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

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ABSTRACT

Nitric oxide (NO) is an important vasodilator and regulator in the cardiovascular system and this link was the subject of a Nobel prize in 1998. However, NO also plays many other regulatory roles including thrombosis, immune function, neural activity and gastrointestinal function. Low concentrations of NO are thought to have important signaling effects. In contrast, high concentrations of NO can interact with reactive oxygen species, causing damage to cells and cellular components. A less recognized site of NO production is within skeletal muscle, where small increases are thought to have beneficial effects such as regulating glucose uptake and possibly blood flow, but higher levels of production are thought to lead to deleterious effects such as an association with insulin resistance. This review will discuss the role of NO in skeletal muscle during and following exercise including in mitochondrial biogenesis, muscle efficiency and blood flow with a particular focus on its potential role in regulating skeletal muscle glucose uptake during exercise.

INTRODUCTION

People with type 2 diabetes exhibit postprandial glucose intolerance, due to a reduction in skeletal muscle and liver insulin resistance and pancreatic cell insufficiency. The 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 hours 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 nNOS (NOS1, the primary NOS isozyme in skeletal muscle fibres) 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/PKG (PKG: cGMP-dependent protein kinase) 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 Figure 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 through which NO signals glucose uptake during exercise, specific therapeutics can be designed for people with type 2 diabetes to mimic the contraction signalling 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 NITRIC OXIDE / NITRIC OXIDE SYNTHASE**

In skeletal muscle the primary isoform of NOS expressed is nNOSµ which is an alternatively spliced isozyme of neuronal NOS (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 inducible NOS (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 nNOSμ and eNOS in rats (3) and nNOSμ 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 nNOSμ 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 several fold higher levels of NO that may then downregulate nNOSμ expression. Low concentrations of NO appear to have important signaling effects, but high concentrations of NO (from iNOS) can interact with reactive oxygen species, 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), although 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. As mentioned above, 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 nNOS in addition to nNOSµ 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 nNOSµ and nNOSβ suggest that nNOSβ is a critical regulator of the structural and functional integrity in skeletal muscle (59).

SKELETAL MUSCLE NITRIC OXIDE 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 nNOSµ and eNOS in rodent skeletal muscle, nNOSµ is the primary source of skeletal muscle NO during contraction in mouse muscle (39) and in contracting muscle cells (25, 58, 69).

EFFECTS OF NITRIC OXIDE 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 GLUT-4 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/CaMK, protein kinase C, reactive oxygen species (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 L-NMMA into the
defemoral artery during cycling exercise at 60% VO$_2$ 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 minutes after the
contractions or exercise was ceased so therefore relate to post-exercise/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 utilised may also contribute to
differences in results between studies. Indeed, Hellsten and colleagues have shown in
contracting primary muscle cells that NO is only released at higher intensities (69), 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).

Since inhibitors can have non-specific effects it is necessary that experiments be performed where the influence of nNOSµ is removed by other means such as examining nnosµ⁻/⁻ mice. This may be quite a complex undertaking because there is evidence of several other alternatively spliced isoforms of nNOS in addition to nNOSµ in skeletal muscle (59). In addition, knocking out nNOSµ can result in compensatory increases in eNOS protein expression in skeletal muscle (75). Surprisingly, enos⁻/⁻ mice have higher skeletal muscle glucose uptake during treadmill exercise (40). It is likely, however, that this was due to a greater reliance on anaerobic metabolism of glucose because of the relative hypoxia during exercise as a result of the observed lower muscle blood flow (% of cardiac output) during exercise and the impaired mitochondrial function in these mice and indeed plasma lactate levels were higher during running in these mice (40).

How does NO signal skeletal muscle glucose uptake during contraction?

Having provided strong evidence that NO plays an essential role in the regulation of skeletal muscle glucose uptake during contraction in mice (48, 49), rats (64) and during exercise in humans (6, 29), we have now begun to try to determine the pathways downstream of NO/NOS that are involved (See Figure 1).
NO can bind to a haem group on soluble guanylate cyclase (sGC), which is expressed in skeletal muscle (32, 73), producing cGMP and subsequently activating PKG (82) (Figure 1). Skeletal muscle cGMP concentration increases during contraction of mouse muscle \textit{ex vivo}, and this increase is abolished by NOS inhibition and is also absent in nNOS knockout mouse muscle (39). NO donors raise cGMP levels and increase glucose uptake (3, 14, 39) while sGC inhibition prevents this increase in cGMP and glucose uptake (14, 82, 83). Indeed we have found that the NO donor diethylenetriamine/NO (DETA/NO) increases glucose uptake in C57Bl6 mouse EDL muscles \textit{ex vivo} and this is prevented by the specific (19) sGC inhibitor 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ; 10 μM) (48).

Interestingly, the same concentration (10 μM) of the sGC inhibitor ODQ that blocked NO donor-induced glucose uptake in non-contracting muscle had no effect on glucose uptake during contraction (48), unlike the NOS inhibitor L-NMMA which attenuated glucose uptake. In addition, the PKG inhibitor Rp-8-Br-PET-cGMPS also did not affect muscle glucose uptake during contraction (48). These results suggest that NO may be activating glucose uptake during contraction via a cGMP/PKG-independent mechanism(s). However, these findings need to be 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 post-translational modifications of proteins via S-nitrosylation, S-glutathionylation and tyrosine nitration (See Figure 1). Nitric oxide can produce post-translational modifications of thiol (-SH) groups on cysteine residues 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 (eg 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 (GSNO) (11, 44). Furthermore, superoxide and NO can react to form peroxynitrite, which modifies protein thiol 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 a ~260 KDa band increased during contraction of rodent muscle and during exercise in human skeletal muscle (47, 48, 50). DTT, a thiol specific reducing agent (62) attenuated contraction-stimulated glucose uptake and S-glutathionylation of this band in mouse 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 are 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 a ~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 non-specific effects, such as increasing resting tension (48). It is possible that tyrosine nitration was increased because of the likely high RONS produced during contraction *ex vivo* since the preparation is bubbled with carbogen, and in the absence of an intact blood supply, there is likely a hypoxic core in the muscle and the contractions result in high 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 GLUT-4 translocation. Recent studies have provided evidence that Akt-substrate of 160 KDa (AS-160, 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 nNOSµ 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 (eg 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, nNOSμ 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 nNOSμ (yet express normal levels of eNOS) and nNOSμ KO 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 nNOSμ (30). Importantly, restoring dystrophin – and thus sarcolemmal nNOSμ – 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 nNOSμ expression (7, 77). It is interesting to note that as with mdx mice, people with type 2 diabetes (5), who have reduced nNOS 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 is
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 prior to 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 demonstrated in L6 myoblasts that NO donors and also cGMP analogues increase mitochondrial biogenesis, mitochondrial volume and also oxygen consumption (54). 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, 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 \( \alpha_1 \) isoform (43) and perhaps by calcium (46). Therefore, it is reasonable to hypothesise 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 PGC-1α (76). Furthermore, eNOS and nNOS KO 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 (non-contraction) 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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Figure legend

Figure 1. Potential nitric oxide signaling in regards to skeletal muscle glucose uptake during exercise. S-gluta=S-glutathionylation; TN= tyrosine nitration; S-nitro= S-nitrosylation; ONOO⁻ is peroxynitrite.
FIGURE 1

Contraction

ROS → ONOO− → S-gluta → TN

NO− → cGMP → S-nitro → PKG activity

Glucose transport and glucose uptake