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Exercise training induces depot-specific remodelling of protein secretion in skeletal muscle and adipose tissue of obese male mice

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Short title: Myokine and adipokine profiling with exercise training

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Abstract

Acute exercise induces changes in circulating proteins which are known to alter metabolism and systemic energy balance. Skeletal muscle is a primary contributor to changes in the plasma proteome with acute exercise. An important consideration when assessing the endocrine function of muscle is the presence of different fibre types, which show distinct functional and metabolic properties and likely secrete different proteins. Similarly, adipokines are important regulators of systemic metabolism and have been shown to differ between depots.

Given the health-promoting effects of exercise, we proposed that understanding depot-specific remodelling of protein secretion in muscle and adipose tissue would provide new insights into inter-tissue communication and uncover novel regulators of energy homeostasis. Here, we examined the effect of endurance exercise training on protein secretion from fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus muscle, and visceral and subcutaneous adipose tissue.

High-fat diet-fed mice were exercise trained for six weeks, while a Control group remained sedentary. Secreted proteins from excised EDL and soleus muscle, inguinal and epididymal adipose tissues were detected using mass spectrometry.

We detected 575 and 784 secreted proteins from EDL and soleus muscle, and 738 and 920 proteins from inguinal and epididymal adipose tissue, respectively. Of these, 331 proteins were secreted from all tissues, while secretion of many other proteins was tissue and depot specific. Exercise training led to substantial remodelling of protein secretion from EDL, while soleus showed only minor changes. Myokines released exclusively from EDL or soleus were associated with glycogen metabolism and cellular stress response, respectively. Adipokine secretion was completely refractory to exercise regulation in both adipose depots.

This study provides an in-depth resource of protein secretion from muscle and adipose tissue, and its regulation following exercise training, and identifies distinct depot-specific secretion patterns that are related to the metabolic properties of the tissue of origin.

Keywords: exercise, endocrine, adipokine, myokine, mitochondrial function

New and Noteworthy: This present study examines the effects of exercise training on protein secretion from fast-twitch and slow-twitch muscle as well as visceral and subcutaneous adipose tissue of obese mice. While exercise training leads to substantial remodelling of protein secretion from fast-twitch muscle, adipose tissue is completely refractory to exercise regulation.

Introduction

Exercise is an effective strategy for the treatment of obesity and obesity-associated metabolic comorbidities, including non-alcoholic fatty liver disease and type 2 diabetes (1). These health benefits are mediated by a myriad of acute effects and chronic adaptations such as increases in energy expenditure, enhanced mitochondrial capacity and function and other molecular adaptations in skeletal muscle that promote more efficient lipid metabolism, improved insulin action, and changes in circulating factors secreted from muscle (2) and other exercise-responsive tissues (3-6). Research efforts utilising mass spectrometry proteomics approaches have uncovered an unappreciated depth of protein secretion from many tissues and across various (patho)physiological conditions. While highlighting the general complexity of endocrine regulation, it is apparent that skeletal muscle is a particularly active endocrine organ, secreting hundreds of proteins and metabolites (2). These muscle-released proteins/peptides, known as myokines, regulate a diversity of functions, including lipid and glucose metabolism, adipose tissue browning, and muscle hypertrophy (7-11).

Many health benefits of exercise training have been attributed to increased release of myokines, which may, at least partly, prevent or reverse obesity and its metabolic comorbidities (12). In this respect, IL-6, IL-15, irisin, meteorin-like and β-aminoisobutyric acid (BAIBA) are secreted from skeletal muscle in response to acute exercise (3), and induce numerous metabolic effects in muscle and other tissues. For example, IL-6 increases muscle glucose uptake and fatty acid oxidation via an AMPK-dependent pathway (10, 13), and enhances systemic insulin sensitivity and fatty acid turnover in healthy/elderly humans (10, 14). Similarly, irisin induces "browning" of white adipocytes, increases energy expenditure and reduces adiposity (15), while IL-15 is associated with improvements in insulin sensitivity, systemic fat oxidation, and protection from diet-induced obesity (16). In non-obese humans, skeletal muscle accounts for ~40% of total body weight, constituting the largest organ in the body. Given that myokine secretion is increased in response to acute exercise (12), it is therefore not surprising that these factors are likely to contribute to the changes in the plasma proteome during and immediately after exercise (17, 18), although this has yet to be definitely shown. However, emerging

data indicate that the protein secretome of skeletal muscle may not be highly responsive to exercise training (19).

Skeletal muscle is composed of different fibre types that possess distinct functional and metabolic properties (20). Muscle fibres can be broadly characterized as fast-twitch (glycolytic type 2A, type 2X, and 2B) or slow-twitch (oxidative type 1) based on the expression of myosin heavy chain isoforms (21), and show major functional differences, including speed of contraction and fatiguability, mitochondrial composition and oxidative capacity, capillary density, and substrate storage and utilization (reviewed in (22)), and distinct proteomes and phospho-proteomes that reflect these major functional traits (22-24). In light of these fibre specific properties, there is the likelihood of differences in protein secretion from specific muscle fibre types, although this remains unresolved.

In addition to myokine release from skeletal muscle, protein secretion from other tissues, and specific cell types within these tissues, is altered following exercise training (19). We have recently described changes in hepatokine secretion following exercise training in mice, and identified syndecan-4 as a novel regulator of hepatic lipid metabolism (5), while others have shown that acute and/or chronic exercise regulates the release of cardiokines, osteokines, immune cytokines, and adipokines from adipose tissue (3, 12, 19). There has been intense interest in adipokine secretion because of the powerful metabolic and anti-obesogenic effects of various adipokines, including leptin and adiponectin (25, 26), the reported differences in adipokine secretion from different types of adipocytes (*e.g.*, brown vs white), and even differences in protein secretion from adipocyte progenitor cells of different origins (27-29). Given that visceral adipose tissue is associated with increased risk for developing cardiometabolic diseases while subcutaneous adipose tissue is considered protective (30), we hypothesize that protein secretion from these depots may vary in response to exercise training, and might explain some of the observed detrimental or protective metabolic effects of visceral and subcutaneous adipose tissue, respectively.

