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# Mechanisms associated with hypoxia- and contraction-mediated glucose transport in muscle are fibre-dependent

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## ABSTRACT

The purpose of this study was to examine the effects of hypoxia and muscle contractions on rates of 2-deoxyglucose (2-DG) transport in red and white portions of the gastrocnemius muscle of the rat. 2-DG transport was measured during the last 10 min of a 60-min hindlimb perfusion in male Wistar rats (≈ 300 g), with or without muscle contractions of one limb. The medium was gassed with either 95% oxygen and 5% carbon dioxide or 95% nitrogen and 5% carbon dioxide to achieve normal or hypoxic conditions, respectively. Muscle contractions began after 30 min of perfusion and consisted of isometric muscle actions (200-ms trains, 100 Hz; one train per second) for two sets of 5 min, with 1-min rest between sets. 2-DG transport in white gastrocnemius was higher ($P < 0.05$) than basal during hypoxia (4.8-fold) and following contractions using oxygenated or hypoxic medium (4.6-fold and 5.4-fold, respectively; $n = 6$ for each group). 2-DG transport was not different ($P > 0.05$) between these stimulated conditions. Similarly, 2-DG transport in red gastrocnemius was 5.1- and 4.8-fold higher ($P < 0.05$) than basal during hypoxia and following contractions in oxygenated medium, respectively. However, 2-DG transport following contractions during hypoxic conditions in red gastrocnemius was, unlike white gastrocnemius, higher (8.9-fold over basal; $P < 0.05$) than in all other conditions. These results suggest that mechanisms associated with hypoxia- and muscle contraction-mediated glucose transport are fibre type-dependent, with additive effects of the two stimuli in fast-twitch, oxidative fibres.

**Keywords** 2-deoxyglucose transport, exercise, hindlimb perfusion, metabolism.

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The regulation of glucose transport into skeletal muscle has been well studied (Holloszy & Hansen 1996). Muscle contractile activity has been shown to elevate rates of glucose transport in skeletal muscle (Holloszy & Narahara 1967, Nesher *et al.* 1985), and this elevation persists depending on fibre type for varying periods of time following the activity (Ploug *et al.* 1987). Apart from studies involving muscle contractions, others have disclosed that several physiological conditions exist where an increased need for glucose uptake can also stimulate glucose transport. For example, studies have demonstrated that hypoxia stimulates glucose transport in muscle (Henriksen *et al.* 1990, Cartee *et al.* 1991), and like exercise or *in vitro* muscle contractions, this effect appears to be additive to the effect of insulin (Ploug *et al.* 1987, Henriksen *et al.* 1990, Gao *et al.* 1994, Youn *et al.* 1994). This additive effect of insulin and muscle contractions or hypoxia on glucose transport has been generally accepted to represent differing pathways mediating elevated muscle glucose transport by these stimuli, and possibly the engagement of different GLUT4 transporter pools. Although insulin-mediated glucose transport has been extensively studied and appears to be mediated by PI-3 kinase activity following hormone/receptor complexation (Cheatham *et al.* 1994; see Cheatham & Kahn 1995 for review), less is known about non-insulin-mediated pathways.

It is generally accepted, however, that elevated glucose transport mediated by muscle contractions and hypoxia operates independently of PI-3 kinase (Cartee *et al.* 1991, Goodyear *et al.* 1995, Lee *et al.* 1995, Lund *et al.* 1995). Cartee *et al.* (1991) studied the effects of hypoxia and *in vitro* muscle contractions on 3-O-methylglucose transport in epitroclearis muscles of the rat. Their results suggested that the pathway for hypoxia- or muscle contraction-mediated glucose transport is the

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same, as the effect of the combined stimuli was no greater than of either stimulus alone (Cartee *et al.* 1991). Additionally, it appears that the elevation of glucose transport by these two stimulants is probably mediated by calcium in this predominately white, fast-twitch muscle.

