Skeletal muscle nitric oxide signaling and exercise: a focus on glucose metabolism

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People with type 2 diabetes exhibit postprandial glucose intolerance, due to skeletal muscle and liver insulin resistance and pancreatic cell insufficiency. Skeletal muscle insulin resistance results from an impairment in skeletal muscle insulin signaling and also reductions in the vasodilatory effects of insulin. Importantly, although skeletal muscle insulin-stimulated glucose uptake is impaired in people with type 2 diabetes (12), skeletal muscle glucose uptake during exercise is normal in these individuals (29). This is because the regulation of contraction-stimulated glucose uptake in skeletal muscle differs from insulin-stimulated glucose uptake (81), and it appears that the contraction pathway is intact in people with type 2 diabetes.

Exercise is so effective in people with type 2 diabetes that blood glucose levels can decrease to within normal levels during 45 min of intense (70% of work maximum) cycling exercise (53). Furthermore, skeletal muscle also becomes more sensitive to insulin for 24–48 h after an acute exercise bout.

The factor(s) regulating skeletal muscle glucose uptake during exercise/contraction are not entirely clear. We have substantial evidence that nitric oxide (NO) production by neuronal nitric oxide synthase (nNOS1, the primary NOS isozyme in skeletal muscle fibers) is involved. However, there are conflicting results from other laboratories concerning the role of NO in contraction-stimulated glucose uptake, and these will be discussed. Although the cGMP/cGMP-dependent protein kinase (PKG) signaling pathway is generally considered to be the major downstream target of NO, NO can act through a number of cGMP-independent mechanisms, including S-nitrosylation, S-glutathionylation, and tyrosine nitration (see Fig. 1). Indeed, we have early indications in mice that the cGMP/PKG pathway may not be involved in the NO-dependent regulation of skeletal muscle glucose uptake during contraction (48). Once there is a better understanding of the mechanisms though which NO signals glucose uptake during exercise, specific therapeutics can be designed for people with type 2 diabetes to mimic the contraction signaling pathway. Such an agent could assist with blood glucose control of people with type 2 diabetes who are either unable or unwilling to exercise regularly.
Skeletal Muscle NO/NOS

In skeletal muscle, the primary isof orm of NOS expressed is nNOS, which is an alternatively spliced isozyme of nNOS (or NOS1) (16, 45, 59, 65). While NO can also be produced by the endothelial (eNOS; NOS3) and inducible (iNOS; NOS2) isoforms, eNOS is expressed at low levels in skeletal muscle and is mainly associated with the vascular endothelium (16), and there is essentially no expression of iNOS in healthy skeletal muscle (45, 65). In rodent skeletal muscle, the expression of NOS isoforms is similar to that of humans although there is evidence of greater eNOS expression (31). Interestingly, exercise training has been shown to increase skeletal muscle nNOS and eNOS in rats (3) and nNOS in humans (45).

We have shown that skeletal muscle nNOS protein levels are reduced in people with insulin resistance/type 2 diabetes (5). Although speculative, it is possible that the reduced expression of nNOS in people with insulin resistance/type 2 diabetes (5) is due to the increase in iNOS expression that is seen in diabetic human skeletal muscle (71) since iNOS produces severalfold higher levels of NO that may then downregulate nNOS expression. Low concentrations of NO appear to have important signaling effects, but high concentrations of NO (from iNOS) can interact with reactive oxygen species (ROS), causing damage to cells and cellular components. It appears likely that the increase in iNOS in diabetes is due to an inflammatory process (71), and indeed the increase in iNOS in skeletal muscle may well play a causative role in muscle insulin resistance (60). This is because muscle iNOS and inflammation are increased in people with diabetes, and, in line with this theory, global deletion of iNOS (inos−/−) in mice is protective against diet-induced insulin resistance (60). There appears to be a complex interplay between NOS and insulin sensitivity since there is also some evidence that enos−/− and nnos−/− mice are insulin resistant (68), and we have been unable to detect iNOS protein expression in skeletal muscle from these mice (75).

