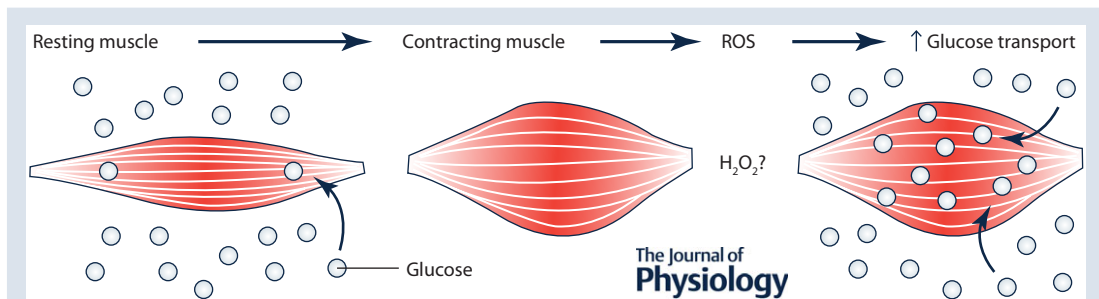**TOPICAL REVIEW**

# Role of reactive oxygen species in regulation of glucose transport in skeletal muscle during exercise

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**Abstract**  Glucose derived from extracellular sources serves as an energy source in virtually all eukaryotic cells, including skeletal muscle. Its contribution to energy turnover increases with exercise intensity up to moderately heavy workloads. However, at very high workloads, the contribution of extracellular glucose to energy turnover is negligible, despite the high rate of glucose transport. Reactive oxygen species (ROS) are involved in the stimulation of glucose transport in isolated skeletal muscle preparations during intense repeated contractions. Consistent with this observation, heavy exercise is associated with significant production of ROS. However, during more mild to moderate stimulation or exercise conditions (*in vitro*, *in situ* and *in vivo*) antioxidants do not affect glucose transport. It is noteworthy that the production of ROS is limited or not observed under these conditions and that the concentration of the antioxidant used was extremely low. The results to date suggest that ROS involvement in activation of glucose transport occurs primarily during intense short-term exercise and that other mechanisms are involved during mild to moderate exercise. What remains puzzling is why ROS-mediated activation of glucose transport would occur under conditions where glucose transport is highest and utilization (i.e. phosphorylation of glucose by hexokinase) is low. Possibly ROS production is involved in priming glucose transport during heavy exercise to accelerate glycogen biogenesis during the initial recovery period after exercise, as well as altering other aspects of intracellular metabolism.

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**Abstract figure legend** At rest glucose transport into muscle is low. Intense contractions result in formation of reactive oxygen species (ROS); one of these (possibly $H_2O_2$) can lead to increased glucose transport.

**Abbreviations**     AMPK, AMP-dependent protein kinase; BTS, *N*-benzyl-*p*-toluene sulphonamide; EDL, extensor digitorum longus; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; SOD, superoxide dismutase.

## Introduction

More than a century ago, increased glucose uptake was observed in the equine masseter muscle during chewing (Chauveau & Kaufmann, 1887). Studies in the 1950 s confirmed that contractions increased muscle glucose uptake in rats and dogs (Goldstein *et al.* 1953; Huycke, 1955) and in the 1960 s and 1970 s measurements of muscle glucose uptake were performed in humans (Reichard *et al.* 1961; Sanders *et al.* 1964; Havel *et al.* 1967; Whichelow *et al.* 1968; Jorfeldt & Wahren, 1970; Wahren *et al.* 1971; Ahlborg *et al.* 1974). It is generally implied or implicitly stated that extracellular glucose is an important fuel for muscle function or performance, especially under conditions of prolonged submaximal exercise (Richter & Hargreaves, 2013). A link between reduced extracellular glucose (hypoglycaemia) and fatigue was identified in early (Levine *et al.* 1924; Christensen, 1939) and later studies as well (Coyle *et al.* 1983), although hypoglycaemia does not always lead to fatigue during prolonged submaximal exercise (Felig *et al.* 1982).