The aim of this study was to determine the effects of endurance exercise training on protein secretion from skeletal muscles and adipose tissues of obese mice, with a focus on understanding the differences in myokine responses between fast-twitch EDL muscle and slow-twitch soleus muscle and adipokine secretion from visceral and subcutaneous adipose tissues.

Methods

Animal experimental procedures and exercise training studies

Mouse experiments were approved by the University of Melbourne Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology animal ethics committee, and procedures conformed to the guidelines of the National Health and Medical Research Council of Australia regarding the use and care of experimental animals. The experimental procedures for this cohort of mice have been recently described in detail (5). Briefly, eight-week-old male C57BL/6J mice (sourced from Animal Resources Centre, Canning Vale, Australia) were maintained in a temperature-controlled room (22 °C ± 1 °C) with a 12-hour light/dark cycle, and had *ad libitum* access to food and water. Mice were fed a high fat diet (HFD, High Fat Rodent Diet SF04-001, 43% energy from fat, Specialty Feeds, Australia) for 6 weeks, then assigned to a Sedentary or Exercise group, matched for body weight, and maintained on the HFD for a further six weeks. Following an initial maximal velocity test, mice in the Exercise group underwent a progressive endurance exercise training program for 6 weeks, as described (5). Mice were anesthetised two days after the last bout of exercise by cervical dislocation to mitigate the effects of acute exercise on protein secretion. Visceral and subcutaneous adipose tissue (~ 30 mg from each depot, for each preparation one single piece of adipose tissue was used)) were dissected, then Extensor digitorum longus muscle (EDL) and soleus muscle were carefully excised from tendon-to-tendon assuring that the whole muscle remained intact.

Collection of secreted factors

EDL and soleus muscle, and visceral and subcutaneous adipose tissue explants were incubated in carbogenated EX-CELL® 325 PF CHO serum-free medium (Sigma-Aldrich, Australia) for 4 h at 37°C. At the end of the incubation period, the medium containing secreted factors was centrifuged at 300 x g for 10 mins and the supernatant stored at -80°C until proteomic assessment. Previous studies have shown that muscles remain stable for over 12 hours under the same conditions (31, 32).

Proteomics processing and mass spectrometry

Sample processing for mass spectrometry assessment of secreted factors has been described in detail (5, 33). Briefly, proteins within the incubation medium were purified using Amicon Ultra 4 centrifugal filter devices (Merck, USA), protein content determined (Pierce^{TM} BCA protein assay, Thermo Scientific, USA), and proteins reduced with 10 mmol/L TCEP for 20 mins at 65°C, then washed with 8 mol/L urea. Proteins were alkylated through addition of 10 mmol/L chloroacetamide followed by wash steps with 8 mol/L urea and 50 mmol/L ammonia bicarbonate (pH=8.5). Proteins were digested with trypsin (1 µg trypsin / 100 µg of protein) overnight at 37 °C, and eluted with ammonia bicarbonate and 0.5 mol/L NaCl. Peptides were acidified with formic acid, purified with Millipore® C18 Ziptips (Sigma Aldrich, USA), and dried using a Speedvac concentrator (Eppendorf Concentrator Plus, Germany). The purified peptides were reconstituted in 0.1% formic acid, sonicated for 10 mins, centrifuged at 16,000 x g and transferred to liquid chromatography vials for analysis.

Peptides were separated and identified by nano electrospray ionisation liquid chromatography tandem mass spectrometry (Nano-ESI-LC-MS/MS) on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA), as described in detail (33). All generated files were processed with MaxQuant (v1.6.2.10) (34), and database searching was performed, as described (33). The MaxQuant output was processed further through Perseus (v1.6.6.0) (35). Bioinformatic assessment of the proteome data sets was performed using Ingenuity Pathway Analysis (IPA, QIAGEN) (36) and Reactome Pathway Analysis (37). Of note, we analysed our data with *in silico* digestion of trypsin and hence the majority of our peptides sequenced are unlikely to be the result of protein degradation. To detect peptide degradation products, a peptidomic analysis is required which enriches for low-molecular weight endogenous peptides and analyses the data without *in silico* digestion.

Statistical analysis

The values were Log_2 normalised and underwent two sample t-test (correcting for multiple hypothesis testing using Benjamini-Hochberg set at FDR < 5%) to obtain a list of significantly regulated proteins based on $q < 0.05$.

Results

Skeletal muscle and adipose tissue show depot-specific protein secretion.

We recently reported that exercise training led to a reduction in diet-induced body weight gain and reduced adiposity, improvements in maximal running capacity, improved mitochondrial function in skeletal muscle and reduced liver triglyceride content, with these measures highlighting the efficacy of the exercise program (5). We further reported exercise-induced adaptations in proteins secreted from hepatocytes, with upregulated proteins associated with pathways related to lipid metabolism, and downregulated proteins associated with hepatic steatosis, oxidative stress and liver cancer (5). Using these same mice, we herein focus on protein secretion from fast-twitch EDL muscle and slow-twitch soleus muscle, and visceral and subcutaneous adipose tissue explants, as well as exercise-induced adaptations in protein secretion in these tissues. There are four main muscle types: slow-twitch oxidative type 1, fast-twitch oxidative glycolytic type 2A, fast-twitch glycolytic type 2X, and fast-twitch glycolytic type 2B, with most muscle depots containing a combination of fast- and slow-twitch fibres (21). However, in soleus muscle, 96% of fibres express the type 1 MyHC isoform (slow-twitch oxidative), while EDL muscle consists primarily of fast-twitch glycolytic type 2B fibres (76%), followed by type 2A (19%) and type 1 (5%) (38). Therefore, for the purpose of this comparison, we refer to soleus and EDL as slow-twitch oxidative and fast-twitch glycolytic muscles, respectively.

