Nitric oxide stimulates glucose transport and metabolism in rat skeletal muscle *in vitro*

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1. The effects of the nitric oxide (NO) donor sodium nitroprusside (SNP) on the rates of glucose transport and utilization and its interaction with insulin were investigated in rat soleus muscle *in vitro*. SNP stimulated the rate of 2-deoxyglucose transport and insulin-mediated (100 μ -units/ml) rates of both net and [14 C]lactate release and the rate of glucose oxidation. The effects of SNP were independent of the concentration-dependent effects of insulin on glucose metabolism. 2. SNP stimulated the insulin-stimulated rates of net and [14 C]lactate release and glucose oxidation in a concentration-dependent manner. The rate of [14 C]lactate release was also stimulated by another NO donor, (Z)-1-(N-[aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino)-diazene-1-ium-1,2-diolate (spermine NONOate). 3. SNP at 5, 10 and 15 mM inhibited the insulin-stimulated rate of glycogen synthesis and this rate was further decreased at 20 and 25 mM SNP. SNP did not affect the rate of glycogen synthesis in the absence of insulin. 4. Haemoglobin, which is a NO scavenger, prevented the stimulation of the rates of [14 C]lactate release by

SNP or spermine NONOate. 5. The cGMP content was increased maximally (by approx. 80-fold) within 15 min by SNP (15 mM). The cGMP content, raised maximally by SNP, was significantly decreased by the guanylate cyclase inhibitor LY-83583 (10 μ M). The cGMP analogue 8-bromo-cGMP (100 μ M) significantly increased the rate of net lactate release. 6. LY-83583 significantly inhibited SNP-stimulated rates of 2-deoxyglucose transport, [14 C]lactate release and glucose oxidation. Methylene Blue (another guanylate cyclase inhibitor) also inhibited SNP-stimulated rates of [14 C]lactate release. 7. The results suggest that in rat skeletal muscle: (a) nitric oxide (from SNP or spermine NONOate) increases the rate of glucose transport and metabolism, an effect independent of insulin; (b) SNP inhibits insulin-mediated rates of glycogen synthesis; (c) SNP stimulates cGMP formation, which mediates, at least partly, the effects on glucose metabolism; (d) nitric oxide-mediated stimulation of glucose utilization might occur in fibre contraction. The implications of the effects of NO on glucose metabolism are discussed.

INTRODUCTION

Increases in the rates of glucose uptake and metabolism in skeletal muscle occur by an insulin-mediated and/or contraction-stimulated pathways [1]. The phosphatidylinositol-3-kinase inhibitor, wortmannin, decreases insulin-mediated, but not contraction-stimulated, glucose utilization in skeletal muscle, which suggests that distinct signalling pathways exist [2,3]. Wortmannin decreases only insulin-mediated glucose utilization [2,3]. Thus contraction might employ a phosphatidylinositol-3-kinase-independent (assuming that muscle contains no wortmannin-resistant phosphatidylinositol-3-kinase) mechanism to stimulate glucose utilization, although undoubtedly some common signalling steps are used by both contraction and insulin [2].

Results from our laboratory demonstrate that superoxide dismutase stimulates glucose utilization in isolated rat soleus muscle preparations *in vitro* [4]. Superoxide dismutase lowers the number of reactive oxygen species, which are generated abundantly in the muscle preparations incubated *in vitro* [5]. We hypothesize [4] that reactive oxygen species suppress the activity of an endogenous agent that was responsible for increased glucose utilization. Reactive oxygen species inactivate nitric oxide (NO) [6,7].

A significant nitric oxide synthase (NOS) activity is found in skeletal muscle cells [6,8,9]. NOS is also present in skeletal muscle

cell lines [10,11]. Neuronal-type NOS (nNOS) is expressed at higher levels in skeletal muscle than in brain of humans [12]. nNOS is found at higher activity in muscles composed of type II fibres [6], which are better designed for rapid contraction. Because muscles that are almost wholly populated with type II fibres are less well vascularized, this clearly demonstrates that the activity is not solely endothelial NOS. The endothelial type-NOS (eNOS) is also expressed in skeletal muscle [8]. In skeletal muscle from mice with X-chromosome-linked muscular dystrophy (*mdx*) both the content of dystrophin and the activity of particulate NOS are low [7]. NOS binds to dystrophin, which locates NOS to the sarcolemmal membrane, although some NOS is located within muscle mitochondria [13].

