Metabolic and molecular changes associated with the increased skeletal muscle insulin action 24 to 48 hours after exercise in young and old humans

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Abstract
The molecular and metabolic mechanisms underlying the increase in insulin sensitivity (i.e. increased insulin-stimulated skeletal muscle glucose uptake, phosphorylation and storage as glycogen) observed from 12 to 48 h following a single bout of exercise in humans remain unresolved. Moreover, whether these mechanisms differ with age is unclear. It is well established that a single bout of exercise increases the translocation of the glucose transporter, GLUT4, to the plasma membrane. Previous research using unilateral limb muscle contraction models in combination with hyperinsulinaemia has demonstrated that the increase in insulin sensitivity and glycogen synthesis 24 h after exercise is also associated with an increase in hexokinase (HKII) mRNA and protein content, suggesting an increase in the capacity of the muscle to phosphorylate glucose and divert it towards glycogen synthesis. Interestingly, this response is altered in older individuals for up to 48 h post exercise and is associated with molecular changes in skeletal muscle tissue that are indicative of reduced lipid oxidation, increased lipogenesis, increased inflammation, and a relative inflexibility of changes in Intramyocellular lipid (IMCL) content. Reduced insulin sensitivity (insulin resistance) is generally related to IMCL content, particularly in the subsarcolemmal (SSL) region, and both are associated with increasing age. Recent research has demonstrated that ageing per se appears to cause an exacerbated lipolytic response to exercise that may result in SSL IMCL accumulation. Further research is required to determine if increased IMCL content affects HKII expression in the days after exercise in older individuals, and the effect of this on skeletal muscle insulin action.
Introduction - Effect of acute exercise on insulin action in human skeletal muscle

Elevated pre-exercise muscle glycogen content is essential for optimal exercise performance (1), and nutritional strategies to increase muscle glycogen content are widespread. Administering carbohydrate immediately post-exercise, when muscle glycogen content is <150 mmol·(kg dm)^{-1}, has been reported to increase muscle glycogen synthesis, often supercompensating to far above basal levels, in a biphasic manner (2-7). During the initial period of recovery there is a rapid, insulin-independent, increase in muscle glycogen resynthesis lasting around 30 to 60 min, followed by a secondary, slower insulin-dependent phase, lasting several hours to days (6, 8). The rapid phase is most likely attributed to an exercise-induced increase in plasma membrane GLUT4 content (the protein responsible for the facilitated transport of glucose into skeletal muscle; 9, 10), and an increase in glycogen synthase (GS) activity induced by a low muscle glycogen concentration (which appears to be a far more potent regulator of glycogen synthase activity than insulin or muscle contraction; 2, 11-13). Insulin is thought not to play a significant role in this rapid phase, as post-exercise somatostatin infusion designed to suppress the endogenous secretion of insulin, has no effect glycogen synthesis during this period (6). On the other hand, the slow phase of glycogen synthesis is inhibited by somatostatin infusion (6) and, therefore, is most likely due to a marked increase in sensitivity of glucose uptake and glycogen synthesis to insulin (14).

This sustained increase in skeletal muscle insulin action following exercise is not only important for glycogen loading in athletes prior to competition or during intense periods of training, but also beneficial in the treatment of type 2 diabetes (T2D), as even a single bout of exercise can increase insulin sensitivity in insulin resistant individuals by reversing a defect in insulin stimulated glucose transport and phosphorylation (15). However, despite a plethora of studies in this area, the exact cellular mechanisms underlying the well documented increase in insulin stimulated skeletal muscle glucose uptake and glycogen synthesis observed up to 48 hours following a single bout of exercise (16, 17) remain unresolved. Surprisingly, little attention has been given to differences with age, despite the global prevalence of T2D being most apparent in older people (18), and the estimation in 2005 that the number of people over 65 years of age with diabetes will have increased 4.5 fold by 2050 (19).

The aim of this review is to focus on potential intracellular mechanisms leading to the increased skeletal muscle insulin sensitivity following an acute bout of exercise, and whether these differ with age. It is beyond the scope of this review to consider all intracellular mechanisms, and we refer the reader to several excellent reviews that detail molecular mechanisms in the first few hours after
exercise in both animal and human models (20, 21). We will also not focus on extracellular mechanisms such as the role of blood flow post-exercise as not much work has been performed in the area, although it is important to note recent reports suggesting a large contribution (around 50%) to improved insulin sensitivity from increased microvascular perfusion in the first few hours after exercise (22). Instead we focus on human studies that have investigated the later phase of enhanced insulin stimulated glucose uptake following exercise (>12 hours) and the premise that transcriptional events during this time period may contribute to the sustained increase in insulin-stimulated glucose transport and glycogen resynthesis.