We first compared protein secretion from the two muscle and two adipose tissue depots in the sedentary / untrained state (*i.e.,* proteins secreted from tissues of sedentary high-fat diet mice). We identified 784, 575, 920, and 738 proteins released from soleus and EDL muscles, and epididymal (visceral) and inguinal (subcutaneous) adipose tissues, respectively (Figure 1). Of these proteins, 13-25% were denoted as classically secreted by the presence of a N-terminal signal peptide (SP), while 75-87% were predicted to be secreted via unconventional processes (*i.e.,* contained within extracellular vesicles (EV)). We found that 331 proteins were secreted from all four tissues, while

each tissue secreted specific proteins that were not secreted from any of the other four tissues. For example, soleus muscle (highlighted in red) and EDL muscle (highlighted in orange) secreted 106 and 30 proteins, respectively, that were not released from the other tissues. Similarly, epididymal adipose tissue secreted 153 proteins (highlighted in blue) and inguinal adipose tissue secreted 39 proteins (highlighted in green) that were not released from any other tissue assessed. Across all tissues, the canonical pathways associated with these tissue-selective secretion patterns were related to 'metabolism', 'signal transduction' and 'the immune system'. EDL muscle showed specific enrichment of proteins associated with 'glycogen breakdown' while proteins released from soleus muscle were associated with 'cellular response to stress' (Figure 1).

EDL muscle releases proteins involved in glycogen breakdown.

Skeletal muscle is a highly active endocrine organ, with secretome analysis of human myocyte culture identifying over 600 myokines (39). Given the diverse metabolic properties of fast-twitch EDL muscle and slow-twitch soleus muscle, particularly in the context of increased capacity for glycolysis and oxidative phosphorylation, respectively (40), and differential expression of proteins predicted to be secreted (41), we next compared protein secretion between these two muscle depots in more detail. There was clear clustering of the EDL and soleus protein secretome as determined by principal component analysis (Figure 2A), and volcano plot analysis highlighted significant differences in protein secretion between groups, with 59 and 245 proteins showing higher secretion from EDL and soleus muscle, respectively (Figure 2B). In soleus muscle, Reactome pathway analysis identified overrepresentation of secreted proteins associated with 'response to stress' and the 'KEAP1/Nrf2 pathway', a pathway associated with antioxidant transcription and protection against oxidative stress (42) (Figure 2C). Secreted proteins enriched in EDL muscle were associated with pathways related to glucose metabolism, most prominently 'gluconeogenesis' and 'glycogen breakdown' (Figure 2D).

While 335 proteins were secreted from both muscles, 133 proteins were uniquely secreted from soleus muscle (*i.e.*, not secreted from EDL muscle) (Figure 2E). These proteins were associated with pathways related to 'neutrophil degranulation', 'response to stress' and 'HSF1 activation', with heat shock transcription factor 1 playing important roles in oxidative stress and protein repair (43) (Figure 2E). This suggests that proteins released from oxidative soleus muscle might play protective roles in the context of cellular stress. On the other hand, proteins uniquely secreted from EDL muscle (*i.e.*, not secreted from soleus muscle) were associated with 'glycogen breakdown' (Figure 2E). Specifically, proteins secreted by EDL muscle included calmodulin 1 (Calm1), glycogen debranching enzyme (Agl), phosphoglucomutase 1 (Pgm1), glycogen phosphorylase kinase (Phk) and glycogen phosphorylase (Pygm), with each of these proteins known to be involved in either increased glycogen breakdown and/or reduced glycogen synthesis (Figure 2F). Given that these proteins do not contain a N-terminal signal peptide (i.e., are not classically secreted), it is likely that they are bound and transported within extracellular vesicles. In addition, Pygm1 was the 7th most abundant protein secreted from EDL muscle (out of a total of 363 proteins), highlighting the potential metabolic role of fast-twitch glycolytic EDL muscle in regulating systemic glycogen metabolism. All experiments were carried out 48 hours after the last exercise bout, and it is therefore unlikely that the release of glycogen metabolism enzymes from EDL muscle is related to intramuscular glycogen depletion, given that muscle glycogen levels are commonly re-established by 24 hours post-exercise (44).

Protein secretion from EDL muscle is highly responsive to exercise training.

We next assessed the impact of endurance exercise training on protein secretion from EDL and soleus muscle. In EDL muscle, we identified a total of 582 secreted proteins, with exercise training leading to a significant increase in secretion of 417 proteins, while only one protein showed a significant reduction in secretion (Haptoglobin, Hb) (Figure 3A). This striking remodelling is similar to previous reports of myokine responsiveness to both acute exercise and exercise training (2, 12). Notably, 64

proteins of mitochondrial origin were increased in secretion from exercised trained EDL muscle (11% of total, highlighted in red, Figure 3A), with most of these mitochondrial proteins originating from the mitochondrial matrix (Figure 3B). Supporting this finding, Ingenuity Pathway Analysis (IPA) pointed to overrepresentation of metabolic pathways associated with 'sirtuin signalling' and the 'TCA cycle' (Figure 3C), with PGC1α, a master regulator of mitochondrial biogenesis, and mTOR, an important regulator of glucose and lipid metabolism, predicted as likely upstream regulators of the exercise-induced response in protein secretion from EDL muscle (Figure 3D). Other predicted upstream regulators responsive to exercise training included the transcriptional repressor KDM5A (RBP2 – retinoblastoma binding protein 2), which has been associated with expression of oxidative stress mediated genes (45) and Nrf1/Nrf2 that control expression of many cytoprotective enzymes (Figure 3D).

Protein secretion from soleus muscle is refractory to exercise training.

In striking contrast to EDL muscle, where 72% of the detected proteins were increased following endurance exercise training (417 of 582), only 3% of the proteins secreted from soleus muscle were significantly regulated by exercise training (24 of 793), with the secretion of 22 proteins being increased and 2 proteins decreased (Figure 4A). Similar to EDL muscle, 99 proteins secreted from soleus muscle were of mitochondrial origin (12% of total, highlighted in red, Figure 4A), with enrichment of proteins commonly present in the mitochondrial matrix and the mitochondrial inner membrane (Figure 4B). Importantly, of the 22 proteins that were significantly increased in secretion following endurance exercise training, 17 proteins were of mitochondrial origin (Figure 4A).

The exercise-induced secretion of mitochondrial proteins is specific to skeletal muscle.