NO is released from resting skeletal muscle preparations *in vitro* [14]. Prior electrical stimulation of extensor digitorum longus preparations causes a stimulation of NO release [14]. Because contraction stimulates both rates of glucose transport and metabolism [1–4] and NO release [14] in skeletal muscle, we tested the hypothesis that NO acts as a mediator to cause increased glucose utilization. The direct effects of exogenous NO, generated from the nitric oxide donor sodium nitroprusside (SNP), on rates of 2-deoxyglucose transport, [14 C]-labelled and net lactate release, glucose oxidation and glycogen synthesis were measured in isolated incubated rat soleus muscle preparations. SNP might also give rise to cyanide ions, which can be removed by the addition of rhodanese and sodium thiosulphate to the

Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside; spermine NONOate, (Z)-1-(N-[aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino)-diazene-1-ium-1,2-diolate.

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incubation medium [15]. SNP does not spontaneously decompose or hydrolyse to release cyanide but the contact of SNP with biological tissues can initiate the formation of cyanide ions [15]. Consequently, the effect of SNP on muscle glucose metabolism was measured in the presence of rhodanese and sodium thiosulphate. The effects of a second NO donor, (Z)-1-(N-[aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino)-diazene-1-ium-1,2-diolate (spermine NONOate) on the rate of [14 C]lactate release was also measured. Whether SNP or spermine NONOate act via NO was examined by testing the effects of haemoglobin, which is an NO scavenger [16]. NO activates the soluble form of guanylate cyclase, increasing the rate of cGMP formation [17,18]. Guanylate cyclase activity is inhibited by LY-83583 [19] or Methylene Blue [20]. Thus we determined whether LY-83583 affected SNP-mediated effects on cGMP levels and the rate of 2-deoxyglucose transport, and whether LY-83583 or Methylene Blue inhibited SNP-stimulated rates of [14 C]lactate release in soleus muscle preparations *in vitro*.

MATERIALS AND METHODS

Animals

Male Wistar rats (140–160 g; Harlan-Olac, Bicester, Oxon., U.K.) were kept in the animal house of the Department of Biochemistry, University of Oxford, in controlled conditions ($23 \pm 1^\circ\text{C}$; 12 h light/12 h dark cycle) and received standard laboratory chow and water *ad libitum*. Food was withdrawn for 15 h before experimentation. Water was always provided *ad libitum* to all rats.

Materials

Enzymes, chemicals, biochemicals and radiochemicals were purchased from sources previously given [21–23], except for SNP, Methylene Blue, rhodanese, sodium thiosulphate, 8-bromo-cGMP, LY-83583 (all from Sigma), haemoglobin (ICN Biomedicals), spermine NONOate (L.C. Laboratory, Alexis Corporation, Nottingham, Notts., U.K.) and cGMP assay kits (Amersham, Little Chalfont, Bucks., U.K.)

Incubation procedures and analysis

Rats were killed by cervical dislocation, and stripped solei were prepared as described previously [21–23]. The tendons of the muscles were ligated, rapidly weighed and tied at the resting length *in situ* on stainless steel clips, then placed in 25 ml Erlenmeyer flasks containing 3 ml of oxygenated Krebs–Ringer bicarbonate buffer plus 10 mM Hepes (pH 7.4)/5.5 mM glucose/1 % (w/v) BSA/insulin (10 μ -units/ml). Flasks were sealed and aerated continuously with O_2/CO_2 (19:1). When the effects of LY-83583 or Methylene Blue were being investigated, these compounds were also present in the preincubation medium. After preincubation of muscles for 30 min at 37°C in an oscillating water bath (100 rev./min), the muscle strips were transferred to similar vials containing similar medium with added [14 C]glucose (0.5 $\mu\text{Ci}/\text{ml}$), insulin (at varied concentrations; see legends to Figures or Tables) and other reagents (e.g. SNP, spermine NONOate, Methylene Blue, LY-83583, rhodanese, haemoglobin and sodium thiosulphate). LY-83583 was dissolved in DMSO, in which case a similar volume of DMSO was added to the control incubation medium. The flasks were sealed and re-gassed for the initial 15 min period in a 1 h incubation. At the end of the incubation, muscles were blotted