Do changes in insulin signaling modulate improved post-exercise insulin action?
It appears that augmentation of the classical insulin signalling cascade may not be involved in the positive effect of exercise on post-exercise insulin action and glycogen synthesis, as many studies have demonstrated that a single bout of exercise does not increase IRS-1 tyrosine phosphorylation, IRS-1 associated PI3K activity, serine phosphorylation of Akt and glycogen synthase kinase 3 (GSK3) in response to insulin for up to one day after exercise (23-28). An overview of these molecular events can be found in several excellent reviews (e.g. 21) and is beyond the scope of the present review. However, it is worth mentioning that immediately after acute exercise, during the insulin independent phase of muscle glucose uptake and glycogen resynthesis, the phosphorylation (activation) of TBC1D4/AS160 (a downstream target of Akt) was increased in rat (29-31) and human muscle in the absence of insulin (22, 32). This suggests that TBC1D4/AS160 phosphorylation could play a potential role in increased insulin sensitivity when muscle is subsequently stimulated by insulin several hours later. Indeed, Treebak et al. (32) demonstrated the increased phosphorylation of TBC1D4/AS160 was associated with increase glucose uptake and glycogen resynthesis in response to insulin 4 h following a single bout of one-legged exercise compared to the non-exercised leg. Moreover, TBC1D4/AS160 was still phosphorylated 27 h after a bout of exercise in rats when insulin sensitivity remained enhanced (30), although the rats had been deprived of food for this time and had low muscle glycogen content, which may have also contributed to the improved insulin action (14).

Why is glucose uptake, phosphorylation, and storage enhanced by insulin post-exercise?
It is well established that a single bout of exercise increases the transcription (32) and plasma membrane translocation and content (33-35) of GLUT4. A single bout of exercise also increases skeletal muscle hexokinase II (HKII) activity, transcription and protein content for a number of hours after the end of exercise (28, 36-38). HKII is the predominant hexokinase isoform in skeletal muscle,
where it phosphorylates internalized glucose thus ensuring a concentration gradient across the plasma membrane and sustained glucose transport into muscle and substrate (glucose-6-phosphate; G6P) for glycogen synthesis or glycolysis. As mentioned above, low muscle glycogen content increases GS activity post exercise (22, 27), and may independently also contribute to increased glucose uptake as low muscle glycogen concentration has been observed in most studies in humans reporting enhanced insulin action following exercise (15, 27, 39). However, it is interesting to note that recent observations have demonstrated enhanced skeletal muscle insulin action the day following a single bout of exercise when muscle glycogen content had returned to pre-exercise levels. For example, a recent study by our group performed euglycaemic hyperinsulinaemic clamps 22 hours after 90 min of one-legged cycling exercise at 60% VO\textsubscript{2}max in healthy human participants to ensure that muscle glycogen in the exercised leg had returned to a similar content of the non-exercised leg (28). A major strength of the one-legged exercise protocol is that it also ensures both limbs are exposed to the same circulating metabolic milieu and thus, any differences in the molecular adaptations observed between the exercised and non-exercised legs can be attributed to contraction per se. Skeletal muscle glycogen content measured biochemically in muscle biopsy samples was similar in the exercised and non-exercised legs before the clamp (471 ± 30 vs. 463 ± 50 mmol-(kg dm)\textsuperscript{-1}, respectively), but increased during the clamp in the exercise leg, such that it was 17% greater than the non-exercised leg (527 ± 20 mmol-(kg dm)\textsuperscript{-1} vs. 449 ± 35 mmol-(kg dm)\textsuperscript{-1}). This clearly demonstrated improved insulin action in the absence of glycogen depletion. Prior exercise was associated with increased basal HKII mRNA expression and protein content at 22 hours, but not GLUT4 mRNA, suggesting an increased capacity (through upregulation of HKII content) of muscle to phosphorylate and divert glucose towards glycogen storage is an important contribution to the insulin sensitive phase of skeletal muscle glycogen synthesis the day after a bout of exercise. In support of this finding, HKII, but not GLUT4, overexpression in mice increased insulin-stimulated whole body and skeletal muscle glucose uptake (40). However, it is important to note that inhibition of protein synthesis in rats using cyclohexamide for 3.5 h after a bout of exercise does not affect insulin-stimulated glucose uptake, suggesting that an increase in HKII protein content does not play a role in insulin action the immediate hours post-exercise (41).