We next compared the exercise-induced changes in protein secretion between EDL and soleus muscle (including all secreted proteins – significant and non-significant), and report a significant positive correlation (R^2=0.249, p<0.001), highlighting that many secreted proteins are regulated in a similar manner following endurance exercise training, despite the attenuated response in soleus muscle (Figure 4C). Importantly, the most highly exercise-induced proteins in both muscle depots were of mitochondrial origin (Figure 4C). To determine whether this 'mitochondrial secretion pattern' was generalizable to other tissues, we assessed exercise-induced changes in secretion of mitochondrial proteins from hepatocytes. Hepatocytes secreted a total of 228 mitochondrial proteins (out of 899 secreted proteins identified across both groups, as recently reported (5)), primarily originating from the mitochondrial matrix and the mitochondrial inner membrane (Figure 4E). However, secretion of most of these mitochondrial proteins (170 proteins, 75%) was decreased following exercise training (Figure 4D), suggesting that the release of mitochondrial proteins is not a general response to exercise training.

Epididymal and inguinal adipose tissue show distinct protein secretion patterns.

Exercise training has been reported to alter adipokine secretion (4). However, this body of evidence is limited by inferences of reported secretion patterns from analysis of intracellular gene/protein expression and not protein secretion *per se*, via the assessment of adipokines in the circulation that may originate from other tissues, and a lack of knowledge of depot-specific protein secretion (4, 46). In this respect, while visceral adipose tissue is associated with increased risk for developing cardiometabolic diseases, subcutaneous adipose tissue is considered protective (30), with these distinct metabolic features likely related to depot-specific adipokine secretion and subsequent endocrine effects. To expand our understanding of depot-specific adipokine secretion, we first compared protein secretion from epididymal (EPI; visceral depot) and inguinal (ING; subcutaneous depot) adipose tissue, in the basal state (i.e., in sedentary mice).

There was clustering of proteins secreted from EPI and ING as determined by PCA (Figure 5A), and volcano plot analysis highlighted significant differences in protein secretion between groups (Figure 5B). From a total of 601 proteins identified in both groups, 149 and 27 proteins showed higher secretion from EPI and ING, respectively (Figure 5B). For the EPI-enriched secreted proteins, Reactome pathway analysis pointed to overrepresentation of pathways associated with the 'nervous system', 'response to stress' and 'ROBO receptors' (Figure 5C), receptors involved in cell migration and organization of the actin cytoskeleton, while the ING-enriched secreted proteins were associated with 'platelet activation' and 'triglyceride metabolism' (Figure 5D). Specifically, we found increased secretion of hormone-sensitive lipase (Lipe) and monoglyceride lipase (Mgll), two essential lipolytic enzymes, and fatty acid binding protein 3 (Fabp3), a protein increased by cold exposure and thought to be characteristic of beige/brown adipocytes (47). Given the absence of a N-terminal signal peptide in these proteins, they are most likely released within extracellular vesicles. In this respect, various lipolysis-related proteins and FABPs have been previously shown to be present within plasma EV (19, 48) and specifically within adipose tissue-derived EV (19, 49, 50). It is unclear why these lipolytic proteins are released from adipose tissue, however, we hypothesize that they may impact lipid metabolism in peripheral tissues. Furthermore, while 450 proteins were secreted from both adipose tissue depots, 129 proteins were only secreted from EPI (Figure 5E), with these proteins being associated with 'axon guidance', 'metabolism' and 'response to stress' (Figure 5E). Only 20 proteins were specifically secreted from ING, with these proteins being associated with 'metabolism', 'signal transduction' and 'RTK (receptor tyrosine kinase) signalling' (Figure 5E). RTKs are abundant cell surface receptors, including the insulin receptor, the FGF21 receptor, and various growth factor receptors (e.g., EGFR, VEGFR, FGFR) that are involved in diverse metabolic signalling pathways implicated in energy homeostasis. Overall, while protein secretion from EPI is associated with cellular stress, the ING-enriched and ING-selective secretion patterns point towards a more pronounced role of subcutaneous adipose tissue in regulating systemic and/or peripheral fatty acid mobilization and maintenance of energy homeostasis in an endocrine manner.

Protein secretion from epididymal and inguinal adipose tissue is refractory to endurance exercise training.

In contrast to the exercise training effects on protein secretion from skeletal muscle and hepatocytes (5), both epididymal adipose tissue (Figure 6A-B) and inguinal adipose tissue (Figure 6C-D) were completely refractory to exercise training, with no clustering within the PCA and no significant changes as shown by volcano plot analysis. Lastly, we compared the exercise-induced changes in protein secretion between EPI and ING, and found a significant positive correlation ($R2=0.289$, $p<0.001$), highlighting that many secreted proteins are regulated in a similar manner following endurance exercise training, despite the absence of significant changes between groups (Figure 6E). Taken together, these results highlight a tissue-specific pattern of adaptations in protein secretion in response to endurance exercise training, with significant regulation in oxidative slow-twitch and glycolytic fast-twitch muscle, but not in adipose tissue.

Discussion

Metabolic tissues, including muscle, liver and adipose tissue, communicate with each other through endocrine signals, with proteomics studies describing changes in myokine (2), hepatokine (51) and adipokine (52) secretion in various (patho)physiological conditions. In the context of acute exercise and chronic exercise training, intense research efforts have uncovered the complexity of endocrine regulation in skeletal muscle (2, 12), but also across a range of other tissues (3, 12, 19). Here we extend on these previous studies and perform the first comparison of protein secretion between fast-twitch EDL and slow-twitch soleus muscle, as well as between visceral and subcutaneous adipose tissue, both in the basal state and following exercise training. We show that fast-twitch EDL muscle has a pronounced endocrine signature related to glycogen metabolism, with selective release of major enzymes responsible for glycogen breakdown. In contrast, slow-twitch soleus muscle shows selective release of proteins involved in antioxidant response, highlighting that proteins released from soleus muscle might play protective roles in the context of cellular stress. While myokine secretion from both muscle depots, and particularly from EDL muscle, was responsive to exercise training, showing increased release of mitochondrial proteins, adipokine release from both adipose depots was completely refractory to exercise training.