The subcellular distribution of NOS can influence its activity and expression. The lack of dystrophin in duchenne muscular dystrophy results in nNOS in the cytoplasm and a downregulation of its expression. There is evidence that eNOS is associated with the mitochondria (31). There are early indications that there are alternatively spliced isoforms of NOS in addition to nNOS that are expressed in skeletal muscle (59). nNOSβ has been shown to be localized to the Golgi complex in mouse skeletal muscle cells, and studies in mice lacking both nNOSβ and nNOSβ suggest that nNOSβ is a critical regulator of the structural and functional integrity in skeletal muscle (59).

Skeletal Muscle NO During Contraction/Exercise

Isolated rat muscle produces NO basally indicating that NOS is constitutively active (2). Ex vivo contraction of isolated rat muscle results in an increase in NO concentration in the incubation media (2), and we have shown that skeletal muscle NOS activity increases during ex vivo contractions in mouse muscle (48), during in situ contractions in rats (64), and during in vivo exercise in humans (42). Despite the expression of both nNOS and eNOS in rodent skeletal muscle, nNOS is the primary source of skeletal muscle NO during contraction in mouse muscle (39) and in contracting muscle cells (25, 58, 69).

Effects of NO Production During Contraction/Exercise

Glucose uptake. Muscle glucose uptake increases greatly during exercise, due to increases in both skeletal muscle glucose extraction and blood flow. Glucose is transported into skeletal muscle cells during contraction by the GLUT4 glucose transporter. The signaling pathways associated with insulin-stimulated glucose uptake are fairly well understood. Less is known about the regulation of contraction-stimulated glucose uptake, but potential mediators include calcium/calmodulin-dependent kinase, protein kinase C, ROS, AMP-activated protein kinase (AMPK), and NO (3, 29, 48, 63). It is likely that more than one regulator is involved in the control of skeletal muscle glucose uptake during exercise, and that some redundancy exists.

We have shown in humans that local infusion of the NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA) in the femoral artery during cycling exercise at 60% VO2 peak (moderate intensity) substantially attenuated the increase in leg glucose uptake in healthy individuals (∼30%) and in people with type 2 diabetes (up to 75%) (6, 29). Importantly, the NOS inhibitor had no effect on total leg blood flow, blood pressure, or arterial insulin and glucose concentrations during exercise (6, 29). Moreover, local infusion of a NOS inhibitor during in situ contractions in rats attenuates glucose uptake without affecting skeletal muscle microvascular (capillary) blood flow (64).

These results indicate that NOS inhibition attenuates increases in glucose transport into the muscle cell during contraction rather than affecting glucose delivery to the muscle (which is determined largely by blood flow) (64). However, some studies from other laboratories have yielded conflicting results (14, 20, 23, 26). It is possible that one reason for the difference in results between groups could be in relation to the timing of the glucose uptake measurements compared with when the contraction was undertaken. In some studies (14, 23), the glucose transport/uptake measurements usually were performed at least 20 min after the contractions or exercise was ceased and therefore relate to postexercise/contraction glucose uptake, as opposed to glucose uptake during contraction. In contrast, we measure skeletal muscle glucose uptake during contraction/exercise in our rodent and human studies (6, 29, 48, 64). The intensity of skeletal muscle contraction/exercise utilized may also contribute to differences in results between studies. Indeed, Silveira and colleagues (69) have shown in contracting primary muscle cells that NO is only released at high intensities, while Inyard et al. (26) found little effect of NOS inhibition on muscle glucose uptake during low-frequency contractions in rat muscle; at high frequencies a halving of glucose uptake was observed with NOS inhibition (although not significant). Similarly, in humans, there is no effect of NOS inhibition on glucose uptake during low-intensity (10 watts) leg kicking exercise (20), but clear inhibitory effects of NOS inhibition on glucose uptake are observed during moderate-intensity (19 watts) leg kicking exercise (52).

