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EXERCISE SERUM INCREASES GLUT4 IN HUMAN ADIPOCYTES

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NEW FINDINGS

What is the central question of this study?

Do circulating factors mediate exercise-induced effects on adipose tissue GLUT4 expression?

What is the main finding and its importance?

Serum (10%) obtained from human volunteers immediately after a single exercise bout increased GLUT4 protein levels in human adipocytes in culture. This result suggests that circulating factors may mediate exercise effects on adipose tissue GLUT4 and prompt further effort to identify the specific factor(s) and tissue(s) of origin.

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ABSTRACT

In this study we tested the hypothesis that circulating factors generated during exercise increase adipose tissue GLUT4 expression. Serum was obtained from eight healthy subjects before and after 60 min of cycling exercise and primary adipocytes were cultured from stromal vascular fractions that were isolated from subcutaneous abdominal adipose tissue samples from two healthy, male volunteers. Exposure of human primary adipocytes to 10% serum obtained after exercise for 48 hr increased GLUT4 protein expression, on average, by 12% compared with exposure to 10% serum obtained at rest before exercise. GLUT4 mRNA levels were increased after 12 hr of exposure to exercise serum but were unchanged after 6 and 24 hr of exposure. Our results suggest that circulating factors may mediate exercise effects on adipose tissue GLUT4 expression and encourage further efforts to identify the potential factor(s), tissue(s) of origin and physiological relevance.

Key words: exercise serum, GLUT4, adipocytes

1. INTRODUCTION

Exercise has profound effects on all physiological systems within the body (Hawley, Hargreaves, Joyner & Zierath, 2014) and regular physical activity/exercise training results in enhanced functional capacity and improved health and well-being, as a consequence of adaptations in multiple tissues and organs. In recent years, it has been demonstrated that contracting skeletal muscle, and potentially other tissues, release biologically active proteins, nucleic acids and metabolites ("myokines/exerkines") that may mediate some of the systemic benefits of regular exercise (Safdar, Saleem & Tarnopolsky, 2016; Whitham & Febbraio, 2016). It was shown previously that a combination of metabolites that increase in human plasma after exercise increases Nur77 gene expression in C2C12 myotubes in vitro (Lewis et al., 2010) suggesting an exercise-induced increase in circulating bioactive molecules. Adipose tissue is involved in metabolic regulation through its role in energy substrate availability, glucose homeostasis and insulin action and

the glucose transporter GLUT4 may be an important mediator of these latter processes (Herman & Kahn, 2006). Exercise has been shown to increase adipose tissue GLUT4 expression in rodents (Ferrara et al., 1998; Hirshman et al., 1993; Stallknecht et al., 1993) and exercise-induced metabolic adaptations in adipose tissue may potentially be mediated by circulating factors (Stanford & Goodyear, 2016). The aim of our study was to examine whether serum obtained from exercised subjects increased GLUT4 expression in human primary adipocytes.

2. METHODS

2.1 Ethical approval. The study was approved by the Human Research Ethics Committee of the University of Melbourne (HREC ID 1748590) and conformed with the standards set by the *Declaration of Helsinki*, except for registration in a public database. Eight healthy, male subjects (age: 21.4 $\pm$ 0.6 yr; body mass: 71 $\pm$ 4 kg; VO_2 peak: 43 $\pm$ 2 $ml.kg^{-1}.min^{-1}$, mean $\pm$ SD) participated in the study after providing their informed, written consent.

2.2 Exercise. The subjects exercised on a stationary cycle ergometer for 60 min at a power output requiring ~70% of their previously determined peak pulmonary oxygen uptake (VO_2 peak). Subjects were not engaged in regular exercise training and reported to the laboratory in the morning after an overnight fast and having abstained from alcohol, caffeine, tobacco and physical activity for at least 24 hr. Venous blood samples were obtained from a forearm vein before and during the last minute of exercise, placed in BD Vacutainer Rapid Serum® tubes, spun in a centrifuge and the serum (resting – Rest; exercise-conditioned serum – EX) frozen at -80°C for later use.