In support of this notion, Cartee *et al.* (1991) demonstrated that muscle calcium stimulates glucose transport during muscle contractions or under hypoxic conditions in a similar manner. For example, caffeine, a mobilizer of intracellular calcium, potentiates glucose transport, but dantrolene, a calcium mobilization inhibitor, attenuates non-insulin-stimulated elevations of glucose transport. These results demonstrate that calcium may be a potent modulator of glucose transport without insulin. However, recent evidence from Wojtaszewski *et al.* (1996) suggested that high doses of wortmannin, a phosphatidylinositol 3-kinase inhibitor, attenuated glucose transport to varying degrees in skeletal muscle following muscle contractions, depending on the fibre composition. In that study, wortmannin did not attenuate the increase in glucose transport with hypoxia, suggesting that some independence between glucose transport stimulating pathways may exist at least in some fibre-types. Therefore, the purpose of this study was to examine the influence of muscle fibre-type composition on the interaction between hypoxia and contractions in glucose transport stimulation. Rat hindlimbs were perfused and glucose transport was measured in the deep red and superficial white portions of the predominately fast-twitch gastrocnemius muscles following hypoxia and/or electrical stimulation.

## METHODS

All experimental procedures used in the current investigation were approved by the animal care and use committee of Copenhagen University. Fed male Wistar rats ($n = 34$ total; $n = 12$ for hypoxia vs. contractions experiment) weighing ≈ 300 g were anaesthetized by injection of Na-pentobarbital (5 mg $[100\ g]^{-1}$ body wt., i.p.), and then surgically prepared for hindlimb perfusion as described by Ruderman *et al.* (1971). A cell-free Krebs–Henseleit medium (pH 7.4) was perfused through the hindlimb via the abdominal aorta and the perfusate was collected from the inferior vena cava. Once flow was established, the animal was sacrificed by a lethal intracardial injection of Na-pentobarbital. The first 80 mL of perfusate was discarded, and then the medium was recycled yielding a final volume of 130 mL. The glucose-free medium contained 2 mM pyruvate and 4% bovine serum albumin (Sigma Chemical, St. Louis, MO) that was dialysed for 48 h against 20 volumes of Krebs–Henseleit solution at 4 °C. The Krebs–Henseleit medium used for perfusions was maintained at 37 °C and gassed with either a 95% $O_2$/5% $CO_2$ (normoxic) or for ≈ 2 h with 95% $N_2$/5% $CO_2$ (hypoxic) mixture. Also, dialysis of bovine serum albumin solutions used for hypoxic experiments was performed with hypoxic buffers.

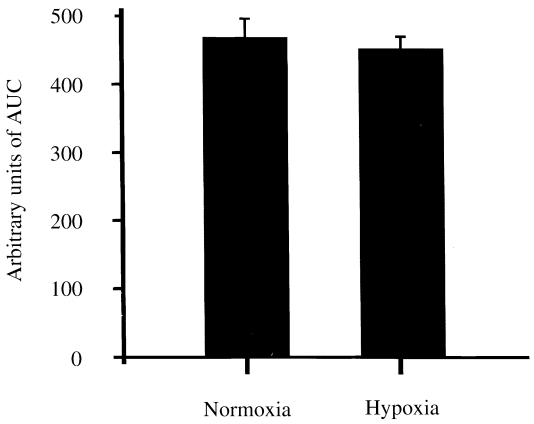


**Figure 1** Isometric force production during electrically stimulated muscle contractions. Data are expressed as arbitrary units of area under the force curve for normoxic ($n = 6$) and hypoxic ($n = 6$) conditions. Values are expressed as mean ± SE. There were no differences between means ($P > 0.05$).

The perfusion period was conducted for 60 min following the initial washout period. The electrical stimulation period, when applicable, occurred at the 30-min time period of the protocol (see below for protocol). We chose to conduct the electrical stimulation at this time owing to pilot observations that contractile activity was virtually non-existent at the end of the perfusion protocol under hypoxic conditions. However, no differences in glucose transport were observed whether transport was measured immediately after the 11-min electrical stimulation protocol or 9 min later, at the end of the perfusion period (data not presented). Although muscle contractions appear slightly attenuated during hypoxic conditions (see Fig. 1), this attenuation was not significant ($P > 0.05$). Furthermore, the stimulation protocol used during the present investigation has been shown to elicit maximal contraction-mediated glucose transport during normoxic conditions (Ploug *et al.* 1987, Ploug *et al.* 1993).