EDL muscle consists of fast-twitch glycolytic type 2B (76%), type 2A (19%) and type 1 fibres (5%) and thereby have greater capacity for glycolytic metabolism to generate energy (38). In contrast, soleus muscle is composed of 96% of slow-twitch oxidative type 1 fibres (38), that contract more slowly and display a higher content of oxidative enzymes, with these fibre-type specific metabolic differences being associated with differences in mitochondrial composition and oxidative capacity, substrate storage and utilization, and speed of muscle contraction and/or fatiguability (17, 22, 41). These broad metabolic differences are accompanied by distinct transcriptome profiles, particularly in respect to expression of genes coding for mitochondrial function and lipid/glucose metabolism (41,

53). Given these distinct metabolic properties, it is not surprising that EDL and soleus muscle differ substantially in the type of proteins that are secreted from these muscle depots. EDL muscle selectively released proteins related to glycogen breakdown, most likely contained within EVs given the absence of a N-terminal signal peptide. Glycogen is an important energy store in muscle, which can be quickly and efficiently accessed during states of increased energy demand, such as exercise, and is largely regulated by activation of glycogen phosphorylase b and glycogen debranching enzyme, providing glucose-6-phosphate for glycolysis (54). The increased secretion of proteins important for glycogen breakdown from EDL muscle could simply reflect the increased content of these proteins in fast-twitch muscle (41). This supposition is supported by the observation that the composition of many circulating EVs (55), including those derived from muscle (56), can predict their tissue of origin.

Little is known about the selective secretion of enzymes involved in glycogen metabolism from muscle, and their potential impact in distant peripheral tissues, including the liver as one of the major glycogen storage sites in the body (57). Previous studies have isolated EVs secreted from cultured myotubes or muscle *ex vivo*, however, there is little information describing the protein and metabolite composition of muscle-derived EVs. Previous studies investigating protein secretion from C2C12 myotubes and differentiated human myotubes identified 600-950 secreted proteins, including various glycolysis and glycogen metabolism enzymes (56, 58-60), supporting the notion that these proteins can be released from muscle, most likely contained within extracellular vesicles, given the absence of a secretory signal peptide. EVs released from any cell/tissue type can conceivably signal to and/or be taken up by many cell types (61), however, recent studies show more directed targeting of EVs to recipient cells/tissues, effects mediated by the presence of specific tetraspanins and the EV surface composition (62). A recent comparison assessing the systemic delivery of EV derived from C2C12 myotubes, melanoma cells or bone marrow-derived dendritic cells, showed that myotube-derived EVs displayed the most pronounced accumulation in the liver (63). Similarly, EVs secreted during

exercise-induced muscle damage are largely targeted to the liver (64). Given that muscle EVs can dock and fuse with a variety of target cells, and importantly deliver functional protein cargo (60, 65), this suggests that proteins secreted from EDL muscle (and potentially other fast-twitch muscle fibres) may deliver protein cargo that facilitates efficient glycogen metabolism in the liver. This would align with the marked increase in hepatic glucose production that occurs during exercise, and the requirement for liver glycogenolysis to maintain both substrate supply to contracting tissues and blood glucose levels (66, 67).

A second notable observation from these studies was the enrichment of mitochondrial proteins secreted from skeletal muscle after exercise training, which was particularly prominent in the EDL. This is not surprising given that enhancement of mitochondrial function in skeletal muscle is a pronounced adaptation following exercise training (20), and that (as detailed above) the composition of circulating EV mirrors intracellular protein expression/composition of their respective tissue of origin (55, 56). Supporting these findings, a recent study assessing correlations of plasma proteins with skeletal muscle mitochondrial function in humans reported specific clusters of plasma proteins that were associated with energy metabolism, oxidative stress and mitochondrial function (68). Similarly, various studies have reported increased mitochondrial protein content in plasma following endurance exercise training (69) or contained within plasma extracellular vesicles after acute exercise (64). Given that the exercise-responsive increase in secretion of mitochondrial proteins in our study was confined to skeletal muscle, and not the hepatocytes or adipose tissue, it is likely that increases in mitochondrial proteins in the circulation following exercise training are related to release from skeletal muscle, as recently suggested (17, 18).

These observations raise several questions, such as, why are mitochondrial proteins secreted from skeletal muscle, particularly during exercise; what are their target tissue/s; and if protein cargo is

functional, can this impact mitochondrial function in recipient cells? It has been suggested that secretion of mitochondrial proteins is a 'metabolic stress response' to excessive oxidative stress (70) or inflammation (71, 72). In this respect, it is well known that exercise training leads to increased oxidative stress and damage in muscle, with simultaneous adaptations in antioxidant defence and cellular stress systems (73). This is in line with our finding that soleus muscle showed enrichment of secreted proteins associated with cellular response to stress, and the antioxidant KEAP1/Nrf2 pathway, with EDL muscle also showing overrepresentation of exercise-regulated proteins associated with the Nrf2-pathway. Given the likely increase in oxidative stress with exercise training, future studies are required to assess oxidative damage and functional performance of mitochondrial proteins secreted from muscle, and to determine whether muscle EV release is a mechanism to remove damaged mitochondrial components to maintain intracellular quality control, as recently suggested (74).

Previous studies have suggested that exercise exerts its beneficial effects partly through alterations in adipokine release, particularly increased secretion of anti-inflammatory adipokines and decreased secretion of proinflammatory cytokines (4). In this respect, given that visceral adipose tissue is commonly associated with increased risk for developing metabolic disease while subcutaneous adipose tissue is considered protective (30), we hypothesized that inguinal adipose tissue would show changes in protein secretion with exercise training that would be considered metabolically beneficial and/or protective. Surprisingly, in contrast to the pronounced changes in protein secretion from skeletal muscle, adipokine release from both adipose tissue depots was completely refractory to exercise training. However, the conclusions from many previous studies are drawn from analysis of intracellular changes in proteins with a N-terminal secretory peptide, and thereby inferences of protein secretion, or assessment of adipokines in the circulation, and thereby inferences that adipose tissue is the major tissue mediating such changes (4, 46). In addition, conclusions drawn from studies examining the effects of exercise training on serum/plasma adipokines (e.g., adiponectin and leptin) have been confounded by a concomitant loss of fat mass (reviewed in 75). We overcame these issues

by directly assessing protein secretion from adipose tissue explants following exercise training. However, a potential limitation of this *ex vivo* approach is the absence of hormonal and neuronal input, which could affect protein secretion. Furthermore, exercise might induce acute effects on protein secretion from adipose tissue which are potentially not sustained beyond the hours after the last exercise bout.