Insight into the role of HKII in skeletal muscle insulin sensitivity the days after a bout of exercise can also be gleaned from studies using the glucose analogue 3-O-methylglucose, which is taken up by muscle but not further metabolized and therefore independent of HKII, GS, and presumably glycogen content. For example, Cartee et al (14) demonstrated increased insulin-stimulated transport of 3-O-methylglucose 18 and 48 h after exercise in rat muscle in the glycogen depleted
state, but not after 18 h in the glycogen supercompensated state, despite the increased glycogen synthesis. This latter observation would suggest that glycogen supercompensation is not reflected by insulin stimulated 3-O-methylglucose uptake and is dependent on glucose metabolism, perhaps by HKII. Collectively, these studies suggest that increased insulin stimulated glucose uptake the day(s) after a single bout of exercise is mediated by enhanced capacity for glucose phosphorylation and storage, that is independent of the prevailing muscle glycogen content. Although this would suggest that muscle glycogen content per se is not important during the insulin-sensitive period post exercise, it does not rule out the possibility that glycogen depletion could trigger adaptations within the muscle that sustain the improvement in insulin sensitivity for several days. Indeed, glycogen depletion has been suggested to affect metabolic gene expression (42, 43) and activation of key insulin signalling proteins (44). Although the molecular mechanisms responsible for the upregulation of HKII content following an acute bout of exercise are unclear, possible candidates include the activation of transcription factors such as the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC1α (45), sterol regulatory binding protein 1c (SREBP1c) (46, 47) and peroxisome proliferator-activated receptor-α (PPARα) (48).

Is glucose diverted from oxidation to storage?
The pyruvate dehydrogenase complex (PDC) plays a central role in the oxidation and, therefore, fate of disposed muscle glucose. The PDC is covalently regulated by two competing enzymes, a Ca²⁺-dependent phosphatase (PDP), which dephosphorylates the pyruvate dehydrogenase (PDH) component of the complex and transforms the enzyme to the active form, and a kinase (PDK), which catalyses the ATP-dependent phosphorylation of PDH and inactivates the enzyme complex (49). The magnitude of PDC activation (PDCa) is central to the control of acetyl-CoA delivery to the TCA cycle and, thus, carbohydrate oxidation in skeletal muscle. We have previously demonstrated that inhibition of PDC activation (via a high fat diet (50) or intravenous L-carnitine infusion (51)) under insulin stimulated conditions results in a diversion of disposed glucose from oxidation to storage, and is associated with a selective up-regulation of PDK4, but not PDK2, content (the two predominant PDH kinase isoforms in skeletal muscle; 50, 52). Interestingly, an increase in PDK4 mRNA expression has been observed in human skeletal muscle for up to 12 hours after prolonged exercise (although PDK4 protein content was not measured; 53-55). One potential explanation is that a post-exercise induced upregulation of PDK4 would inhibit PDC, which would facilitate a diversion of glucose uptake to storage under insulin-stimulated conditions and, thus, partly explain the routinely observed post-exercise increase in insulin stimulated glycogen synthesis. As the regulation of PDK4 expression occurs primarily at the level of transcription (56) and generally
changes in mRNA are fairly rapid and precede changes in its protein content by several hours (52, 53), it is likely that any functional impact of changes in PDK4 protein on muscle PDCa activity and insulin-stimulated glycogen storage would occur the day after an acute bout of exercise. We have tested this hypothesis using the one-legged exercise protocol described previously (28) and found that neither PDK4 protein content nor activation of the PDCa by insulin were affected by exercise performed the previous day, suggesting that the PDC may not play an important role in facilitating the post-exercise increase in insulin-stimulated glucose uptake and glycogen synthesis. Nevertheless, further research in this area is required.