When appropriate, additional groups were added to make comparisons between hypoxia at 60 vs. 80 min. This made it possible to verify that a maximal effect of hypoxia was observed after 60 min. Briefly, rats were perfused during hypoxic conditions for an additional 20 min with the radiolabelled substrates being added during the last 10 min. Muscles were excised, treated and analysed as stated below. Partial pressures of oxygen were always measured at 40 and 60 min of the perfusion protocol, as well as at 80 min during the longer experiments, from the 'arterial' line feeding into

the abdominal aorta. All samples were collected using a sealed syringe and immediately analysed using an Acid–Base Laboratory from Radiometer (Copenhagen, Denmark).

Indirect electrical stimulation of the rat hindlimb was conducted as previously described (Ploug *et al.* 1984, 1987). Briefly, the animals were electrically stimulated via the sciatic nerve using a hook electrode. The knees of the animals were immobilized using a steel pin that was placed directly under the tibiopatellar tendon and secured to a fixed platform. A steel braided cord, fixed to an isometric force transducer (Harvard Instruments, Millis, MA) was attached to the achilles tendon. The force transducer was connected to an Astro-Med recorder (Atlan Tol Industries, West Warwick, RI) so that comparisons between contractions during hypoxia or normoxia could be made (see Fig. 1). Isometric force is expressed as arbitrary units of area under the curve. The electrical stimulation protocol consisted of 200-ms trains of 100 Hz, with each impulse lasting 0.1 ms. Trains were delivered at a rate of 1 $s^{-1}$ over a 5-min period. The protocol consisted of two 5-min periods, separated by one minute of rest.

For measurements of glucose transport, 130 $\mu$Ci each of [$^3$H] 2-deoxyglucose and [$^{14}$C] sucrose (Amersham, England) were added to the medium, yielding 1 $\mu$Ci $mL^{-1}$ for each radiolabel. Exposure to the labelled substances during the perfusion protocol was 10 min for all treatments. Subsequently, the superficial white and deep red portions of the gastrocnemius from each leg were rapidly excised and freeze-clamped to the temperature of liquid nitrogen, and later assessed for rates of glucose transport. Briefly, an aliquot of the recycled cell free perfusion medium was precipitated with an equal volume of ice-cold perchloric acid, and following centrifugation, the supernatant analysed for specific radioactivity. Intracellular accumulation of 2-DG was calculated from the radioactivity of [$^3$H]-2-DG in muscle homogenates and the specific radioactivity of the perfusion medium using [$^{14}$C]-sucrose as an extracellular marker.

Differences between treatments were determined using ANOVA or two-tailed *t*-test. Differences were considered statistically significant when $P < 0.05$. When significant *F* ratios were observed, a Student–Newman–Kuels test was used to determine differences between means. All data are expressed as mean ± SE.

## RESULTS

Maximal rates of 2-DG transport in both portions of the gastrocnemius were obtained when the arterial partial pressure of oxygen was below 65 mmHg for a 60-min period (see Fig. 2). Rates of 2-DG transport were similar with further reductions of the partial pressure of oxygen at least down to 34 mmHg. Tissues were considered hypoxic when arterial partial pressures of oxygen were less than 60 mmHg for 60 min.