Because inhibitors can have nonspecific effects, it is necessary that experiments be performed where the influence of nNOS is removed by other means such as examining nnos−/− mice. This may be quite a complex undertaking because there is evidence of several other alternatively spliced isoforms of NOS in addition to nNOS in skeletal muscle (59). In addition, knocking out nNOS can result in compen-
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Skeletal muscle glucose uptake during exercise is regulated by nitric oxide (NO). NO activates soluble guanylyl cyclase (sGC), which increases cGMP levels and subsequently activates protein kinase G (PKG) (Fig. 1). This increase in cGMP promotes glucose uptake during contraction, which is abolished by a NO donor (48). It has been suggested that NO plays a role in the regulation of skeletal muscle glucose uptake during contraction (49), but this has not been confirmed by measuring cGMP content, sGC activity, and PKG activity under these conditions.

There is evidence that NO-mediated GLUT4 translocation and glucose uptake into adipocytes occurs via a cGMP-independent pathway (27). Potential cGMP-independent NO signaling processes include posttranslational modifications of proteins via S-nitrosylation, S-glutathionylation, and tyrosine nitration (see Fig. 1). NO can produce posttranslational modifications of thiol (–SH) groups on cysteine residues (S-nitrosylation), which appears to be of functional relevance and importance (44). Indeed, S-nitrosylation is involved in cGMP-independent signaling effects of NO in vascular smooth muscle cells, human endothelial cells, intact hearts, and skeletal muscle (57, 67, 78, 80). In addition, proteins associated with glucose transport regulation [e.g., protein kinase B (Akt/PKB)] are susceptible to S-nitrosylation in skeletal muscle (80). However, white light exposure of muscles, which breaks S-nitrosylation (S-nitrosothiols) bonds (51), has no effect on skeletal muscle glucose uptake during ex vivo contractions (48). Although this suggests that S-nitrosylation does not play a role in the regulation of skeletal muscle glucose uptake during contraction, further studies are needed to determine whether contraction increases S-nitrosylation in skeletal muscle and, if so, whether white light prevents this increase in S-nitrosylation.

S-glutathionylation (also referred to as S-glutathionation), like S-nitrosylation, is now recognized as a signaling event analogous to phosphorylation (11). S-glutathionylation occurs when oxidative stress results in glutathione binding to cysteine residues of proteins, and some S-glutathionylation reactions occur following glutathione being nitrosylated to S-nitrosoglutathione (11, 44). Furthermore, superoxide and NO can react to form peroxynitrite, which modifies protein thioul groups to promote both S-nitrosylation and S-glutathionylation (44). Although we are not aware of this being examined previously, it is possible also that a degree of uncoupling of nNOS occurs in skeletal muscle during exercise, which would result in some superoxide being produced from nNOS. We found S-glutathionylation of an ~260-kDa band increased during contraction of rodent muscle and during exercise in human skeletal muscle (47, 48, 50). Dithiothreitol, a thiol-specific reducing agent (62), attenuated contraction-stimulated glucose uptake and S-glutathionylation of this band in mouse extensor digitorum longus (EDL) muscles (48). We examined by mass spectroscopy the proteins of ~260 kDa that were S-glutathionylated with exercise, but it appears that none of these proteins is involved in glucose uptake although more work is required to clarify this.

Peroxynitrite not only modifies thiol groups promoting S-nitrosylation and S-glutathionylation, but at higher concentrations it can also irreversibly modify side chains of amino acids, especially tyrosine to form nitrotyrosine (tyrosine nitration) (22). At pathophysiological concentrations peroxynitrite is detrimental to cell function, but at physiological levels peroxynitrite can upregulate an array of signaling enzymes (56). We found an increase in tyrosine nitration of an ~35-kDa band in mouse EDL muscle following ex vivo contraction, and the peroxynitrite scavenger urate (33) attenuated the increase in both glucose uptake and this tyrosine nitration (48). However, it was clear that urate was having nonspecific effects, such as increasing resting tension (48). It is possible that tyrosine nitration was increased because of the likely high reactive oxygen and nitrogen species produced during contraction ex vivo, since the preparation is bubbled with carbon, and in the absence of an intact blood supply, there is likely a hypoxic core in the muscle and the contractions result in high oxygen tension in the muscle and the contractions result in high oxygen tension.