## Glucose transport and carbohydrate utilization

The extent to which glucose transport contributes to carbohydrate utilization in muscle depends on the experimental conditions (e.g. intensity, duration and mode of exercise, muscle mass involved, glycogen and blood flow analyses, etc.). Almost 30 years ago, it was shown that during maximal leg cycling exercise (corresponding to ∼100% of maximal oxygen uptake ($\dot{V}_{O_2max}$), leg glucose uptake increased approximately 50-fold *vs.* rest, but virtually none of the glucose that entered the muscle was metabolized (metabolized and/or utilized denotes glucose phosphorylation by hexokinase followed by entry into glycolysis), presumably due to inhibition of hexokinase by glucose-6-P (Katz *et al.* 1986). Thus under conditions of intense short-term exercise, despite maximal rates of glucose transport, the contribution of extracellular glucose to muscle carbohydrate utilization is negligible. Indeed during the initial phase of moderate cycling exercise (first 40 min at 75% of $\dot{V}_{O_2max}$), where high rates of glucose uptake are achieved, extra-cellular glucose contributes to only about 5% of muscle carbohydrate utilization. And even

during the latter half of the exercise (the last 35 min), extracellular glucose accounted for less than 20% of carbohydrate utilization. These estimates were based on direct measurements of muscle glucose uptake (leg blood flow measurements and arterial and femoral venous glucose concentrations) and muscle glycogen contents (Katz *et al.* 1991). The contribution of extracellular glucose to carbohydrate utilization increases as the glycogen store is depleted and this becomes especially clear when performing exercise with initial high or low muscle glycogen content (Gollnick *et al.* 1981). In summary, extracellular glucose contributes little to muscle carbohydrate utilization during moderate and heavy exercise. Therefore the use of carbohydrate supplements will be of limited benefit under many exercise conditions when glycogen stores are full or adequate (Coyle *et al.* 1986). Only when muscle glycogen stores reach low levels will carbohydrate supplements have positive results on muscle performance (Coyle *et al.* 1986).

## Limitations of glucose metabolism

What limits glucose metabolism in skeletal muscle has been an issue of debate. Three specific sites or processes have been suggested: (1) glucose delivery, (2) glucose transport, and (3) glucose phosphorylation by hexokinase. Richter and Hargreaves have discussed this issue in depth and the reader is referred to their recent review for details (Richter & Hargreaves, 2013). Various experimental approaches have been employed, including alterations in the expression of enzymes or transport proteins (e.g. hexokinase, glycogen synthase, GLUT1 and GLUT4), or use of isotopic glucose analogues and calculations based on specific assumptions in mice, as well as increasing extracellular glucose concentrations, to assess what limits glucose metabolism. Depending on the manipulation and the conditions studied, it has been concluded that all three mechanisms can be limiting (Schultz *et al.* 1977; Furler *et al.* 1991; Ren *et al.* 1993; Manchester *et al.* 1996; Azpiazu *et al.* 2000; Wasserman, 2009; Richter & Hargreaves, 2013). Thus conclusions regarding the limiting step are likely to depend on the assumptions made and conditions of study. Noteworthy was the important observation that mice exhibit muscle glycogen concentrations that are only about 10% of those in humans (Coyle *et al.* 1986; Katz *et al.*

1991; Pederson *et al.* 2004; Zhang *et al.* 2008). Further, mice that lack glycogen synthase, and therefore lack glycogen as well, are able to run as well as wild-type (WT) mice (Pederson *et al.* 2005). Thus caution should be used in extrapolating results from such models to intact humans. Briefly, it appears that during the onset of moderate exercise or during short-term intense exercise in humans, glucose phosphorylation limits glucose metabolism. This conclusion is based on measurements of accumulation of free glucose in the muscle, and it appears that the free glucose derives from the extracellular space (from glucose transported into muscle) rather than release from intracellular glycogen (via debranching enzyme) (Katz *et al.* 1986, 1991; Sahlin *et al.* 1989).

