide Y-mediated hypertension in some people (Klemann et al., 2016).

Despite the widespread presence of DPP-IV in the body, the actual functions of DPP-IV in most tissues is poorly understood. The purpose of this review is to investigate the current knowledge of DPP-IV in and around muscle and the current knowledge of the actions of DPP-IV on the muscle.

DPP-IV AND SKELETAL MUSCLE BLOOD FLOW

Neuropeptide Y is a powerful vasoconstrictor that plays an important role in skeletal muscle blood flow regulation in humans (Zukowska et al., 2003), dogs (Buckwalter et al., 2004, 2005), and rodents (Jackson et al., 2005a; Evanson et al., 2011; Al-Khazraji et al., 2015). One of the most well understood purpose of DPP-IV is the truncation the sympathetic vasoconstrictor, neuropeptide Y, into a form that does not result in Y1-receptor mediated vasoconstriction. DPP-IV is bound to the endothelium (Lewandowski et al., 1998; Kitlinska et al., 2002; Pala et al., 2012) and the smooth muscle (Chung et al., 2010; Evanson et al., 2011, 2012) of arterioles. DPP-IV is also found bound to the smooth muscle membrane of the abluminal surface of skeletal muscle arterioles (Chung et al., 2010), where it truncates approximately 40% of the neuropeptide Y (NPY_{1-36}) released from the sympathetic neurons, to become NPY_{3-36} (Evanson et al., 2011, 2012). This results in reduced NPY-mediated vasoconstriction and, in turn, improved blood flow to the skeletal muscle (Jackson et al., 2005a; Dubinion et al., 2006; Neidert et al., 2018). The effect of DPP-IV on NPY may be greater in females compared to males. Jackson et al. (2005b) saw a decrease of 34% in vascular conductance after blocking DPP-IV and aminopeptidase P. Work from my lab (Evanson et al., 2012) explored the idea that estrogen may be the cause of this difference between males and females with regard to DPP-IV. Sixty days of being ovariectomized with or without estrogen replacement did not change DPP-IV activity or alter neuropeptide Y degradation (Evanson et al., 2012). However, Younan et al. (2019) investigated the role of DPP-IV in liver inflammation after ovariectomy in rats. They found that DPP-IV inhibition reduced liver inflammation, but this study did not measure DPP-IV activity directly. In a recent study, we looked at normally menstruating women versus post-menopausal women and saw no difference in plasma DPP-IV activity (Kluess et al., 2019), demonstrating that the effect of estrogen appears to be consistent across species.

EVIDENCE THAT DPP-IV IS RELEASED FROM THE MUSCLE

Although plasma or serum DPP-IV is a widely used sampling location to measure DPP-IV, it is difficult to attribute plasma DPP-IV exclusively to muscle released DPP-IV. However, in a group of 111 people we found a positive relationship between plasma DPP-IV and lean mass measured by Dual X-ray Absorptiometry. This relationship explained about 14% of the variation, suggesting that in young healthy people a portion of the DPP-IV in the plasma likely does come from the muscle (Neidert et al., 2016c).

The evidence that muscle can release DPP-IV is quite recent. Raschke et al. (2013) showed that DPP-IV is released by skeletal muscle cell culture during differentiation. Neidert et al. (2016b) demonstrated that DPP-IV was released from skeletal muscle cell culture with the application of whey protein isolates, but only DPP-IV mRNA increased with exercise-like modulators such as caffeine, and hydrogen peroxide. Whey protein also increased DPP-IV activity in the bathing medium from an intact skeletal muscle *in situ* (Neidert et al., 2018). However, in this model we could not distinguish between skeletal muscle-released and smooth muscle-released DPP-IV. There are no studies to date investigating the release of DPP-IV from contracting muscle. It is very difficult to do this work *in vivo* because you cannot distinguish muscle released DPP-IV from DPP-IV released from other sources.