and rapidly frozen in liquid N_2 . The concentration of lactate in the incubation medium was determined spectrophotometrically [23] and [14 C]glucose incorporated into glycogen (glycogen synthesis) was measured [21–23]. Rates of glycogen synthesis were measured in terms of μmol of glucosyl units/h per g wet wt. In other experiments the incubation medium was acidified with perchloric acid (6 %, w/v) before collection of $^{14}\text{CO}_2$ in plastic centre wells containing 0.2 ml of 1:1 (v/v) phenylethylamine/methanol [21]. The amount of $^{14}\text{CO}_2$ formed was calculated from the specific radioactivity of extracellular glucose. This calculation of formation of $^{14}\text{CO}_2$ does not take into account any dilution of [14 C]pyruvate by endogenously available pyruvate. In these experiments for lactate release we measured two rates: net, which is lactate generated potentially from both intracellular glycogen and extracellular glucose, and [14 C]lactate release derived only from extracellular glucose. Any significant differences between these rates probably indicates that glycogenolysis is activated in the isolated incubated muscle preparation. Also, in previous studies we have shown that the rate of [14 C]lactate release is a good indication of the rate of glucose transport [23]. Radiochemical lactate was analysed by separation of [14 C]lactate in the incubation medium on ion-exchange columns [24]. Rates of 2-deoxyglucose transport were measured as previously described [23].

The content of cGMP was measured with a cGMP enzyme immunoassay system (Amersham). Briefly, after incubation muscles were rapidly frozen in liquid N_2 . Muscles were pulverized before homogenization in 0.5 ml of ice-cold 10 % trichloroacetic acid/methanol [10 % (w/v) trichloroacetic acid in 10 % (v/v) methanol]. Each homogenate was extracted three times with 5 vol. of water-saturated diethyl ether. The extract was freeze-dried before reconstitution in assay buffer [50 mM sodium acetate (pH 5.8)/0.02 % BSA/0.005 % thimerosal]. The assay of cGMP was conducted in accordance with the manufacturer's instructions for the assay of acetylated cGMP.

RESULTS

Effects of SNP on rates of lactate release, glycogen synthesis and glucose oxidation

We measured the effects of various concentrations of SNP on insulin-stimulated (100 μ -units/ml) rates of net lactate release from isolated incubated rat soleus muscle preparations (Figure 1, left panel). SNP, over a range of concentrations from 1 to 25 mM, significantly increased the rate of net lactate release. This rate of net lactate release was markedly higher at the highest concentration of SNP (25 mM). SNP, at all concentrations (see Figure 1, right panel), significantly increased the rate of insulin-mediated [14 C]lactate release. The highest concentration of SNP (25 mM) did not further increase the insulin-stimulated rate of [14 C]lactate release (Figure 1, right panel).

The effects of SNP on insulin-stimulated rates of glycogen synthesis are given in Figure 2. A submaximal concentration of insulin (100 μ -units/ml typically stimulates the rate of glycogen synthesis approx. 2-fold compared with basal values (see Table 1). SNP, at concentrations greater than 5 mM, inhibited the insulin-stimulated rate of glycogen synthesis. The rate of glycogen synthesis was decreased by approx. 1.5 $\mu\text{mol}/\text{h}$ per g at 5, 10 and 15 mM SNP but was decreased by approx. 3 $\mu\text{mol}/\text{h}$ per g at 20 and 25 mM SNP (Figure 2).

SNP, at 1, 15 and 25 mM, significantly stimulated the insulin-mediated (100 μ -units/ml) rate of glucose oxidation in soleus muscle preparations *in vitro* in a concentration-dependent manner (Figure 3).

Figure 1 Effect of various concentrations of SNP on insulin-stimulated rates of net lactate release (left panel) and [¹⁴C]lactate release (right panel) from isolated incubated rat soleus muscle preparations

All muscle preparations were incubated in the presence of 100 μ -units of insulin/ml. Results are presented as means $\pm$ S.E.M. for between six and 23 separate experiments. Statistically significant differences from results from incubations in the absence of SNP are indicated by * $P < 0.05$, *** $P < 0.001$.

Figure 2 Effect of various concentrations of SNP on insulin-stimulated rates of glycogen synthesis in isolated incubated rat soleus muscle preparations

All muscle preparations were incubated in the presence of 100 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for between six and 23 separate experiments. Statistically significant differences from results from incubations in the absence of SNP are indicated by *** $P < 0.001$.

Insulin, at 100 and 10000 μ -units/ml, significantly increased the rates of net lactate release, [¹⁴C]lactate release and glycogen synthesis (Table 1).