There are many well documented differences between subcutaneous and intra-abdominal adipose tissues, including differences in lipid and glucose metabolism, mitochondrial (dys)function, oxidative stress and inflammation (76-78), but to the best of our knowledge, there is only one direct comparison of protein secretion that was based on multiplex panels and not untargeted mass spectrometry-based proteomics (79). Despite the absence of exercise training adaptations, there were substantial differences in adipokine secretion between the two adipose tissue depots, which aligns with previous reports in humans highlighting depot-specific differences in adipokine secretion (79) and EV secretion/composition (29). Specifically, proteins secreted from epididymal adipose tissue were associated with cellular stress response, which is consistent with the notion that visceral adipose tissue shows a more pronounced pro-inflammatory phenotype (80-82), and that visceral (abdominal) adiposity increases the risk for developing cardiometabolic disease (83). In contrast, inguinal adipose tissue released proteins associated with fatty acid mobilization and adipose tissue beiging (82), consistent with subcutaneous adipose tissue being more responsive to beiging stimuli (84), further suggesting that such secreted proteins may be transported to other tissues to impact fatty acid metabolism and act via autocrine / paracrine signalling to cause browning, which would be an interesting point for future investigation.

In conclusion, this study provides an in-depth resource describing protein secretion from skeletal muscles and adipose tissues and the regulation following endurance exercise training in mice. We

identify distinct differences in protein secretion from slow-twitch soleus and fast-twitch skeletal muscle and highlight the impact of exercise training on the release of mitochondrial proteins from muscle, particularly EDL muscle. We further show that protein secretion from subcutaneous and visceral adipose depots show pronounced depot-specific differences, yet protein secretion from both adipose tissue depots is completely refractory to endurance exercise training. These findings can be utilised to delineate the likely contribution of skeletal muscle and adipose tissue to the plasma proteome in sedentary states and with exercise training.

Supplemental Material

The proteomics data and accompanying Qiagen IPA and Reactome Pathway Analysis are available on Figshare at the following DOI: 10.6084/m9.figshare.23708583

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD043929.

Figure legends

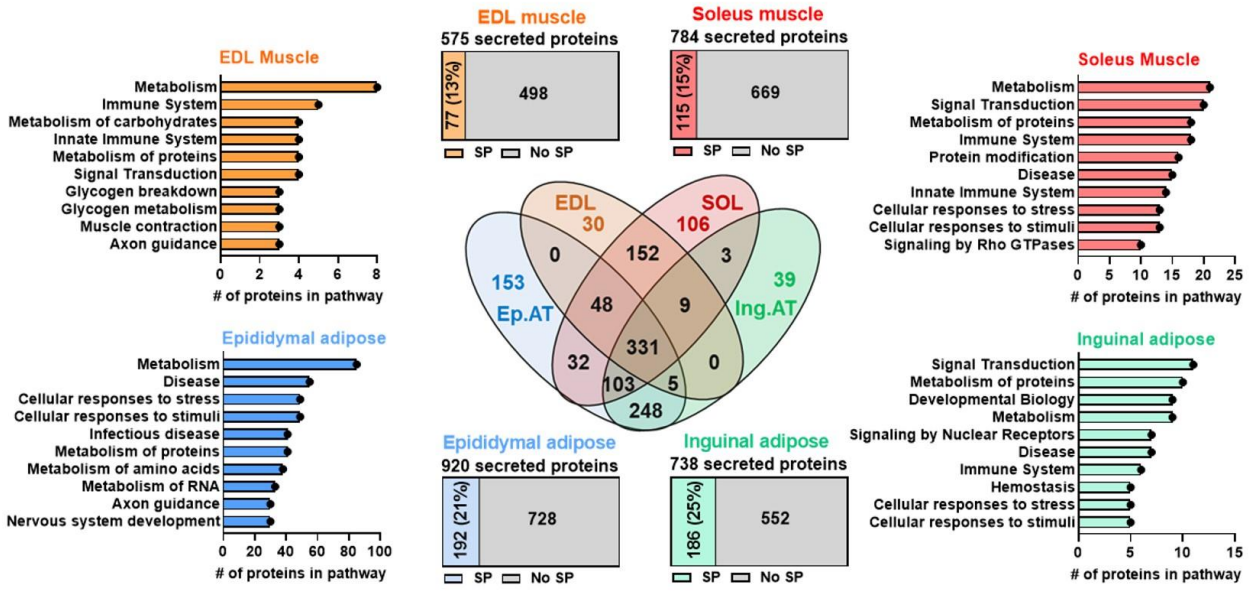

Figure 1. Comparison of protein secretion from intact tendon-to-tendon soleus (red) and EDL muscle (orange), and epididymal (blue) and inguinal (green) adipose tissue explants, obtained from sedentary mice fed a high-fat diet for 12 weeks. For each tissue, shown are total number of secreted proteins, as well as number and percentage of proteins containing a secretory signal peptide (SP). The Venn diagram highlights communal and tissue-specific patterns of protein secretion from all tissues, with canonical pathways associated with the tissue-specific secretion patterns further highlighted.

