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Potential role of nitric oxide in contraction-stimulated glucose uptake and mitochondrial biogenesis in skeletal muscle

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Summary

1. This review will discuss the potential role of nitric oxide (NO) in the (i) regulation of skeletal muscle glucose uptake during exercise and (ii) the activation of mitochondrial biogenesis after exercise.

2. We have shown in humans that local infusion of a NO synthase (NOS) inhibitor during exercise attenuates increases in skeletal muscle glucose uptake without influencing blood flow. Recent studies from our laboratory in rodents support these human findings although rodent studies from other laboratories have yielded conflicting results.

3. There is clear evidence that NO increases mitochondrial biogenesis in non-contracting cells and also that NO influences basal skeletal muscle mitochondrial biogenesis. There have, however, been few studies examining the potential role of NO in the activation of mitochondrial biogenesis following an acute bout of exercise or in response to exercise training. Early indications are that NO is not involved in regulating the increase in mitochondrial biogenesis that occurs in response to exercise.

4. Exercise is considered the best prevention and treatment option for diabetes, but unfortunately, many people with diabetes do not or can not exercise regularly. Alternatives therapies are therefore critical to effectively manage diabetes. If skeletal muscle NO is found to play an important role in regulating glucose uptake and/or mitochondrial biogenesis, pharmaceutical agents designed to mimic these exercise effects may improve glycaemic control.

Skeletal muscle glucose uptake during exercise

Skeletal muscle accounts for over 80% of whole body insulin-stimulated glucose uptake. People with type 2 diabetes have normal levels of the Glucose Transporter GLUT-4 in their skeletal muscles but GLUT-4 translocation and glucose uptake in response to insulin is reduced.[1] Importantly, however, skeletal muscle GLUT-4 translocation[2] and glucose uptake during exercise is normal in people with diabetes.[3] Indeed, blood glucose levels can decrease to normal levels during 45 min of intense exercise in people with type 2 diabetes.[4]

The signalling pathway(s) associated with contraction-stimulated glucose uptake are not fully understood, but are known to differ from insulin pathways. For example wortmannin, a phosphatidylinositol 3-kinase (PI3 kinase) inhibitor, blocks insulin-stimulated glucose uptake in skeletal muscle but it has no effect on contraction-stimulated glucose uptake. Possible candidates regulating glucose uptake during exercise include calcium-calmodulin dependent protein kinase II (CaMKII), protein kinase C, AMP-activated protein kinase (AMPK), reactive oxygen species and NO[5-7] (See Figure 1). 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. The first half of this review will focus on the potential role of NO/NO synthase (NOS) in contraction-stimulated glucose uptake in skeletal muscle.

Does NO/NOS regulate contraction-stimulated glucose uptake?

The production of NO is catalysed by NOS enzymes which convert L-arginine to L-citrulline and NO. NOS activity increases in skeletal muscle during *in situ* contractions[8] and during *in vivo* exercise in rats[9] (Figure 1). The resulting increase in NO release from contracting muscle[10] and contracting cells[11] can be prevented by NOS inhibition.[8-10] NO binds to the haem group of soluble guanylate cyclase (sGC), producing the second messenger cyclic guanosine monophospate (cGMP) (Figure 1). cGMP increases during *in vitro* muscle contractions in mice and this increase in cGMP is prevented by NOS inhibition.[12]

In skeletal muscle, neuronal (n) NOS is the primary source of NO production during contraction.[11-13] Indeed, skeletal muscle cGMP concentration increases during contraction in endothelial (e) NOS knock-out mice but not nNOS knock-out mice.[12] There is very little expression of inducible (i) NOS in normal skeletal muscle.[14,15] We found that infusion of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) in to the femoral artery during cycling exercise substantially attenuated the increase in leg glucose uptake in healthy young individuals[6] and especially in people with type 2 diabetes.[3] Importantly, local infusion of the NOS inhibitor had no effect on leg blood flow, arterial blood pressure, or insulin and glucose concentrations during exercise.[3,6] These studies suggest that NO is required for normal increases in glucose uptake during exercise in humans. We also found that infusion of the NOS precursor L-arginine (0.5 g/min) during prolonged exercise in healthy young men significantly increased glucose disposal during exercise.[16] It has been shown that L-arginine increases basal NO production by rat skeletal muscle *in vitro*[10] so we assume that the L-arginine infusion increased skeletal muscle NO production during exercise.