## Reactive oxygen species and glucose transport

Background information on free radical chemistry and the role of reactive oxygen species (ROS) in exercise-induced oxidative stress are presented elsewhere (Powers & Jackson, 2008). That ROS are involved in activation of glucose transport in isolated muscle preparations has been reviewed earlier (Balon & Yerneni, 2001; Katz, 2007; Merry & McConell, 2009, 2012; Richter & Hargreaves, 2013). The present brief review will examine literature published since the initial observation that ROS is involved in the activation of glucose transport after intense short-term exercise (Sandström *et al.* 2006). In the latter study, it was found that repeated tetanic contractions in isolated mouse extensor digitorum longus muscle (EDL; fast-twitch, glycolytic) increased glucose transport about threefold and the general anti-oxidant *N*-acetylcysteine (NAC) blocked about half of the increase but did not affect force (Sandström *et al.* 2006). The effect of NAC appeared to be specific for exercise since NAC did not affect basal, hypoxia-, or insulin-mediated glucose transport. Although NAC has anti-oxidant properties, it has additional biological effects, including binding to transition and heavy metal ions and provision of precursor for glutathione synthesis (Samuni *et al.* 2013). Therefore experiments were performed to examine if NAC was functioning as an antioxidant in the isolated EDL muscle. Repeated contractions resulted in increased intracellular ROS levels (using the fluorescent indicator, 5-(and 6)-chloromethyl-2′,7′-dichlorohydrofluorescein) and the ratio of oxidized to reduced glutathione (GSSG/GSH, reflecting increased ROS levels), and both of these increases were attenuated or blocked by NAC, demonstrating that NAC was functioning as an anti-oxidant under the conditions studied (Sandström *et al.* 2006). To identify the species of ROS involved, an additional anti-oxidant, ebselen was used (a selenoorganic compound that induces glutathione peroxidase-like reduction of $H_2O_2$ in the presence of GSH) (Cotgreave *et al.* 1987). Ebselen also inhibited about half of

contraction-mediated glucose transport. This finding implicated $H_2O_2$ or its derivative(s) as the active ROS species. To further study the potential role of $H_2O_2$ as the activating agent, muscles from mice overexpressing mitochondrial $Mn^{2+}$-dependent superoxide dismutase (SOD; to enhance conversion of $O^{\cdot-}$ to $H_2O_2$ in mitochondria) were studied. The strategy was to enhance conversion of superoxide ($O^{\cdot-}$), the parent free radical that is produced in mitochondria, to $H_2O_2$ and thereby increase contraction-mediated glucose transport. Indeed, glucose transport after repeated contractions was increased by about 25% in EDL muscle overexpressing $Mn^{2+}$-dependent SOD compared with values from WT muscle (Sandström *et al.* 2006). In contrast, neither basal nor insulin-mediated glucose transport was affected by SOD overexpression. Addition of $H_2O_2$ to the medium bathing resting muscle also increased glucose transport. Overall the results indicated a role for ROS in activation of glucose transport during intense repeated contractions in isolated mouse muscle and suggested that $H_2O_2$ or its derivative(s) was the activating ROS species.

Interestingly, glucose transport after repeated contractions was almost 45% higher in soleus muscles from mice overexpressing SOD *vs.* muscles from WT mice (Katz, 2007). This may derive from a higher mitochondrial $O^{\cdot-}$ production in soleus owing to a higher mitochondrial volume and/or faster conversion of $O^{\cdot-}$ to $H_2O_2$ because of higher activity of $Mn^{2+}$-dependent SOD in soleus *vs.* EDL muscle (Oh-Ishi *et al.* 1995; Hollander *et al.* 1999). Thus one might expect that contraction-mediated glucose transport should be higher in soleus (type I, more oxidative) than in EDL (type II, more glycolytic) muscle owing to a higher mitochondrial capacity, as well as higher expression of GLUT4 protein in type I *vs.* type II muscle (Richter & Hargreaves, 2013). Indeed during treadmill running in rodents this is the case. Glucose disposal during moderate treadmill running in rats is markedly higher in type I *vs.* type II muscle (James *et al.* 1985). However, glycogen breakdown is substantial in type I muscle but negligible in type II muscle under these conditions (James *et al.* 1985), suggesting that type II muscle was not significantly activated. In contrast, *in vitro* studies, where isolated muscles are activated under identical or similar conditions, show that contraction-mediated activation of glucose transport is actually higher in EDL than in soleus muscle (Higaki *et al.* 2001; Johansson *et al.* 2007; Merry *et al.* 2010*b*; Sylow *et al.* 2013), although sometimes the activation of glucose transport in soleus and EDL muscles can be similar (Jorgensen *et al.* 2004). This appears to be in contrast to the idea that endogenous ROS production is important for activation of glucose transport during exercise. It is noteworthy that type II muscle has lower levels of anti-oxidant buffering systems than oxidative muscle (Lawler *et al.* 1993; Leeuwenburgh *et al.* 1994; Hollander *et al.*