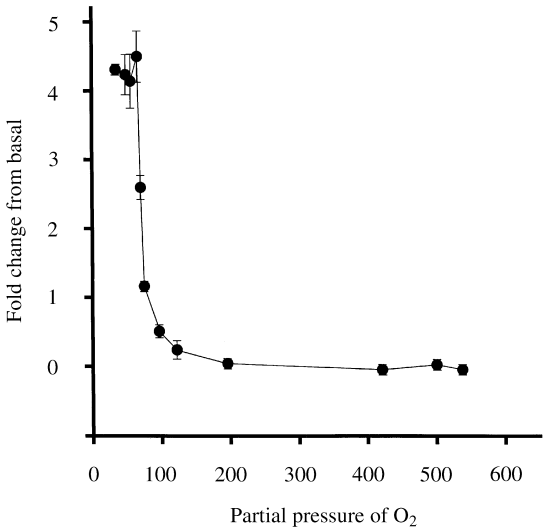


**Figure 2** Alterations of glucose transport with changes in perfusion medium partial pressures of oxygen for red and white gastrocnemius muscle. Data are expressed as the fold-change when compared with basal and were combined for red and white fibres owing to their similar nature ($P > 0.05$). Each data point represents 2–4 animals and was obtained from both rats used in pilot ($n = 22$, for purposes of obtaining a range of partial pressures) and the present investigation ($n = 12$). Values are expressed as mean ± SE.

Rates of 2-deoxyglucose transport for white gastrocnemius (see Fig. 3) were significantly higher ($P < 0.05$) than basal during hypoxia (4.6-fold) or muscle contractions (4.8-fold). These results are similar to the work of Cartee *et al.* (1991), who also showed that a maximal effect of hypoxia on glucose transport is similar to a maximal effect of muscle contractions. Muscle contractions combined with hypoxia also had

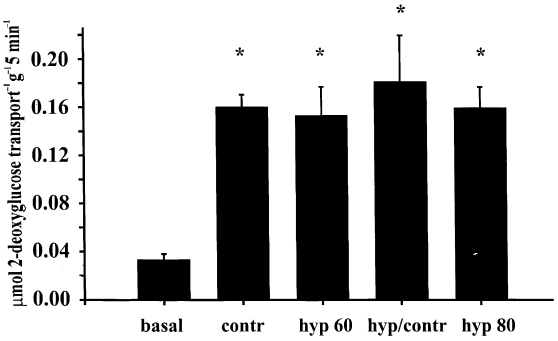


**Figure 3** Rates of 2-deoxyglucose transport in white gastrocnemius at basal, after muscle normoxic contractions (contr), hypoxia for 60 min (hyp 60), after hypoxia and muscle contractions (hyp/contr) and hypoxia for 80 min (hyp 80) ($n = 6$ for all treatments). Values are expressed as mean ± SE. * denotes significantly different from basal values ($P < 0.05$).

higher glucose transport than basal (5.4-fold; $P < 0.05$), but was not different ($P > 0.05$) than either hypoxia or muscle contractions alone. Rates of glucose transport were not different ($P > 0.05$) between 60 and 80 min of hypoxia for this muscle.

Rates of 2-DG transport in red gastrocnemius were significantly higher ($P < 0.05$) following muscle contractions (5.1-fold) or hypoxia (4.8-fold) when compared with basal, normoxic values (see Fig. 4). The independent effects of the two stimulants did not differ ($P > 0.05$). When the stimulants were combined, 2-DG transport was higher (8.9-fold; $P < 0.05$) than basal and in response to either stimulant alone. As with white gastrocnemius, 2-DG transport was not different ($P > 0.05$) between hypoxia for 60 and 80 min.

## DISCUSSION

The most important finding of this study is that mechanisms associated with hypoxia and/or muscle contraction-mediated glucose transport are fibre-type dependent, with additive effects of these stimulants in fast-twitch, oxidative fibres. The finding in fast-twitch oxidative fibres is contrary to the generally accepted view that hypoxia and muscle contractions operate through similar mechanisms. Cartee *et al.* (1991) demonstrated that the effects of hypoxia and *in vitro* muscle contractions were not additive in epitroclearis muscle. The epitroclearis muscle, as with the white gastrocnemius used in the present investigation, is composed predominately of white, fast-twitch fibres. Therefore, it may be that in fast, non-oxidative fibre-types the two stimuli act by the same mechanisms or glucose transport is saturated by each of the stimuli. In contrast, the additivity observed in the present study in oxidative, fast fibres indicates that the two stimuli act, at least in part, by different mechanisms in these fibres (Holloszy & Hansen 1996) or are not individually able to fully recruit glucose transport to capacity. Our finding of different responses in oxidative compared with non-oxidative fibres is especially relevant in light of the fact that the two types of muscle fibres were exposed to the same hypoxic environment.