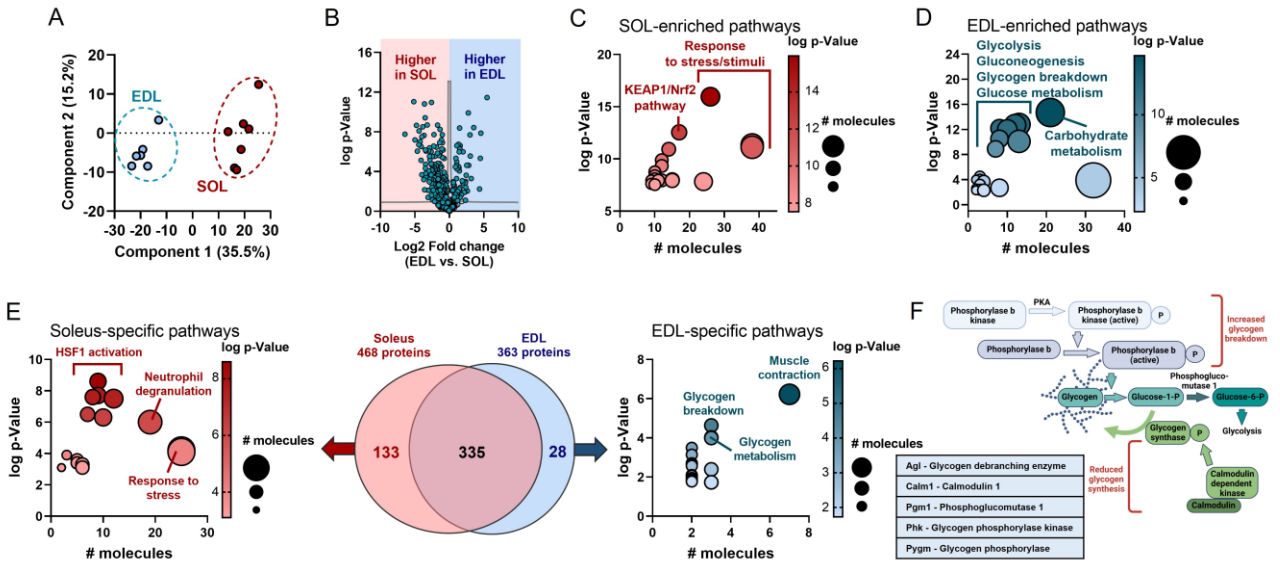

Figure 2. Comparison of protein secretion from intact tendon-to-tendon soleus (red) and EDL muscle (blue) from sedentary mice fed a high-fat diet for 12 weeks. (A) Principal component analysis and (B) volcano plot analysis highlighting significant differences in protein secretion between groups. Reactome pathway analysis showing overrepresentation of pathways in (C) soleus and (D) EDL muscle. (E) Venn diagram showing proteins secreted from both muscle depots, and proteins only found to be secreted from either soleus or EDL muscle (i.e., not found to be released from the other depot), as well as Reactome pathway analysis showing overrepresentation of pathways associated with these depot-specific secretion patterns. (F) Diagram highlighting the proteins secreted by EDL muscle, included calmodulin 1 (Calm1), glycogen debranching enzyme (Agl), phosphoglucomutase 1 (Pgm1), glycogen phosphorylase kinase (Phk) and glycogen phosphorylase (Pygm). For each bubble plot analysis, the x-axis and the size of the pathway bubble represent the number of secreted proteins associated with a specific pathway, and the y-axis and colour intensity highlight the log p-Value of the observed association. Figure 2F was generated using Biorender.

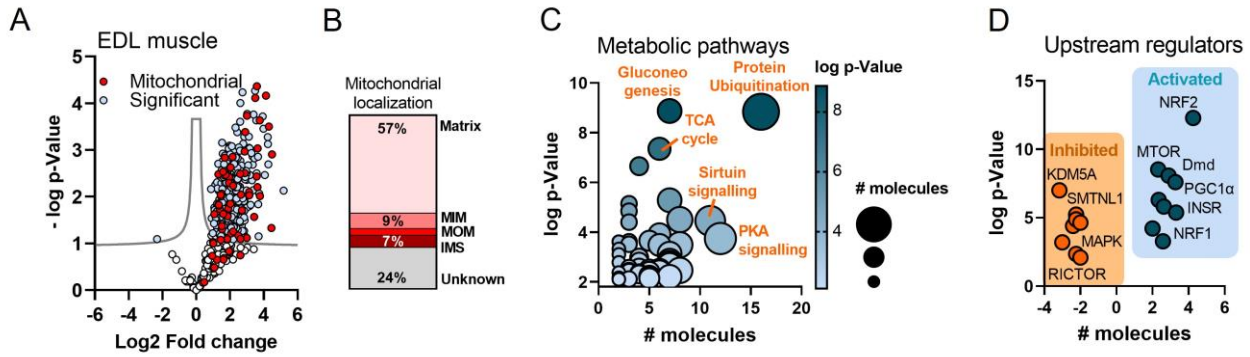

Figure 3. Comparison of protein secretion from glycolytic EDL muscle obtained from sedentary or exercise-trained mice. (A) Volcano plot analysis showing significant differences in protein secretion with exercise (blue) and highlighting secretion of mitochondrial proteins (red). (B) Intracellular origin of secreted mitochondrial proteins. Ingenuity pathway analysis showing overrepresentation of (C) metabolic pathways, and (D) predicted upstream regulators, associated with exercise regulation of protein secretion in EDL muscle. For each bubble plot analysis, the x-axis and the size of the pathway bubble represent the number of secreted proteins associated with a specific pathway, and the y-axis

and colour intensity highlight the log p-Value of the observed association. IMS, intermembrane space; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; PKA, protein kinase A; TCA, tricarboxylic acid.