1999; Zhang *et al.* 2007; Anderson & Neufer, 2006). Consequently, EDL may be more sensitive to oxidants. Support for this idea comes from the observation that exposure of soleus and EDL (or epitrochlearis, also a type II muscle) muscles to exogenously administered $H_2O_2$ (or $H_2O_2$ produced by exogenously administered glucose oxidase) results in a greater degree of inactivation of aconitase (an enzyme of the tricarboxylic acid cycle in mitochondria), as well as increased phosphorylation of signalling molecules (e.g. insulin receptor, insulin receptor substrate-1 and protein kinase B (Akt)) and activation of glucose transport in EDL/epitrochlearis muscle (Kim *et al.* 2006; Zhang *et al.* 2007; Jensen *et al.* 2008; Zhang *et al.* 2010). Interestingly, glycolytic muscle actually has a higher capacity than oxidative muscle to generate $H_2O_2$ in mitochondria (Anderson & Neufer, 2006). This should be considered together with the observation that the ROS-dependent activation of glucose transport in EDL muscle is associated with activation of AMP-dependent protein kinase (AMPK) (see below) (Sandström *et al.* 2006). Since activation of AMPK ($\alpha$2 isoform) during repeated contractions is less in soleus than in EDL muscle (Jorgensen *et al.* 2004), this can also contribute to the lower degree of contraction-mediated glucose transport in soleus muscle. Exogenous $H_2O_2$ was shown to selectively activate the $\alpha$1 isoform of AMPK in epitrochlearis muscle (Toyoda *et al.* 2004), and contraction-mediated activation of this isoform is similar in soleus and EDL muscle (Jorgensen *et al.* 2004). On the other hand, others have shown that $H_2O_2$ does not activate either isoform in isolated EDL muscle (Higaki *et al.* 2008), but activates both $\alpha$1 and $\alpha$2 isoforms in isolated soleus muscle (Jensen *et al.* 2008). In our hands exogenous $H_2O_2$ increases total AMPK activity in isolated EDL muscle threefold (Sandström *et al.* 2006). Such differential results make it difficult to reach a conclusive statement regarding the effects of $H_2O_2$ on AMPK. What appears clear is that contraction-mediated glucose transport is less in isolated soleus than in EDL, possibly owing to a decreased sensitivity to endogenously produced ROS.

Contraction-mediated glucose transport is believed to involve activation of AMPK, although under some conditions a dissociation between AMPK activation and glucose transport has been reported (for references see Sandström *et al.* 2006; Richter & Hargreaves, 2013). Initially it was found that intense repeated contractions induced about a 10-fold increase in AMPK activity and phosphorylation in isolated EDL muscle. Pre-incubation of muscle in NAC blocked about 50% of the increase in AMPK activation (similar to the effect of NAC on contraction-mediated activation of glucose transport) (Sandström *et al.* 2006). Thus the results indicated that endogenously produced ROS resulted in activation of AMPK and this led to the acceleration of glucose transport during repeated contractions (Sandström *et al.* 2006).