It should be noted that in our human studies we did not examine whether exercise increased NOS activity and

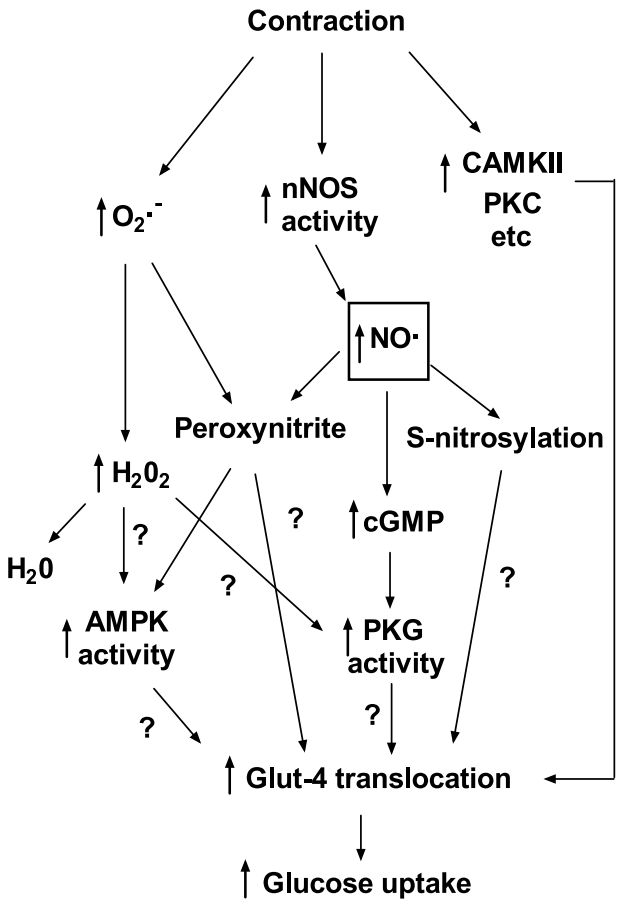

Figure 1. The potential regulation and regulating pathways of skeletal muscle glucose uptake during contraction/exercise with a focus on nitric oxide. *Superoxide (O_2•$^-$), Nitric oxide (NO•), neuronal isoform of NO synthase (nNOS), calcium-calmodulin dependent protein kinase II (CaMKII), Protein kinase C (PKC), hydrogen peroxide (H_2O_2), AMP-activated protein kinase (AMPK), protein kinase G (PKG), cyclic guanosine monophosphate (cGMP).*

whether this was prevented by NOS inhibition or that NOS activity was increased by L-arginine infusion during exercise. Future studies are warranted to address this cognitive gap. We have followed up these human studies with rodent experiments which allow a more thorough examination of mechanisms. In recent preliminary findings we have indications that nNOS knock-out mice display essentially no increase in glucose uptake during *in vitro* contractions (Linden *et al.* unpublished observations[*]) and that NOS inhibition (by L-NMMA) attenuates skeletal muscle glucose uptake during *in vitro* contractions in C57Bl6 mice (Merry *et al.* unpublished observations[†]). Since there is no blood flow during *in vitro* muscle contractions, these results confirm that the effects of a reduction/prevention of NO production (NOS inhibition or nNOS knock-out mice) on glucose uptake are independent of the potential effects of NO on blood flow. Indeed, we also found that NOS inhibition, which prevented the contraction-induced increase in skeletal muscle NOS activity, attenuated increases in glucose uptake during *in situ* contractions in rats without affecting muscle capillary blood flow, suggesting that NOS inhibition was affecting the skeletal muscle fibres *per se*.[8] Taken together with our human findings, these studies suggest that NO is essential for normal increases in skeletal muscle glucose uptake during exercise.

It is worth noting that rat studies from other laboratories have yielded conflicting results (e.g. Higaki *et al.*, 2001,[17] Roberts *et al.*, 1997[18]). For example, two studies found NOS inhibition prevented increases in contraction-stimulated glucose uptake[5,18] but two other studies reported no effect of NOS inhibition on contraction-stimulated glucose uptake.[17,19] In general, the rodent studies in this area of inquiry have been difficult to interpret, since in these studies, unlike in our human studies and our recent rodent studies where glucose uptake is measured *during* exercise, glucose transport/uptake measurements were usually made 20 or more minutes *after* contractions or treadmill exercise. The potential reasons for these contradictory findings in the studies using rodents have been discussed elsewhere.[20]

Downstream signalling of NOS/NO in terms of glucose uptake

It is important to examine the potential mechanisms of how NO increases glucose uptake during contractions. To date, the only studies examining phenomena downstream of NOS in relation to glucose uptake were conducted in non-contracting muscles.[21,22] Clearly, one can not assume that the regulation of muscle glucose uptake at rest is similar to the regulation during exercise.