SNP (15 mM), in the absence or presence of insulin [i.e. at 1 (basal concentration), 100 or 10000 μ -units/ml] stimulated the rates of both net and [¹⁴C]lactate release (Table 1). SNP (15 mM) did not affect the rate of glycogen synthesis in the absence of insulin but markedly inhibited the insulin-stimulated rate of glycogen synthesis (Table 1).

If any cyanide ions were generated by the contact of SNP with skeletal muscle [15], these ions would be dissipated by the addition of rhodanese (1.3 units/ml) and sodium thiosulphate (1 mM) to the incubation medium [15]. The stimulation of the rate of [¹⁴C]lactate release by SNP was unaffected by the presence of rhodanese and sodium thiosulphate (Table 2).

Effects of haemoglobin on insulin-stimulated rates of [¹⁴C]lactate release

Haemoglobin is known to bind avidly and to nullify rapidly the effects of NO and thus to act as a scavenger [16]. SNP or spermine NONOate (both at 1 mM) both significantly increased the rate of [¹⁴C]lactate release (Table 3). Haemoglobin (100 μ M) did not affect the rate of [¹⁴C]lactate release (Table 3). Incubation of rat soleus muscle preparations with either SNP (1 mM) or spermine NONOate (1 mM) in combination with haemoglobin (100 μ M) resulted in rates of [¹⁴C]lactate release that were not significantly different from those obtained in the absence of either SNP or spermine NONOate (see Table 3).

Effect of SNP on cGMP content and of inhibition of guanylate cyclase on SNP-stimulated rates of 2-deoxyglucose transport, [¹⁴C]lactate release and glucose oxidation; effect of 8-bromo cGMP

Soleus muscle preparations were incubated in the absence or presence of SNP (15 mM) at different times before the content of cGMP was measured. The content of cGMP was significantly elevated by 2.5 min, and by 15 min it reached a maximal value (Figure 4). The content of cGMP was increased approx. 80-fold by 15 mM SNP at 15 min, and the cGMP content remained maximally elevated for the duration of the incubation period (i.e. at 20 and 60 min).

SNP (15 mM) stimulated the cGMP content (290 ± 37 pmol/g wet wt.), and this increase was significantly decreased by 10 μ M LY-83583 (105 ± 6 pmol/g wet wt.; $P < 0.001$); values given

Table 1 Effects of SNP on rates of net lactate release and [^{14}C]lactate release and glycogen synthesis in isolated incubated rat soleus muscle preparations

Net and [^{14}C]lactate release and glycogen synthesis were measured as described in the Materials and methods section. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences (Student's *t* test) from results in the absence of SNP are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Insulin concn. (μ -units/ml)	Rate of [^{14}C]lactate release (μ mol/h per g wet wt.)		Rate of net lactate release (μ mol/h per g wet wt.)		Rate of glycogen synthesis (μ mol/h per g wet wt.)	
	Without SNP	With SNP (15 mM)	Without SNP	With SNP (15 mM)	Without SNP	With SNP (15 mM)
0	4.16 $\pm$ 0.25	7.39 $\pm$ 0.93**	5.72 $\pm$ 0.49	10.73 $\pm$ 1.49**	1.12 $\pm$ 0.18	1.21 $\pm$ 0.25
1	3.83 $\pm$ 0.40	8.48 $\pm$ 0.85***	6.79 $\pm$ 0.72	12.82 $\pm$ 2.34**	1.65 $\pm$ 0.34	0.94 $\pm$ 0.21*
100	6.98 $\pm$ 0.40	13.18 $\pm$ 0.65***	9.48 $\pm$ 0.37	18.42 $\pm$ 1.97***	2.76 $\pm$ 0.22	2.08 $\pm$ 0.30*
10000	10.35 $\pm$ 0.85	16.40 $\pm$ 0.79***	13.40 $\pm$ 0.62	20.62 $\pm$ 1.74***	6.93 $\pm$ 0.40	3.37 $\pm$ 0.53***

Figure 3 Effect of various concentrations of SNP on insulin-stimulated rates of conversion of [$\text{U-}^{14}\text{C}$]glucose into $^{14}\text{CO}_2$ in rat soleus muscle preparations *in vitro*

All muscle preparations were incubated in the presence of 100 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least 12 separate experiments. Statistically significant difference from results from incubations in the absence of SNP are indicated by * $P < 0.05$, *** $P < 0.001$.