**Do these responses change as we age?**

Despite a plethora of studies investigating mechanisms underpinning the increase in insulin stimulated skeletal muscle glucose uptake and glycogen synthesis observed up to 48 hours following a single bout of exercise, surprising little attention has been given to differences with age. Age *per se* does not appear to cause insulin resistance (57-59), but older individuals are more likely to be associated with insulin resistance due to increased abdominal adiposity and reduced physical activity (57, 58), along with declines in muscle mass (60, 61). Of note, intramyocellular lipid (IMCL) is often higher in older individuals, particularly in subsarcolemmal (SSL) regions (62, 63), and has been strongly associated with insulin resistance (64-67). It would appear that the SSL pool may play a role in buffering/trafficking of FFA influx (68), and its juxtaposition with the site of insulin signaling at the sarcolemma would also support a role for perturbed SSL buffering/trafficking impairing insulin action. Although this is yet to be supported experimentally, an improvement in insulin sensitivity has been previously observed with reduced SSL but not intramyofibrillar (IMF) IMCL after 10–12 weeks of exercise training (64, 65), which may also provide insight to the athletes paradox where trained individuals have high IMCL content (69). We (63) and others (70) have recently demonstrated that age *per se* results in an excessive rate of appearance of plasma fatty acids during exercise. Whether this resulted in impaired insulin action post exercise was not investigated, but SSL IMCL accumulated during exercise in overweight insulin-resistant older individuals (63). Indeed, Pehmøller et al (71) provided mechanistic evidence, albeit in young individuals, that the insulin resistance associated with 7 hours of lipid infusion (to provide excessive fatty acids) in a non-exercised leg was not fully reversed by a single bout of prior exercise in the contralateral leg. Nevertheless, despite recent research also highlighting that the microvascular response to exercise may be blunted in older individuals (72), it remains unknown if post-exercise insulin action in human skeletal muscle is impaired by age *per se*.
Interestingly, it appears that the HKII transcriptional response is altered in older individuals for up to 48 hours post exercise. For example, we have recently demonstrated that HKII mRNA expression gradually increases at 12, 24, and 48 hours following 45 min of acute resistance type exercise in healthy young men (73). However, despite a rapid increase in expression after 12 hours, HKII mRNA was lower 24 and 48 hours after exercise in healthy older individuals, despite performing the same relative amount of work. In accordance with previous studies (62, 63, 74), the IMCL content was ~2-fold higher at rest (before exercise) in old vs. young but remained unchanged during 12 to 48 hours of the recovery period in the former group, indicating a relative inflexibility in its turnover that is often observed in insulin resistant individuals (75). In contrast, and in line with other studies (76-79) the IMCL content gradually increased during recovery in the young subjects such that the pre-exercise differences between groups were no longer present after 48 hours of post-exercise recovery. It is important to note that HKII protein content and/or activity were not measured in the study of Tsintzas et al (73), and further research is required to determine any role in post-exercise insulin action in older individuals. Furthermore, post-exercise insulin sensitivity was also not measured. However, the older individuals presented with a marked transcriptional response (highlighted in Figure 1) that was consistent with insulin resistance. For example, acute resistance exercise performed by healthy old individuals, when compared to young, led to molecular changes in skeletal muscle during the recovery period favouring reduced lipid oxidation (characterised by reduced LPL, ACAT1 and PPARa), increased lipogenesis (characterised by reduced ATGL and increased FAS and PPARg), and impaired insulin signalling (characterised by increased PI3KR1 and reduced Akt2). Moreover, these changes appeared to be preceded by an exaggerated inflammatory response at 12 hours (characterised by increased expression of COX2, IL6, IkBAlpha and CREB1), that is often linked to impaired skeletal muscle lipid metabolism (80) and the development of insulin resistance (81).

**Conclusion**

The molecular and metabolic mechanisms underlying the increase in insulin sensitivity (i.e. increased insulin-stimulated skeletal muscle glucose uptake, phosphorylation and storage as glycogen) observed for up to several days following a single bout of exercise in humans are clearly multifactorial. It is well established that a single bout of exercise increases the translocation of the glucose transporter, GLUT4, to the plasma membrane. It would also appear that glycogen depletion and enhanced GS activity predominate in the first few insulin-insensitive hours post exercise, followed by activation of distal insulin signaling pathways, and subsequent molecular adaptations after ~24 hours to increase glucose phosphorylation via enhanced protein content of HKII. This latter
phase remains an underexplored area of research and, based on transcriptional events, appears to be impaired in older individuals who present with insulin resistance and an inflexibility in IMCL turnover. It remains to be investigated to what extent regular resistance exercise can improve IMCL turnover in skeletal muscle from older individuals and modify this unfavourable molecular signature.

2451 words


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Figure 1. This figure shows the interaction between exercise and ageing (shown in red) and their effects on biological processes [such as intramyocellular lipid (IMCL) accumulation, Insulin Resistance (IR) and proinflammatory response] often linked to ageing (depicted in green). Genes with significant differential changes in their expression in response to acute resistance exercise in old vs. young subjects are shown in grey.

IR=Insulin Resistance. IMCL=Intramyocellular lipid. DAG=diacylglycerol. ACAT1=Acetyl-CoA acetyltransferase1. FASN=Fatty Acid Synthase. NF-kB=Nuclear Factor kappaB. TNFα=Tumour Necrosis Factor alpha. CREB1=Cyclic AMP responsive element binding protein. IL6=Interleukin 6. COX2=Cyclooxygenase 2. PI3KR1=Phosphatidylinositol 3-kinase, regulatory 1 (p85 alpha). LPL=Lipoprotein lipase. ATGL=Adipose Triglyceride Lipase. PPARα=Peroxisome Proliferator Activated Receptor alpha. PPARg=Peroxisome Proliferator Activated Receptor gamma. IkBalpha=Inhibitor of kappaB kinase alpha. Akt2=Protein kinase B/Akt, isoform 2.