In non-contracting isolated skeletal muscles, NO appears to exert its effects by modulating cGMP levels.[21,22] Increases in NO stimulate soluble guanylate cyclase, producing cGMP (Figure 1). In isolated skeletal muscles from mice and frogs, contraction increases muscle cGMP levels and the cGMP analogue 8-bromo-cGMP increases glucose uptake in isolated rat muscles.[21,22] In addition, NO donors such as sodium nitroprusside (SNP) raise skeletal muscle cGMP content[17,21,22] and increase glucose uptake, presumably as a result of the activation of sGC.[21,22] Indeed, addition of LY-83583, an inhibitor of sGC, decreases skeletal muscle cGMP content and reduces basal 2-deoxyglucose transport in isolated rat muscle to a similar extent as with L-NMMA.[22] LY-83583 also completely

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abolishes SNP augmented glucose transport.[22] However, LY-83583, in addition to inhibiting sGC, also inhibits nNOS.[23] It is therefore important that the more specific sGC inhibitor 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ) be used[24] and also that contraction studies be conducted. It is also important that studies examine whether cGMP-dependent protein kinase (PKG) activity is increased with contraction/exercise (Figure 1).

It has become clear that in many cell types NO also signals through cGMP-independent mechanisms, especially *S*-nitrosylation,[25,26] the covalent addition of NO to a cysteine thiol/sulfhydryl group, resulting in *S*-nitrosothiols (Figure 1). *S*-nitrosylation is "increasingly becoming recognized as a ubiquitous regulatory reaction comparable to phosphorylation".[26] Indeed, it has been demonstrated in vascular smooth muscle cells, human endothelial cells, intact hearts and in skeletal muscle that *S*-nitrosylation is involved in cGMP-independent signalling effects of NO[27-30] and that proteins associated with glucose transport regulation (e.g. Akt/PKB) are susceptible to S-nitrosylation in skeletal muscle.[30] It is important that studies examine whether NO increases glucose uptake during contraction/exercise, at least in part, *via* increases in *S*-nitrosylation.

Finally, NO may exert its effects during exercise in part *via* tyrosine nitration by peroxynitrite.[31] Peroxynitrite is formed by a non-enzymatic reaction between NO and the reactive oxygen species (ROS), superoxide (Figure 1). It has been reported that during contraction of myocytes there are increases in both superoxide and NO, and therefore peroxynitrite[11] (Figure 1). Note that this study[11] involved contraction in healthy muscle cells so is indicative of physiological signalling and not a pathological disease process, as may be the case with peroxynitrite. At physiological levels, peroxynitrite upregulates an array of signalling cascades, through the inhibition of phosphatases and direct activation of several different protein kinases (e.g. AMPK and MAP kinases) involved in regulating skeletal muscle glucose uptake.[25,32]

AMPK does not increase glucose uptake *via* activation of nNOS

5-aminoimidazole-4-carboxyamide-ribonucleoside (AICAR), a cell-permeable activator of AMPK, increases rat skeletal muscle glucose uptake, *in vitro* and *in vivo*. Like exercise, AICAR increases the translocation of GLUT-4 to the sarcolemma and appears to act *via* an insulin-independent mechanism. AMPK phosphorylates and activates eNOS[33] and in the heart this plays a role in glucose uptake.[34] A study in rats found that AICAR increased NOS activity and glucose uptake in muscle fibre bundles, and that these effects could be prevented by NOS inhibition. However, we have found that NOS inhibition has no effect on AICAR-activated glucose transport in isolated rat skeletal muscle.[35] In addition, we found that although AMPK phosphorylates nNOS during contraction *in situ* in rats,[8] and during exercise in human skeletal muscle,[36] *in vitro* phosphorylation of recombinant nNOS by purified AMPK has little effect on nNOS activity (Lee-Young *et al.* unpublished observations[‡]). It is unlikely, therefore, that AMPK exerts effects on glucose uptake *via* phosphorylation of nNOS, which, as mentioned earlier, is the main isoform in muscle. Indeed, preliminary evidence indicates that AICAR increases glucose uptake normally in skeletal muscle of nNOS knock-out mice *in vitro* (Linden *et al.* unpublished observations[*]).