Table 2 Effects of rhodanese and sodium thiosulphate on SNP-stimulated [^{14}C]lactate release in soleus muscle *in vitro*

Results are presented as means $\pm$ S.E.M. for at least six separate experiments. SNP (15 mM) was added either alone or in combination with rhodanese (Rh, 1.3 units/ml) plus sodium thiosulphate (STS, 1 mM). Statistically significant differences from results from incubations in the absence of any added chemical are indicated by *** $P < 0.001$.

Addition	Rate of [^{14}C]lactate release (μ mol/h per g)
None (control)	6.46 $\pm$ 0.88
SNP	15.26 $\pm$ 1.11***
Rh plus STS	8.44 $\pm$ 0.90
SNP combined with Rh plus STS	15.84 $\pm$ 0.63***

Table 3 Effects of haemoglobin on SNP- or spermine NONOate-stimulated rates of [^{14}C]lactate release from isolated incubated soleus muscle preparations

Results are presented as means $\pm$ S.E.M. for six experiments. The rate of release of [^{14}C]lactate was measured as described in the Materials and methods section. The nitric oxide donors were either SNP or spermine NONOate; each was added to the medium at a final concentration of 1 mM. Statistically significant differences from results from incubations in the absence of both haemoglobin and a nitric oxide donor are denoted by * $P < 0.05$, ** $P < 0.01$.

Addition	Rate of [^{14}C]lactate release (μ mol/h per g)	
	SNP	Spermine NONOate
None (control)	8.82 $\pm$ 1.07	11.12 $\pm$ 0.64
Nitric oxide donor	13.50 $\pm$ 1.04**	14.46 $\pm$ 1.29*
Haemoglobin (100 μ M)	9.86 $\pm$ 0.53	9.73 $\pm$ 0.61
Haemoglobin plus nitric oxide donor	10.79 $\pm$ 0.81	10.45 $\pm$ 1.07

Figure 4 Time course of the effect of SNP on intracellular cGMP content in rat soleus muscle *in vitro*

Rat soleus muscle preparations were incubated in the absence (■) or presence (◆) of SNP (15 mM). Preparations were incubated in the presence of a basal concentration of insulin (i.e. 10 μ -units/ml insulin). Results are presented as means $\pm$ S.E.M. for three separate experiments.

Table 4 Effect of the guanylate cyclase inhibitor LY-83583 on SNP-stimulated rate of 2-deoxyglucose transport

Results are presented as means $\pm$ S.E.M. for the numbers of experiments given in parentheses. The rate of 2-deoxyglucose (2-DOG) transport was obtained in the presence of a basal concentration of insulin (10 μ -units/ml). Muscles were further incubated with SNP (15 mM) or LY-83583 (10 μ M) or a combination of SNP and LY-83583. Statistically significant differences from results from incubations in the absence of both LY-83583 and SNP are indicated by * $P < 0.05$, ** $P < 0.01$.

Addition	Rate of 2-DOG transport (μ mol/h per g)
None (control)	4.69 $\pm$ 0.34 (12)
SNP	7.35 $\pm$ 0.40 (6)**
LY-83583	4.41 $\pm$ 0.24 (12)
SNP + LY-83583	3.79 $\pm$ 0.29 (6)*

Table 5 Effect of the guanylate cyclase inhibitors LY-83583 or Methylene Blue on the rate of [14 C]lactate release stimulated by SNP

Results are presented as means $\pm$ S.E.M. for the numbers of experiments given in parentheses. The rate of [14 C]lactate release was obtained in the presence of a half-maximal concentration of insulin (100 μ -units/ml). Muscles were further incubated with SNP (15 mM) or a guanylate cyclase inhibitor (10 μ M) or a combination of SNP plus LY-83583 or Methylene Blue, in the presence of 100 μ -units/ml insulin. Statistically significant differences from results from incubations in the absence of both a guanylate cyclase inhibitor and SNP are indicated by * $P < 0.05$, ** $P < 0.01$.