MECHANISM OF RELEASE OF DPP-IV FROM THE MUSCLE

The process of DPP-IV release from the muscle is a multi-step process. DPP-IV starts bound to the endoplasmic reticulum (Klemann et al., 2016). It then migrates to the muscle cell surface and remains in a functional, but membrane-bound state. Hooper et al. (1997a,b) first identified that phosphatidylinositol-specific phospholipase C could cause release of membrane dipeptidases. The idea that DPP-IV could be shed from the membrane by extracellular proteases was further refined by Röhrborn et al. (2014). They showed that the metalloproteases 1, 2, and 14 are involved in shedding DPP-IV from the membranes of smooth muscle and adipocytes. Neidert et al. (2016b) demonstrated that DPP-IV shedding occurs in skeletal muscle myocyte cell culture using whey protein (a source of metalloproteases) (Raulo et al., 2002; Lubetzky et al., 2010). This hypothesis was also confirmed using specific inhibitors for metalloprotease 2 and 9 and a general protease inhibitor. In a follow-up study (Neidert et al., 2018), we used whey protein to stimulate shedding of DPP-IV from skeletal muscle *in situ* and showed an increase in DPP-IV activity in the muscle bathing medium and an increase in skeletal muscle arteriolar diameter. This effect was inhibited by adding a DPP-IV inhibitor to the media bathing the preparation. This finding suggested that one reason for DPP-IV release from the membrane may be the reduction in neuropeptide Y-mediated vasoconstriction (see **Figure 1** for a diagram of the possible mechanism). DPP-IV may also be involved in shortening the half-life of some cytokines such as IL-6 (Ikeda et al., 2013), but there is no conclusive evidence for this. DPP-IV is known to target stromal cell derived factor 1α (Christopherson et al., 2002) and therefore may be involved in targeting T-cells to damaged skeletal muscle. To date this possibility has not been investigated.

EXERCISE AND EXERCISE TRAINING RELATED CHANGES IN DPP-IV

One of the challenges of measuring DPP-IV changes with exercise or exercise training is the ability to sample DPP-IV. The easiest method is to take a blood sample and measure DPP-IV in the plasma or serum. The downside of this is that the source of the change in DPP-IV may be a variety of sources such as adipose tissue, muscle, immune cells and other areas of the body influenced by exercise. A further