2.2 Adipose tissue. Stromal vascular fractions (SVF) were isolated from subcutaneous abdominal adipose tissue samples that were obtained by percutaneous needle biopsy from one male subject (31 yr; 71 kg; BMI: 25 $kg.m^{-2}$). This subject was not engaged in regular exercise training and fasting plasma levels of glucose, insulin and triglyceride were within normal ranges. He reported to the laboratory in the morning after an overnight fast and having abstained from alcohol, caffeine, tobacco

and physical activity for at least 24 hr. Adipose tissue was sampled and studied based on previously published methods (Lee & Fried, 2014). Briefly, isolated SVF were cultured in alpha-minimum essential growth medium, supplemented with 10% foetal bovine serum, until ~100% confluent. Next, cells were chemically differentiated in serum-free differentiation medium (Dulbecco's modified Eagle's medium – F12) until attainment of characteristics of mature adipocytes as demonstrated by the capacity for lipid loading. Mature adipocytes were insulin-starved for 48 hr and then incubated for a further 48 hr in 10% serum obtained either at rest or after exercise. Serum from the eight subjects was used in studies, in duplicate, on SVF-derived adipocytes. Preliminary experiments indicated that 10% serum elicited a significant change in GLUT4 protein expression compared with 5, 20 and 50% serum or no serum addition. In a preliminary experiment, we did not observe any effect of exercise serum on GLUT4 protein expression after 24 hr incubation. In a separate experiment, cells were harvested after 12 hr of serum exposure for the measurement of GLUT4 mRNA, based on a preliminary time-course experiment in which no differences in GLUT4 mRNA levels were observed after 6 and 24 hr of serum exposure, but were increased at the 12 hr time point. Due to technical difficulties, GLUT4 mRNA measurements were only possible on serum from six subjects.

2.3 GLUT4 protein expression. Serum-treated cells were lysed in 100µl RIPA buffer (supplemented with protease and phosphatase inhibitors and 0.1%DTT) and the protein concentration in whole cell lysates was determined using the BCA assay according to manufacturer's instructions (Thermo-Fisher Scientific). Lysates were solubilised in Laemelli's buffer to final concentration of 100mg.ml^{-1}, spun for one min at 13,000g and then heated for three min at 95ºC. Protein (15-30ug per lane) was loaded to 10% and 4-15% TGX stain free, pre-cast gels (Bio-Rad, Hercules, CA, USA) and resolved by electrophoresis. Gels were transferred to PVDF using the Bio-Rad Trans-Blot Turbo® system. GLUT4 protein was measured by immunoblotting with a specific anti-GLUT4 rabbit polyclonal antibody (PA5-23052; Thermo-Fisher Scientific) 1:1000 in TBST containing 5% BSA. Total protein was visualised and quantified by Stain-free total protein normalisation (ChemiDoc, BioRad, Hercules, CA).

2.4 Gene expression. RNA was isolated from samples using PureLink® RNA Mini kit (Ambion, Life Technologies) according to the manufacturer's directions. Single strand cDNA chains were synthesised from purified RNA using i-SCRIPT cDNA synthesis kit (BioRad, Hercules, CA) to a final concentration of 2.5 ng.μl^{-1}. Expression of genes was assessed by real time qPCR using Taqman probes (GLUT4: Hs00168966; TBP1: Hs99999910).

2.5 Statistical analysis. Data from the Rest and EX experiments were compared using paired t-test with significance at the $P<0.05$ level.

3. RESULTS

Exposure of human primary adipocytes to 10% serum obtained after exercise for 48 hr increased GLUT4 protein expression, on average, by 12% compared with exposure to 10% serum obtained at rest before exercise (1.11 $\pm$ 0.17 vs. 0.99 $\pm$ 0.11 arb units, mean $\pm$ SD, Figure 1). GLUT4 mRNA levels were increased after 12 hr of exposure to exercise serum (Figure 2).

4. DISCUSSION

Our results suggest that circulating factors during exercise increase GLUT4 protein in human primary adipocytes after 48 hr of serum exposure. We also observed an increase in GLUT4 mRNA expression after 12 hr of serum exposure; however, there was no significant correlation between the increases in GLUT4 mRNA and protein. We did not change the serum once applied and the adipocytes were exposed to the serum continuously for 48 hr. It is possible that the effect of the serum may have decreased over time if there was time-dependent degradation of the active agent(s). In addition, changing the serum periodically and "pulsing" the adipocytes with fresh serum, a scenario more like that seen with repeated exercise training bouts, may have enhanced the stimulus to increase GLUT4. We did not have an experimental condition in which cells were not exposed to serum and it is possible that there are bioactive molecules in serum that influence adipose tissue GLUT4 expression under resting conditions. Thus, the effects on GLUT4 expression we have observed could

arise from an increase in stimulatory factors, a decrease in inhibitory factors, or a combination of both.