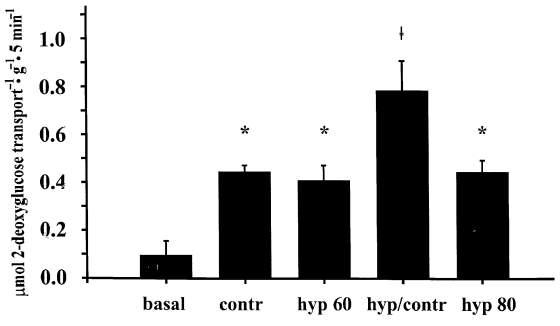


**Figure 4** Rates of 2-deoxyglucose transport in red gastrocnemius at basal, after muscle normoxic contractions (contr), hypoxia for 60 min (hyp 60), after hypoxia and muscle contractions (hyp/contr) and hypoxia for 80 min (hyp 80) ($n = 6$ for all treatments). Values are expressed as mean ± SE. * denotes significantly different from basal values ($P < 0.05$) denotes significantly different from other treatments ($P < 0.05$).

It is generally accepted that additivity represents either separate or divergent pathways leading to the same outcome (see Holloszy & Hansen 1996 for review). Therefore, the observed additivity in response to hypoxia and muscle contractions in the present investigation implies that these stimuli utilize mechanisms, or potentially glucose transporters, that are not shared between pathways. On the other hand, the results of the present study may also reflect that different stimuli can trigger the same pathway, but with different (and additive) capacities. Although the mechanisms have not been elucidated and are the topic of future study, it appears that fast-twitch, oxidative fibres are able to facilitate higher rates of glucose transport in hypoxic environments after muscle contractions than with either stimulant alone.

In the study by Cartee *et al.* (1991) of the interaction between contractions and hypoxia, reintroduction of oxygen prior to contractions was allowed as elevated glucose transport may persist for long periods after hypoxia, even when oxygen is present. However, to exclude the possibility that the lack of additivity observed in those experiments was, after all, owing to the lack of simultaneous presence of both stimuli, we chose another approach where muscle contractions were conducted during a hypoxic state. It was not possible to elicit meaningful contractions at a time when hypoxia-stimulated glucose transport was maximal. Accordingly, we had to electrically stimulate the muscle prior to transport measurements. Therefore, we studied fast-twitch muscle, which exhibits prolonged elevations of glucose transport well after the cessation of muscle contractions (Ploug *et al.* 1987).

The mechanisms involved with the fibre-dependent reversal of glucose transport are not completely understood, but it was clear that our protocol allowed for the examination of glucose transport in these fibres well within the reversal period. However, the importance of maximal contraction effects was minimized as glucose transport was measured during hypoxic conditions that were maximally effective for glucose transport. This fact ensures the validity of the interpretation that in fast-twitch, red muscle the effect of one non-insulin stimulant on glucose transport can be added to the maximal effect of another, indicating additivity. We would have liked to also study slow-twitch oxidative fibres. Unfortunately, however, pilot observations demonstrated that soleus muscle, which

consists predominantly of this fibre-type, has a tendency to contract *in vitro* during hypoxic conditions (data not presented). This tendency made it impossible to distinguish between effects of contractions and hypoxia in this muscle.

In summary, the present investigation demonstrated that hypoxia- and contraction-mediated elevations of glucose transport are additive in fast-twitch, oxidative fibres. Although the mechanisms associated with this finding are not understood at this time, we feel that use of hypoxia as a general tool to represent non-insulin-mediated glucose transport (which includes muscle contractions) should be approached with caution, at least when other than fast-twitch white fibres are being studied. We feel that this work brings important new insight into the potential differences between muscle fibre types as well as how non-insulin-mediated glucose transport may be differentially regulated.

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