Addition	Rate of [14 C]lactate release (μ mol/h per g)	
	Methylene Blue	LY-83583
None (control)	8.85 $\pm$ 0.25 (6)	9.38 $\pm$ 1.34 (5)
SNP	11.80 $\pm$ 0.59 (6)**	12.98 $\pm$ 1.12 (5)**
Cyclase inhibitor	7.04 $\pm$ 0.62 (6)*	3.60 $\pm$ 0.42 (5)**
SNP plus cyclase inhibitor	9.27 $\pm$ 0.41 (6)	6.61 $\pm$ 0.69 (5)*

$\pm$ S.E.M. SNP (15 mM) significantly increased the rate of transport of the glucose analogue 2-deoxyglucose into isolated incubated soleus muscle preparations (Table 4). The guanylate cyclase inhibitor LY-83583 (10 μ M) on its own had no effect on the rate of 2-deoxyglucose transport (Table 4). The SNP-stimulated rate of 2-deoxyglucose transport was decreased by LY-83583 (Table 4). In a similar manner LY-83583 decreased the SNP-stimulated rate of [14 C]lactate release (Table 5). However, LY-83583 on its own significantly inhibited the insulin-stimulated rate of [14 C]lactate release (Table 5). We tested the effects of a second guanylate cyclase inhibitor, Methylene Blue, on the SNP-stimulated rate of [14 C]lactate release (Table 5). Methylene Blue blocked the stimulation of the rate of [14 C]lactate release by both SNP and insulin (Table 5).

Furthermore, LY-83583 significantly decreased the SNP-stimulated rate of glucose oxidation (1.58 $\pm$ 0.08 compared with 0.99 $\pm$ 0.07 μ mol/h per g wet wt; $P < 0.001$) such that it was no longer significantly different from the rate observed in the absence of both SNP and LY-83583 (i.e. the control value).

Also, the effect of the cell-permeant cGMP analogue 8-bromo-cGMP on the rate of net lactate release in isolated incubated soleus muscle preparations was tested. 8-Bromo-cGMP, at 100 μ M, significantly increased the rate of net lactate release [control, 6.10 $\pm$ 0.49 μ mol/h per g ($n = 6$); compare with 8-bromo-cGMP, 7.55 $\pm$ 0.37 μ mol/h per g ($n = 6$); $P < 0.05$].

DISCUSSION

The results from the present study have yielded some novel findings about an alternative mechanism for the stimulation of glucose transport and metabolism in rat skeletal muscle. It is well accepted that skeletal muscle is quantitatively the major site for insulin-mediated glucose disposal [25]. However, it has been realized for a long time that other mechanisms (besides insulin and insulin-like growth factor-1) exist to stimulate glucose transport and metabolism in skeletal muscle (e.g. contraction and hypoxia).

Because a significant amount of NOS activity is present in skeletal muscle cells [6,8,9] and NO is released from isolated incubated muscle preparations [14], it seems likely that these cells have the capacity for endogenous activation of NOS and the production of NO. So far very little is known about what factors or procedures activate NOS in skeletal muscle and NO formation, although it seems likely that contraction of the muscle is one procedure [14]. Because the rate of intracellular formation of NO could not be regulated we decided to generate NO extracellularly by addition of the nitric oxide donors SNP or spermine NONOate to the incubation medium. Initially we measured the dose-dependent effects of SNP (from 1 to 25 mM) on glucose metabolism that had been half-maximally stimulated by insulin (at a concentration of 100 μ -units/ml).

SNP, at all concentrations, significantly stimulated the rates of both net lactate and [14 C]lactate release (Figure 1). The rate of [14 C]lactate release reflects the rate of glucose transport [23]. Only at a high concentration of SNP (i.e. 25 mM) was there a marked difference between the rates of [14 C]lactate and net lactate release, perhaps indicating that high concentrations of SNP stimulate glycogenolysis. It is possible that in the presence of biological tissues cyanide ions are produced from SNP [15]. However, we do not believe that any of the effects of SNP are dependent on cyanide ions because when rhodanese and sodium thiosulphate were present in the incubation medium (to remove cyanide ions), SNP still stimulated the rate of [14 C]lactate release (Table 2). For glucose transport SNP probably acts independently of insulin's effects because the stimulation of the rates of net and [14 C]lactate release were increased in the presence of basal and stimulating concentrations of insulin (Table 1). In a separate experiment, in the presence of a basal concentration of insulin, SNP (15 mM) significantly stimulated the rate of transport of the glucose analogue 2-deoxyglucose (Table 4).