There was variation in the responses to exercise serum (Figures 1, 2) and this is not uncommon in human exercise studies. It is possible that subtle differences in physical fitness, skeletal muscle characteristics and metabolic health status between subjects contributed to this variation. Nevertheless, we observed a small, but significant, increase in adipocyte GLUT4 protein after exercise serum exposure. It is important to assess whether this *in vitro* finding is physiologically relevant *in vivo*. We have recently reported that 10 days of exercise training in young, healthy, untrained subjects does not increase subcutaneous adipose tissue GLUT4 content (Flores-Opazo et al., 2018). In contrast, we have observed that four weeks training in patients with type 2 diabetes, who have lower adipose tissue GLUT4 expression than age-matched healthy subjects, increased adipose tissue GLUT4 protein levels (Hussey et al., 2011). Thus, metabolic health status may impact on the exercise response. Finally, if exercise training does increase adipose tissue GLUT4 it is important to assess the functional consequences in relation to GLUT4 translocation following insulin stimulation, glucose sensing, downstream signalling and adipokine secretion (Herman & Kahn, 2006).

Selective deletion of GLUT4 in adipose tissue results in muscle and hepatic insulin resistance (Abel et al., 2001), potentially via increased plasma levels of retinol binding protein 4 (Yang et al., 2005) or other adipokines. Of note, adipose tissue from patients with obesity and type 2 diabetes has reduced GLUT4 expression (Garvey et al., 1991; Sinha et al., 1991). Overexpression of GLUT4 in adipose tissue results in improved glucose tolerance (Shepherd et al., 1993) and enhanced insulin action (Carvalho, Kotani, Peroni & Kahn, 2005), despite increased fat cell mass, as well as increased levels of novel lipids with antidiabetic effects (Moraes-Viera, Saghatelian & Kahn, 2016). We have previously observed that 4 weeks of exercise training in patients with type 2 diabetes increased adipose tissue GLUT4 expression to levels similar to those in age-, BMI-matched healthy subjects (Hussey et al., 2011). Although local effects on adipose cell size and lipid metabolism could

influence adipose tissue GLUT4 expression, our current results suggest that circulating factors may also contribute to increased adipose tissue GLUT4 expression observed after exercise training.

There is a number of biomolecules that have been shown to be released from contracting skeletal muscle (Safdar, Saleem & Tarnopolsky, 2016; Whitham & Febbraio, 2016), and possibly other organs during exercise, that could be potential mediators of the response we have observed. Circulating catecholamines have previously been implicated in adipose tissue adaptations to exercise (Sutherland et al., 2009), as have various candidate "myokines/exerkines" (Stanford & Goodyear, 2016). Future efforts to identify the specific factor(s), tissue(s) of origin and their physiological significance may contribute to the optimisation of exercise prescription protocols, and/or the development of novel therapeutic strategies, for the management of metabolic diseases.

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AUTHOR CONTRIBUTIONS

M.F-O, M.W and M.H designed the experiment. M.F-O and M.H collected the plasma samples before and after exercise. M.W obtained the human adipose tissue samples. M.F-O and A.R undertook the adipose cell culture experiments and M.F-O completed all biochemical and molecular analyses. M.H wrote the initial draft of the manuscript and all authors contributed to, reviewed and approved the final version of the submitted manuscript.