Fig. 1. Potential nitric oxide signaling in regard to skeletal muscle glucose uptake during exercise. ROS, reactive oxygen species; PKG, protein kinase G; S-gluta, S-glutathionylation; TN, tyrosine nitration; S-nitro, S-nitrosylation; ONOO\(^-\), peroxynitrite.
levels of fatigue, greater than seen in situ and in vivo. These conditions likely produce very high levels of ROS and NO, which would be above that of in vivo contractions. Therefore, further studies are required to clarify the role of tyrosine nitration of proteins in the regulation of glucose uptake during contraction, especially in situ and in vivo.

Studies examining how NO regulates glucose uptake during exercise need to now examine downstream events related to GLUT4 translocation. Recent studies have provided evidence that Akt-substrate of 160 kDa (AS160, also known as TBC1D4) and tre-2/USP6, BUB2, cdc16 domain family member 1 (TBC1D1) may be involved in this process. The role of AS160 and TBC1D1 in GLUT4 translocation is mediated by its GTPase-activating domain and interactions with Rab proteins in vesicle formation, increasing GLUT4 translocation when its GTPase activity is inhibited by phosphorylation. It appears that both of these proteins are phosphorylated in response to both contraction and insulin, and recent research suggests that TBC1D1 is phosphorylated by AMPK and that this may play a role in the regulation of skeletal muscle glucose uptake during contraction (17, 18, 74). Further studies are required to determine whether NOS inhibition and/or a lack of nNOS alters skeletal muscle TBC1D1 phosphorylation during contraction/exercise.

**Blood flow.** In humans, NOS inhibition reduces blood flow at rest and during the immediate recovery from exercise, but it does not attenuate the increase in blood flow during exercise (6, 15, 21, 29, 61). In general, the only human studies that find a reduction in blood flow during exercise actually stopped the exercise to make the measurements (e.g., by venous occlusion plethysmography), so this is more reflective of recovery blood flow (13). This does not mean that NO is not important for regulating blood flow during exercise but instead that other factors are able to compensate for the loss of NO. Indeed, it has been shown that, although NOS inhibition alone has no effect on blood flow, combined NOS inhibition and prostaglandin inhibition attenuates the increase in blood flow during leg exercise in humans (4, 21, 52).

Although NOS inhibition may not affect total blood flow during exercise in humans, this does not rule out the possibility of effects on muscle capillary/microvascular/nutrient blood flow. For this reason, we examined the effect of NOS inhibition on both femoral blood flow and muscle microvascular blood flow (using contrast-enhanced ultrasound) during in situ contractions in anesthetized rats (64). We found that there was no effect of NOS inhibition on the increase in muscle blood volume or microvascular muscle blood flow rate during contraction; however, muscle glucose uptake during contraction was reduced by ~30% (64). This finding is in agreement with Inyard et al. (26) who also found no effect of NOS inhibition on muscle microvascular flow during contraction in anesthetized rats and a reduction in muscle glucose uptake (at high electrical stimulating frequencies, a halving but not statistically significant). This was despite the NOS inhibition being delivered via intravenous infusion, which resulted in large increases in blood pressure and therefore driving pressure (26). These data (26, 64) strongly suggest that NOS inhibitor-induced impairment in muscle glucose uptake during contraction originates within the muscle itself and is not a result of a reduction in supply of glucose or other factors.

Unlike in humans, studies in rodents have generally found that NOS inhibition attenuates increases in skeletal muscle blood flow during exercise (10, 24, 41). Although NO from eNOS in vascular endothelium is involved in the control of blood flow, nNOS in skeletal muscle, which is associated with the sarcolemma via dystrophin (7), also appears to play a role in blood flow. Indeed, mdx mice, which lack dystrophin and sarcolemmal nNOS, (yet express normal levels of eNOS), and nNOS knockout mice have reduced arteriolar dilation in response to contraction in situ (38, 70), and muscle blood flow is also impaired after mild exercise in mice lacking sarcolemmal nNOS (30). Importantly, restoring dystrophin, and thus sarcolemmal nNOS in skeletal muscle of mdx mice improved muscle perfusion during exercise (34). It is currently unknown whether these adverse effects on blood flow are also seen in humans with Duchenne muscle dystrophy, who also lack dystrophin and sarcolemmal nNOS expression (7, 77). It is interesting to note that, as with mdx mice, people with type 2 diabetes (5), who have reduced NOS protein expression, have reduced muscle blood flow during dynamic exercise (29).