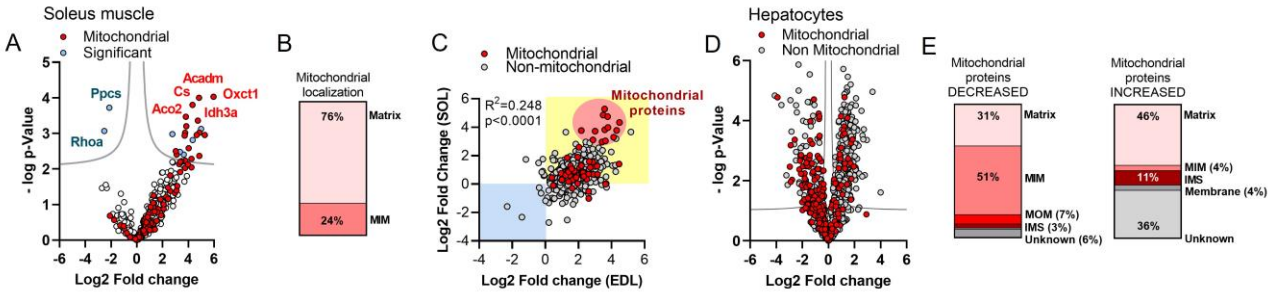

Figure 4. Comparison of protein secretion from oxidative soleus muscle and primary hepatocytes obtained from sedentary or exercise-trained mice. (A) Volcano plot analysis showing significant differences in protein secretion from soleus muscle with exercise (blue) and highlighting secretion of mitochondrial proteins (red). (B) Intracellular origin of secreted mitochondrial proteins in soleus muscle. (C) Association of exercise-induced changes in protein secretion between EDL and soleus muscle, particularly increases in secretion of mitochondrial proteins from both muscle depots (highlighted in red). (D) Volcano plot analysis showing significant differences in protein secretion from primary hepatocytes with exercise (blue) and highlighting secretion of mitochondrial proteins (red). (E) Intracellular origin of secreted mitochondrial proteins increased and decreased in secretion from hepatocytes with exercise. IMS, intermembrane space; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane.

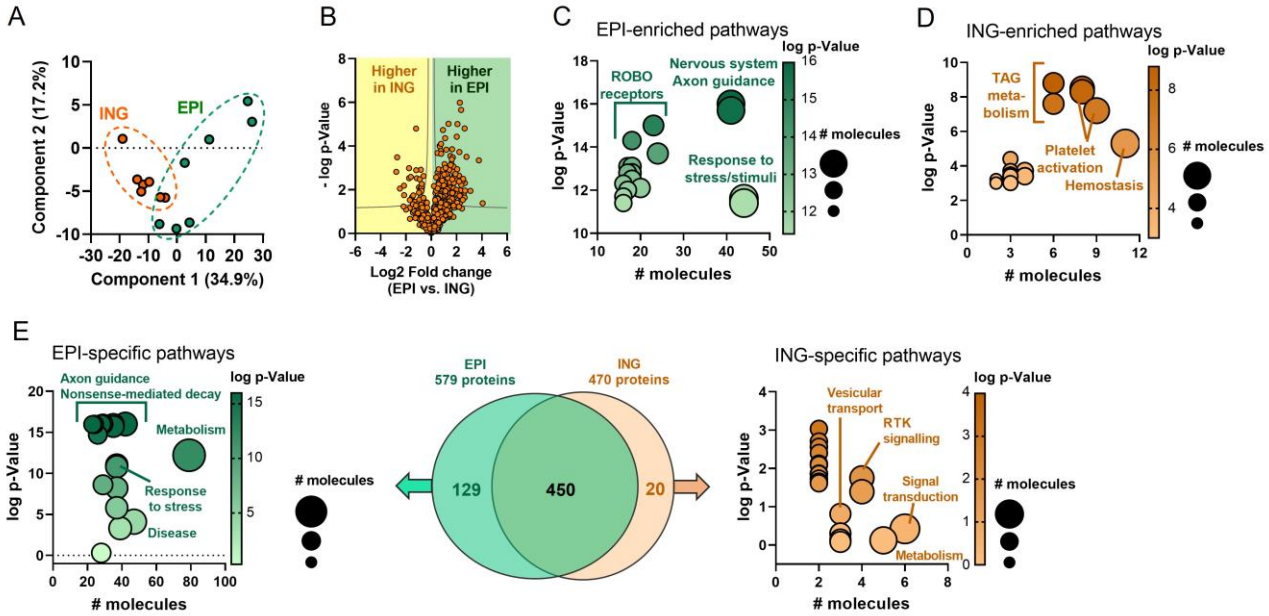

Figure 5. Comparison of protein secretion from epididymal (green) and inguinal (orange) adipose tissue explants from sedentary mice fed a high-fat diet for 12 weeks. (A) Principal component analysis and (B) volcano plot analysis highlighting significant differences in protein secretion between groups. Reactome pathway analysis showing overrepresentation of pathways in (C) epididymal and (D) inguinal adipose tissue. (E) Venn diagram showing proteins secreted from both adipose depots, and proteins only found to be secreted from either adipose depot (i.e., not found to be released from the other depot), as well as Reactome pathway analysis showing overrepresentation of pathways associated with these depot-specific secretion patterns. For each bubble plot analysis, the x-axis and the size of the pathway bubble represent the number of secreted proteins associated with a specific pathway, and the y-axis and colour intensity highlight the log p-Value of the observed association. EPI, epididymal; ING, inguinal; RTK, receptor tyrosine kinase; TAG, triglyceride.

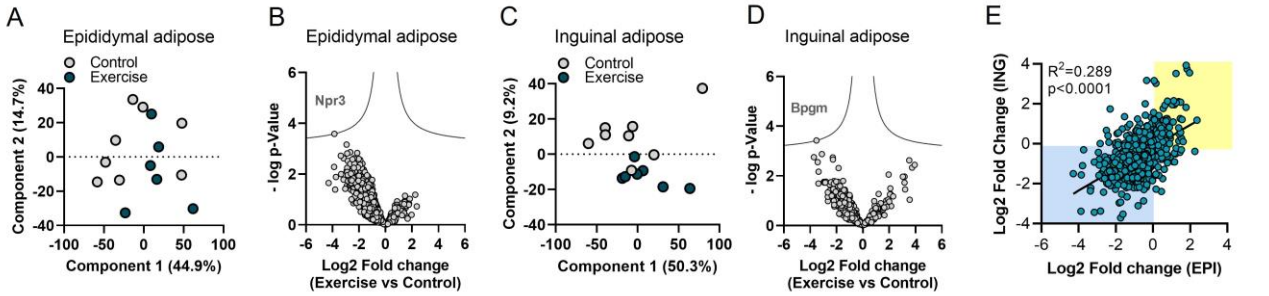

Figure 6. Comparison of protein secretion from epididymal and inguinal adipose tissue explants obtained from sedentary (grey) or exercise-trained (blue) mice. Principal component analysis and volcano plot analysis comparing protein secretion from (A, B) epididymal adipose tissue and (C, D) inguinal adipose tissue. (E) Association of exercise-induced changes in protein secretion between both adipose depots.

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