Mitochondria, exercise and diabetes

The skeletal muscle of people with type 2 diabetes have reduced mitochondrial function, largely due to reduced mitochondrial volume.[37] This reduced mitochondrial volume/function and elevated intramuscular lipids are associated with insulin resistance in skeletal muscle.[38-41] However, it is still unclear if reduced mitochondrial volume/function is a cause or a consequence of insulin resistance.[42,43] Some studies,[39,41,44] but not all,[40] have reported reduced mitochondrial synthesis (biogenesis) in insulin resistant skeletal muscle.

Endurance exercise training increases skeletal muscle mitochondrial volume[45,46] and mitochondrial biogenesis.[45,47-49] It is possible that increases in skeletal muscle mitochondrial biogenesis contribute to the increase in skeletal muscle insulin sensitivity observed after exercise training.[50] Although this has not been extensively examined, evidence suggests that people with type 2 diabetes, who have reduced skeletal muscle mitochondrial biogenesis, respond to exercise training with a normal increase in mitochondrial enzymes and insulin sensitivity.[50]

Many research groups are attempting to determine how exercise increases mitochondrial biogenesis and it appears that the same signals that may be involved in regulating glucose uptake during exercise (*e.g.* AMPK, CaMK and NO) may also be activating gene expression of key markers of mitochondrial biogenesis such as peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PCG-1α), nuclear respiratory factors 1 and 2 (NRF-1, NRF-2) and mitochondrial transcription factor A (mtTFA) after exercise.[51,52] The next section will discuss the potential role of NO in basal and contraction-stimulated mitochondrial biogenesis.

Does NO/NOS play a role in mitochondrial biogenesis after exercise?

It has been shown that NO donors and analogues of the downstream messenger of NO, cGMP, increase mitochondrial biogenesis in muscle cells.[53] Although nNOS is the major NOS isoform in skeletal muscle, both eNOS and nNOS isoforms are expressed within skeletal muscle fibers, with eNOS more abundant in oxidative, and nNOS

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more abundant in glycolytic skeletal muscles in rodents.[12,54-56] NO production, total NOS activity and cGMP, increase during contraction in rodent skeletal muscle[9,10,12] and may therefore be involved in the upregulation of mitochondrial biogenesis after exercise.

We found that ingestion of the non-specific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) for 2 days reduced basal skeletal muscle mitochondrial biogenesis but had no effect on the increase in mitochondrial biogenesis after an acute bout of exercise.[57] Similarly, the increase in mitochondrial biogenesis markers in response to acute exercise and exercise training is intact in nNOS knock-out and eNOS knock-out mice.[58] Therefore, these data suggest there is little involvement of NOS in the activation of mitochondrial biogenesis following exercise. Interestingly, however, rather than basal mitochondrial biogenesis being lower in nNOS knock-out mice muscle it was actually higher,[58] a finding consistent with a previous report of elevated citrate synthase activity in the skeletal muscles of nNOS knock-out mice.[59] The molecular mechanism(s) for elevated basal mitochondrial biogenesis in the muscle of nNOS knock-out mice is unclear, although it is independent of two key signalling proteins for mitochondrial biogenesis; phosphorylation of p38 MAPK and AMPK,[58] which were normal in basal nNOS knock-out mouse skeletal muscle.

Some,[53,60] but not all studies,[58] have also reported reduced basal mitochondrial biogenesis in the skeletal muscles of eNOS knock-out mice. However, methodological differences, including eNOS knock-out mice being generated from different backgrounds and from differently generated eNOS gene deletions[61,62] make comparisons between studies difficult. Further investigations are needed to elucidate a role for eNOS in basal mitochondrial biogenesis in skeletal muscle.

Although more research is required, these studies suggest that NO plays a role in basal mitochondrial biogenesis but not in the increase in mitochondrial biogenesis in response to exercise. Further investigations are required to determine the exact roles of the eNOS and nNOS isoforms in basal mitochondrial biogenesis.

Conclusions

There is good, albeit not fully consistent, evidence that NO is essential for normal increases in glucose uptake during contraction/exercise. Further studies are now required to determine the factors downstream of NO/nNOS that regulate contraction-stimulated glucose uptake. In contrast, although further studies are required it appears that NO/NOS are not important in the activation of skeletal muscle mitochondrial biogenesis following exercise although NO/NOS may play a role in basal skeletal muscle mitochondrial biogenesis.

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