**Oxygen consumption.** NO has been shown to rapidly and reversibly inhibit cytochrome oxidase in isolated rat skeletal muscle mitochondria and in other systems (8, 9, 66). However, despite this, there are conflicting results on the effect of NO on oxygen consumption during exercise. Although several studies have found no effect of NOS inhibition on oxygen consumption during exercise in humans (6, 21, 29, 61), one study found, surprisingly, a reduction (52). Intriguingly, it has been recently shown that the ingestion of nitrate-containing beverages before exercise reduces oxygen consumption (increases efficiency) during exercise in humans (1, 37, 72). Although the mechanism(s) are unclear, it has been proposed that nitrate exerts its effects via NO after nitrate is first converted to nitrite and then to NO. Effects on proton leakage in the mitochondria may be involved (36), although more well-controlled mechanistic studies are required. This research is important because there is preliminary evidence that substances high in nitrate (e.g., beetroot juice) can both increase athletic performance (35) but also improve functional capacity in patients with low aerobic capacities, such as peripheral arterial disease (28).

**Mitochondrial biogenesis.** There is good evidence that NO increases mitochondrial biogenesis in skeletal muscle. Nisoli and colleagues (54) demonstrated in L6 myoblasts that NO donors and also cGMP analogs increase mitochondrial biogenesis, mitochondrial volume, and also oxygen consumption. We (46) and Lira et al. (43) have also shown that NO donors increase markers of mitochondrial biogenesis in L6 myotubes. Interestingly, increases in skeletal muscle cytosolic calcium and NO levels and activation of AMPK are all known to increase mitochondrial biogenesis (43, 46, 55, 79). Furthermore, these NO effects on skeletal muscle mitochondrial biogenesis are mediated, at least in part, by AMPK (43, 46), via the α1A-isofom (43) and perhaps by calcium (46). Therefore, it is reasonable to hypothesize that the increased NO levels in skeletal muscle during exercise are necessary for the increased mitochondrial biogenesis following endurance exercise. However, pharmacological inhibition of NO during exercise does not prevent the increases in markers of mitochondrial biogenesis following acute exercise, such as gene expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (76). Furthermore, eNOS and nNOS knockout mice...
have normal increases in gene expression markers of mitochondrial biogenesis, including PGC-1α, following acute exercise and increased protein abundance of mitochondrial proteins following exercise training (75). Therefore, in summary, although NO appears to play a role in the regulation of skeletal muscle mitochondrial biogenesis under basal (noncontraction) conditions, the increase in NO in skeletal muscle during exercise does not appear necessary for increased mitochondrial biogenesis following exercise.

Summary and Concluding Remarks

It is clear that NO has many roles in skeletal muscle that extend far beyond that of vasodilation. As discussed above, nNOSµ is expressed in skeletal muscle where it is reduced in people with diabetes while being increased with exercise training. There is evidence that a reduction in skeletal muscle nNOSµ is associated with insulin resistance and is responsible for some of the pathology of Duchenne muscular dystrophy. NO appears to play a role in basal skeletal muscle mitochondrial biogenesis but not the increase in mitochondrial biogenesis with exercise. There is good evidence that NO is required for normal increases in skeletal muscle glucose uptake during contraction/exercise in both rodents and humans. The mechanisms involved have not yet been clearly demonstrated with studies examining downstream of NOS, including examination of AS160 and TBC1D1 required. Finally, recent exciting research has demonstrated that nitrate supplementation increases muscle energetic efficiency during exercise, and it has been assumed that this is due to increases in NO. Mechanistic studies to determine if this is indeed the case are necessary.

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