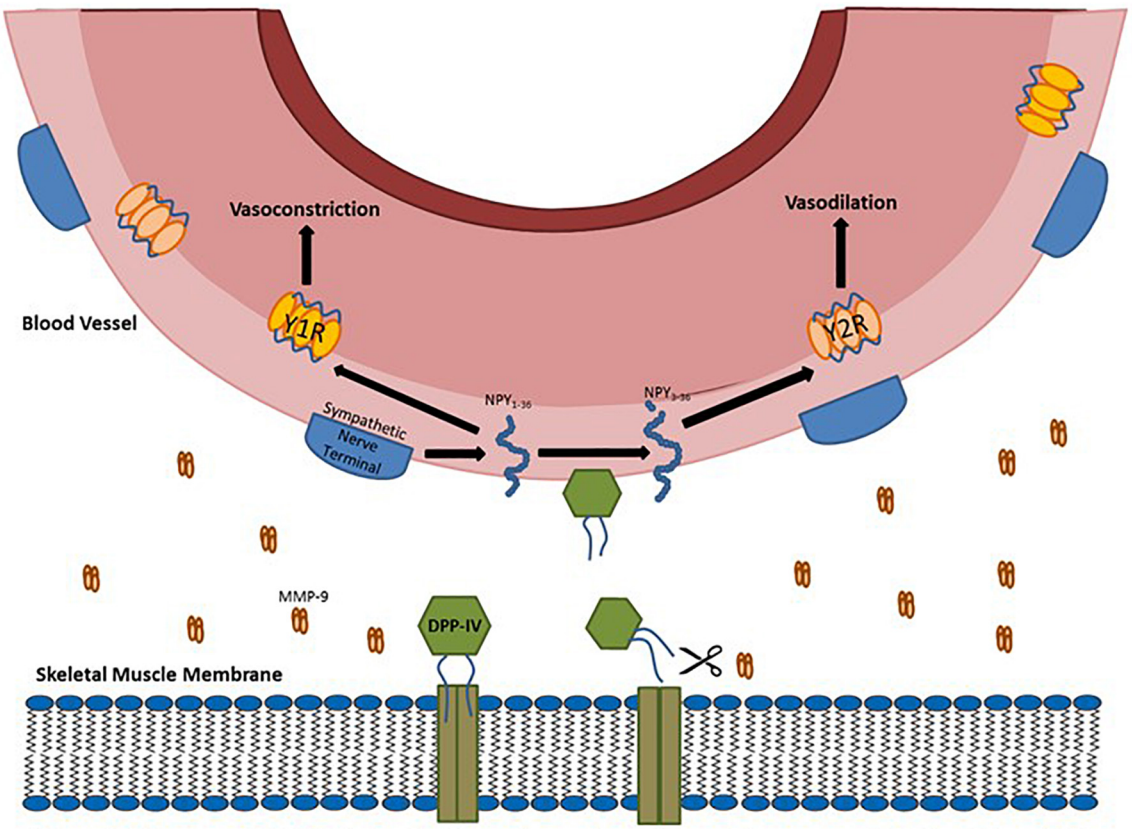

FIGURE 1 | Diagram of DPP-IV being shed from the membrane by metalloproteases (MMP) and the soluble DPP-IV cleaving full length NPY into NPY3-36.

complication in the measurement of DPP-IV activity in the plasma is it is so well buffered. For example, it is well described that DPP-IV in the blood does not change with feeding (Meneilly et al., 2000; Ryskjaer et al., 2006; Neidert et al., 2016a) or acute exercise (Neidert et al., 2016b). However, there are several studies that have attempted to measure DPP-IV changes in the muscle or plasma with acute exercise. In anesthetized rats, the gastrocnemius muscle was electrically stimulated to create four sets of dynamic plantar flexions. Immediately after they were either gavage fed whey protein or no whey protein. From the muscles harvested from the rats, we found an increase in DPP-IV mRNA only when exercise was combined with whey protein feeding (Neidert et al., 2016b). In humans, taking whey protein prior to a maximal exercise test also resulted in elevated DPP-IV in the plasma, but the maximal exercise test alone failed to increase plasma DPP-IV (Kluess and Neidert, 2018). We also performed a resistance exercise protocol designed to cause muscle soreness in the leg muscles. This protocol did cause muscle soreness, but failed to increase serum DPP-IV (Neidert et al., 2016b). We suspected the leg exercise protocols failed to increase DPP-IV because of buffering by other sources of DPP-IV from the legs to the sampling location in the arm. Studies that sample close to the source of the exercising muscles are needed.

There are limited number of studies looking at exercise and DPP-IV inhibition. Takada et al. (2016) treated mice with heart failure with a DPP-IV inhibitor for 4 weeks and found an improvement in peak VO_2. This effect may have something to do with GLP-1 because a GLP-1 inhibitor abolished the improvement in VO_2peak. Further, they saw improved mitochondrial function and fiber type shifts from IIB to I with DPP-IV inhibition. This same positive effect of DPP-IV inhibition has not been seen in humans with heart failure (Bassi and Fonarow, 2018) and in fact, may be harmful in some people with heart failure (Clifton, 2014; Lourenco et al., 2017). Considering the effect that DPP-IV has on muscle blood flow and muscle function, more studies investigating the muscle effects of DPP-IV inhibition are warranted.

There is little literature on the changes in DPP-IV with exercise training. Tanimura et al. (2019) showed improved blood triglycerides and reduced hepatic lipid accumulation in diabetic mice on a high fat diet with exercise training and a DPP-IV inhibitor. There are two studies in humans that measure plasma DPP-IV after exercise training. Lee et al. (2015) studied adolescents with type 2 diabetes and measured a variety of outcomes after 12 weeks of high or low intensity exercise training. The high intensity training group lost weight and saw a decrease in insulin. Both training groups saw an improvement in VO_2max, higher GLP-1 and a reduction in serum DPP-IV. In obese adults

with metabolic syndrome, Malin et al. (2013) saw lower plasma DPP-IV and improved insulin sensitivity with a decrease in body weight and fat mass following 12 weeks of supervised exercise. To date there are no studies in healthy people regarding DPP-IV changes with exercise training.

CONCLUSION

Dipeptidyl peptidase IV is an enzyme with broad effects throughout the body. It is released by a variety of tissues including muscle. In the muscle, DPP-IV is released from the membrane by metalloproteases in the interstitial space. The effects of DPP-IV on muscle include the reduction in neuropeptide Y-mediated vasoconstriction to increase muscle blood flow. Other possible effects of DPP-IV are uninvestigated to date. DPP-IV has many targets that may influence muscle function and many research questions remain.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares 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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