We also examined the effects of SNP on the intracellular fate of [U- 14 C]glucose. At 1 mM SNP there was no effect of SNP on rates of incorporation of [U- 14 C]glucose into glycogen (glycogen synthesis) even though this concentration of SNP stimulated both net and [14 C]lactate release (Figure 1). At higher concentrations of SNP (5, 10 and 15 mM) there was a significant inhibition of insulin-mediated glycogen synthesis and the magnitude of this inhibition was increased further at 20 and 25 mM SNP. β -Adrenoreceptor agonists, such as isoprenaline, inhibit glycogen synthesis and stimulate net lactate release in a similar manner; however, a major difference between SNP and β -adrenoreceptor agonists is that the latter do not stimulate either [14 C]lactate release or the transport of glucose analogues [23] (Figure 1 and Table 4). SNP did not inhibit the rate of glycogen synthesis in the absence of insulin but the rate was inhibited at all concentrations of insulin (Table 1). This suggests that NO can specifically decrease the intracellular signal for insulin's activation of glycogen synthesis, or glycogen synthase might be inactivated by a mechanism involving the NO/cGMP signalling pathway.

SNP greatly stimulated the rate of glucose oxidation (Figure 3). The effect of SNP on glucose oxidation was greater than any

effect that insulin has on this process in soleus muscle preparations *in vitro* [21]. The stimulation of the rate of glucose oxidation seems to conflict with the current perception of the role of NO on respiration. Indeed the location of NOS activity within muscle mitochondria suggests that it might play a role in regulating respiration. NO might interact and reversibly inactivate respiratory enzymes such as cytochrome oxidase [26]. NO inhibits respiration in activated astrocytes [27]. Further studies are required to elucidate the role of NO on respiration in skeletal muscle. For example, it is known that resting soleus muscle preparation oxidizes a substantial amount of endogenous fatty acids and this raises the possibility that fatty acid oxidation might be inhibited to a greater extent by NO, thus driving the cells to oxidize more glucose.

A central issue is whether the mechanism of action of SNP is mediated via cGMP. NO stimulates a soluble form of guanylate cyclase in most tissues [17,18]. Similarly we found that SNP, at 15 mM, caused a rapid and sustained increase (approx. 80-fold) in the content of cGMP (Figure 4). That SNP produced its effects through the stimulation of guanylate cyclase by NO was investigated in three ways. First, haemoglobin is a NO scavenger [16]: activation of the rates of [¹⁴C]lactate release by SNP or spermine NONOate was obliterated by haemoglobin (Table 3). Secondly, we monitored the effects of known inhibitors of guanylate cyclase (i.e. LY-83583 [19] or Methylene Blue [20]) on SNP-stimulated effects. LY-83583 inhibited the rise in cGMP caused by SNP (see Figure 4 and the Results section). Stimulation of glucose oxidation and 2-deoxyglucose transport by SNP was blocked by LY-83583 (see Table 4 and the Results section). Methylene Blue and LY-83583 both inhibited SNP-stimulated [¹⁴C]lactate release (Table 5). Thus the inhibition of guanylate cyclase activity is linked to diminished effects of SNP on cGMP content and glucose transport and utilization. Lastly, the cGMP analogue 8-bromo-cGMP significantly increased the rate of net lactate release. This agrees with results showing that the rate of 2-deoxyglucose transport into isolated incubated rat diaphragm preparation is increased by 8-bromo-cGMP [28].

In summary, the present study suggests that NO acts to stimulate glucose uptake and metabolism in skeletal muscle, providing a novel mechanism that may operate endogenously by the activation of NOS [6,8,9]. This mechanism could be activated by contraction, which is associated with both increased NO release and increased glucose utilization. Indeed, NO effects on glucose metabolism are qualitatively similar to the intracellular fate of glucose during contraction in skeletal muscle cells [1,29]. Interestingly, hypoxia has the same effects as contraction on glucose utilization in skeletal muscle preparations [30]. NO interacts rapidly and is inactivated by superoxide anions [6,7,31]. The concentration of superoxide radicals would be low during hypoxia. Superoxide dismutase preserves the effectiveness of NO [31], which is consistent with our observation of increased glucose utilization in soleus muscle preparation *in vitro* by superoxide dismutase [4]. This raises the possibility that the stimulation of glucose utilization by some agents, for example lipoic acid [31,32], is via their ability to act as antioxidants. If so, there is a need to investigate the activity of the NO/cGMP system in insulin-resistant skeletal muscle. Consequently, glucose transport and

utilization might be therapeutically increased in insulin-resistant skeletal muscle by specific activation of the NO/cGMP system, by, for example, use of antioxidants, induction of NOS in muscle cells or elevation of the intracellular cGMP concentration.