Since the initial observation of ROS involvement in contraction-mediated activation of glucose transport, it was shown that isolated EDL muscle from WT and adenylate kinase-1 knockout mice exhibited a twofold increase in glucose transport after repeated contractions and that NAC blocked about 30% of the increase in both groups (Zhang *et al.* 2008). In another study, the role of cross-bridge function in contraction-mediated glucose transport was investigated. By blocking cross-bridge activity with the myosin II ATPase inhibitor *N*-benzyl-*p*-toluene sulphonamide (BTS), force production during repeated contractions was decreased to ∼5% of control (Sandström *et al.* 2007). Despite the almost complete abolition of force, glucose transport after repeated contractions was similar in both control and BTS groups (∼4-fold that of basal). Importantly, the GSSG/GSH ratio was markedly increased, as was AMPK activity, in both groups. These results confirmed and extended the original observations, indicating that ROS was involved in the activation of AMPK and glucose transport during electrical stimulation of isolated glycolytic muscle (Sandström *et al.* 2007; Zhang *et al.* 2008). Further, the activation of glucose transport does not appear to depend on force generation, which is consistent with earlier work (Holloszy & Narahara, 1965) and discussed in more detail elsewhere (Sandström *et al.* 2007). Others subsequently used BTS, or BTS together with blebbistatin (non-muscle myosin II inhibitor), to study the role of mechanical load on contraction-mediated glucose transport (Blair *et al.* 2009; Jensen *et al.* 2014; Sylow *et al.* 2015). When Jensen *et al.* (2014) used BTS together with blebbistatin and our stimulation conditions on isolated mouse EDL muscle (Sandström *et al.* 2007), they too found that contraction-mediated glucose transport was unaffected by the myosin ATPase inhibitors, although force was almost completely suppressed. However, with more mild stimulation protocols, the myosin ATPase inhibitors suppressed contraction-mediated glucose transport (Jensen *et al.* 2014). Under other experimental conditions (including higher concentrations of BTS), the myosin ATPase inhibitors also partially blocked contraction-mediated glucose transport and activation of signalling molecules (including AMPK) (Blair *et al.* 2009; Sylow *et al.* 2015). The reason for these apparently divergent results is not clear and therefore additional studies are warranted.

McConell and colleagues embarked on a series of experiments to investigate the role of ROS in contraction-mediated glucose transport. First they confirmed that repeated contractions in isolated mouse EDL muscle, under conditions similar to those reported earlier (Sandström *et al.* 2006), resulted in activation of glucose transport that was blocked by ∼50% by NAC (20 mM) (Merry & McConell, 2009). Similar findings were observed in the isolated soleus muscle.

However, in contrast to earlier findings (Sandström *et al.* 2006), contraction-mediated activation of AMPK was not affected by NAC either in EDL or in soleus muscle (Merry & McConell, 2009). Further, repeated contractions of muscles isolated from mice expressing a kinase-dead form of the $\alpha2$ AMPK isoform (AMPK-KD) resulted in normal activation of glucose transport and the activation was also blocked by NAC. However, there was no activation AMPK in AMPK-KD muscles. These findings led the authors to conclude that ROS are involved in activation of glucose uptake during repeated contractions via a mechanism that is independent of AMPK (Merry & McConell, 2009).

In additional studies, McConell *et al.* examined the effects of NAC infusion on glucose uptake in the rat hindlimb *in situ* during repeated contractions, as well as in humans during cycle ergometry ($\sim60\%$ of $\dot{V}_{O_2max}$) (Merry *et al.* 2010*a,c*). The authors studied light to moderate exercise to mimic physiological conditions. In both studies significant increases in glucose uptake or disposal were noted during exercise, but NAC infusion did not affect glucose metabolism during exercise in either study. In neither study was the GSSG/GSH ratio in muscle altered by exercise although *S*-glutathiolynation of an unidentified protein(s) was increased and this increase was blocked by NAC (Merry *et al.* 2010*a,c*). It was concluded that ROS were not significantly involved in activation of glucose transport during moderate exercise conditions (Merry

*et al.* 2010*a,c*). In these studies, the level of NAC was about 20 $\mu$M in rat plasma and about 200 $\mu$M in human plasma. This should be contrasted with a concentration of 20 mM in the medium bathing isolated muscles (Sandström *et al.* 2006; Zhang *et al.* 2008; Merry *et al.* 2010*b*). In this context it was recently demonstrated that the effect of NAC on contractile function of isolated mouse EDL muscle is temperature and concentration dependent (Katz *et al.* 2014). Indeed 1 mM NAC was ineffective, whereas 10 mM NAC had a small but significant effect and 20 mM NAC had a robust effect on force generation (Katz *et al.* 2014). Thus, although the small degree of oxidative stress during moderate exercise (*S*-glutathionlyation of unknown proteins) was blocked by NAC (Merry *et al.* 2010*a,c*), it appears that neither substantial oxidative stress nor antioxidant buffer capacity was achieved in these studies (Merry *et al.* 2010*a,c*). Of interest, exercise at 75% of $\dot{V}_{O_2max}$ results in a robust increase in leg glucose uptake (Katz *et al.* 1991) and at this exercise intensity significant increases in the muscle GSSG content are detected in human muscle (GSSG + GSH remain unchanged) (Zhang *et al.* 2007). However, it is not known if exogenous antioxidants affect glucose uptake under these conditions. Finally one should consider that experiments performed in one species under given conditions may not always be applicable to other conditions or species (e.g. mouse *vs.* human).