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FIGURE LEGENDS

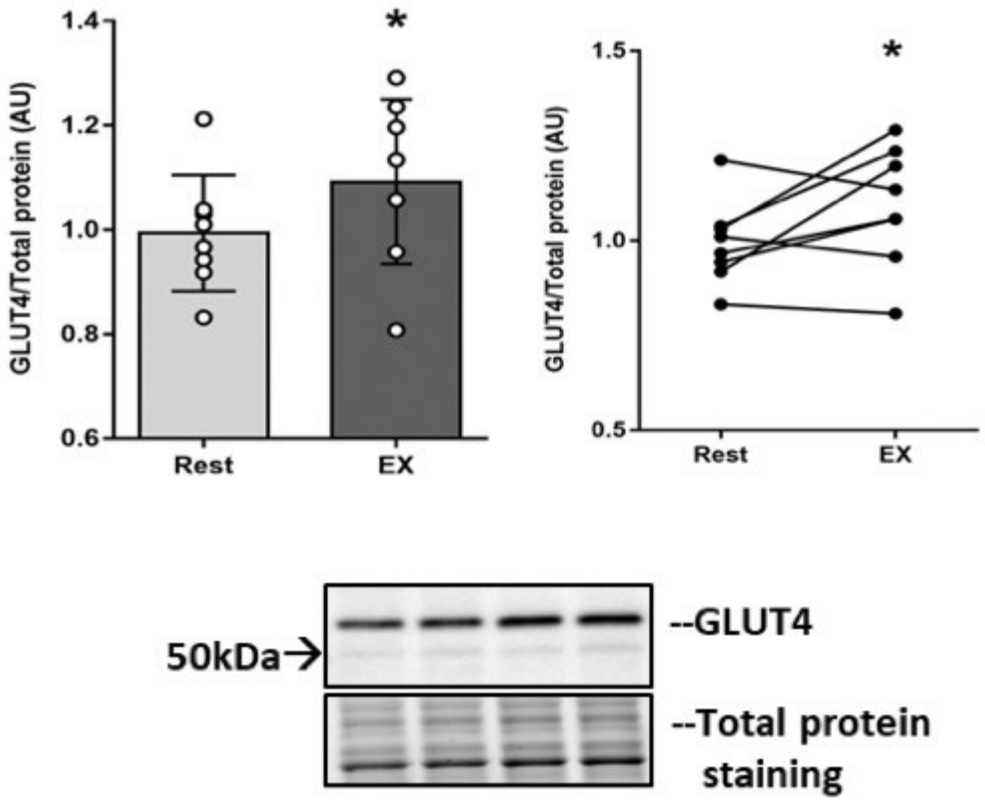

FIGURE 1: GLUT4 protein expression, normalised against total protein, in human primary adipocytes after 48 hr exposure to 10% serum obtained either before (Rest) or after (EX) exercise. Data are means ± SD (n=8). Representative blots for GLUT4 (top panel) and total protein (bottom panel) in two subjects (Rest: lanes 1 and 2; EX: lanes 3 and 4). * denotes different from Rest ($P<0.05$).

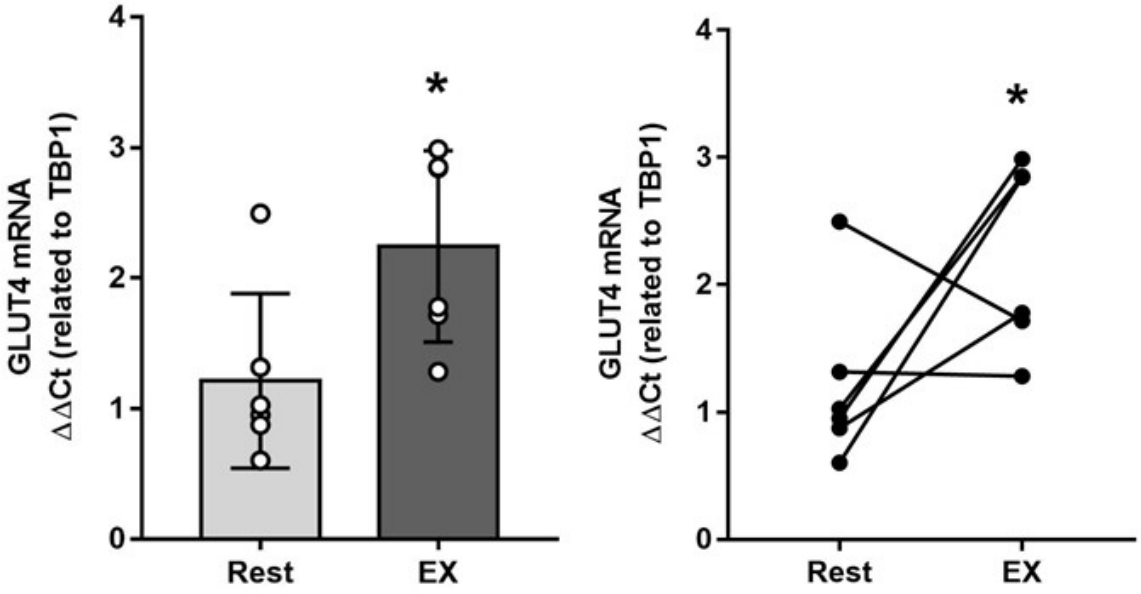

FIGURE 2: GLUT4 mRNA expression in human primary adipocytes after 12 hr exposure to 10% serum obtained either before (Rest) or after (EX) exercise. Data are means ± SD (n=6). * denotes different from Rest (P<0.05).