## Summary and conclusions

From recent reviews it appears that ROS are not involved in activation of glucose transport during light to moderate exercise conditions, but may contribute during intense exercise (Merry & McConell, 2012; Richter & Hargreaves, 2013), as depicted in Fig. 1. In an earlier review, it was pointed out that the effect of ROS on glucose transport would be expected to be most significant during heavy exercise, where oxidative stress is expected to be highest (Katz, 2007). Indeed, the ROS effect may become most apparent at exercise intensities exceeding 75% of $\dot{V}_{O_2max}$ (Gohil *et al.* 1988; Sahlin *et al.* 1991; Wang *et al.* 2006; Zhang *et al.* 2007). The source of the ROS is not clear but it may derive from $O^{\cdot-}$ produced in mitochondria (Katz, 2007), as supported by the experiments on muscle from mice overexpressing mitochondrial $Mn^{2+}$-dependent SOD (see above). But the question remains as to what the physiological significance is of ROS for muscle metabolism and function. During exercise at 100% of $\dot{V}_{O_2max}$, where the effects of ROS are assumed to be near maximal and the highest rates of glucose transport are achieved, virtually none of the glucose is metabolized. The physiological benefit of ROS under these conditions is not clear. Possibly the activation of glucose transport under these conditions is not for glucose supply for energy metabolism during exercise
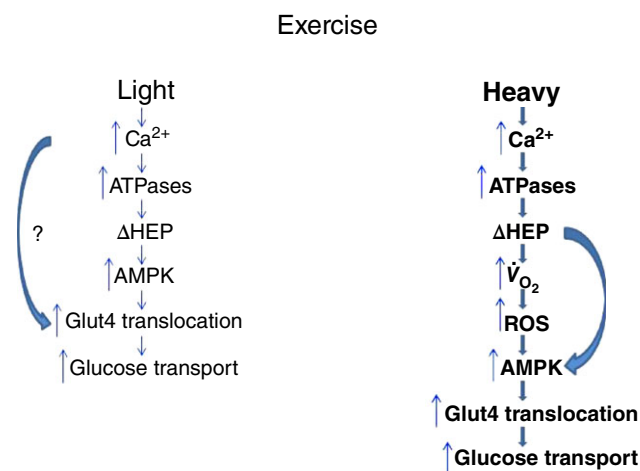


**Figure 1. Scheme for key steps in activation of glucose transport during exercise in skeletal muscle**
Light and Heavy denote exercise intensity. Bold lettering and bold arrows denote greater activities/levels (in Heavy). ΔHEP, change in levels of high energy phosphates (decreases in phosphocreatine and ATP and increases in ADP and AMP); AMPK, AMP-activated protein kinase; $V_{O_2}$, oxygen uptake. Curved arrow in Light denotes a $Ca^{2+}$-dependent enhancement of Glut4 translocation via an unknown mechanism(s) (denoted by ?) in addition to a low degree of activation of AMPK. Curved arrow in Heavy denotes AMP-mediated activation of AMPK. The figure is partially derived from Katz (2007).

but rather for rapid glycogenesis during the initial phase of recovery after exercise. Moreover, the effects of ROS may not be relegated solely to glucose transport but to other intracellular processes as well. The finding that NAC results in marked changes in glucose-6-P (enhanced) and malate (decreased) after just a few near maximal tetanic contractions in isolated muscle indicates that ROS is significantly involved in metabolic processes occurring in the cytosol and mitochondria during intense exercise (Katz *et al.* 2014), in addition to affecting contractile function (Powers & Jackson, 2008). Thus full elucidation of the role of ROS in control of glucose transport and other metabolic events in muscle during exercise awaits further research.

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## Additional information

### Competing interests

None declared.