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REFERENCES

- Nesher, R., Karl, I. E. and Kipnis, D. M. (1985) *Am. J. Physiol.* **249**, C226–C232
- Lee, A. D., Hansen, P. A. and Holloszy, J. O. (1995) *FEBS Lett.* **361**, 51–54
- Yeh, J.-I., Gulve, E. A., Rameh, L. and Birnbaum, M. J. (1995) *J. Biol. Chem.* **270**, 2107–2111
- Young, M. E., Radda, G. K. and Leighton, B. (1996) *Diabetic Med.* **18**, (suppl. 2), S18
- Reid, M. B., Haack, K. E., Franchek, K. M., Valberg, P. A., Kobzik, L. and West, M. S. (1992) *J. Appl. Physiol.* **73**, 1797–1804
- Kobzik, L., Reid, M. B., Bredt, D. S. and Stamler, J. S. (1994) *Nature (London)* **372**, 546–548
- Wink, D. A., Hanbauer, I., Krishna, M. C., DeGraff, W., Gamson, J. and Mitchell, J. B. (1993) *Proc. Natl. Acad. Sci., U.S.A.* **90**, 9813–9817
- Brennan, J. E., Chao, D. S., Xia, H., Aldape, K. and Bredt, D. S. (1995) *Cell* **82**, 743–752
- Kobzik, L., Stringer, B., Balligand, J.-L., Reid, M. B. and Stamler, J. S. (1995) *Biochem. Biophys. Res. Commun.* **211**, 375–381
- Lee, K. H., Baek, M. Y., Moon, K. Y., Song, W. K., Chung, C. H., Ha, D. B. and Kang, M.-S. (1994) *J. Biol. Chem.* **269**, 14371–14374
- Williams, G., Brown, T., Becker, L., Prager, M. and Giroir, B. P. (1994) *Am. J. Physiol.* **267**, R1020–R1025
- Nakane, M., Schmidt, H. H. W., Pollock, J. S., Forstermann, U. and Murad, F. (1993) *FEBS Lett.* **316**, 175–180
- Bates, T. E., Loesch, A., Burnstock, G. and Clark, J. B. (1996) *Biochem. Biophys. Res. Commun.* **218**, 40–44
- Balon, T. W. and Nadler, J. L. (1994) *J. Appl. Physiol.* **77**, 2519–2521
- Smith, R. P. and Kruszyna, H. (1976) *Fed. Proc.* **35**, 69–72
- Tsuura, Y., Ishida, H., Hayashi, S., Sakamoto, K., Horie, M. and Seino, Y. (1994) *J. Gen. Physiol.* **104**, 1079–1098
- Ignarro, L. J. (1989) *FASEB J.* **3**, 31–36
- McDonald, L. J. and Murad, F. (1996) *Proc. Soc. Exp. Biol. Med.* **211**, 1–6
- Mulsch, A., Busse, R., Liebau, S. and Forstermann, U. (1988) *J. Pharmacol. Exp. Ther.* **247**, 283–288
- Takakura, K., Goto, Y. and Muramatsu, I. (1996) *Life Sci.* **58**, 9–17
- Leighton, B., Budohoski, L., Lozeman, F. J., Challiss, R. A. J. and Newsholme, E. A. (1985) *Biochem. J.* **227**, 337–340
- Leighton, B., Kowalchuk, J. M., Challiss, R. A. J. and Newsholme, E. A. (1988) *Am. J. Physiol.* **255**, E41–E45
- Challiss, R. A. J., Lozeman, F. J., Leighton, B. and Newsholme, E. A. (1986) *Biochem. J.* **233**, 377–381
- Hammerstedt, R. H. (1980) *Anal. Biochem.* **109**, 443–448
- Leighton, B. and Cooper, G. J. S. (1990) *Trends Biochem. Sci.* **15**, 295–299
- Brown, G. C. (1995) *FEBS Lett.* **369**, 136–139
- Brown, G. C., Bolanos, J. P., Heales, S. J. and Clark, J. R. (1995) *Neurosci. Lett.* **193**, 201–204
- Pinkett, M. O. and Perlman, R. L. (1975) *Biochim. Biophys. Acta* **399**, 473–477
- Nesher, R., Karl, I. E. and Kidnis, D. M. (1980) *Am. J. Physiol.* **239**, E461–E467
- Azevedo, J. L., Carey, J. O., Pories, W. J., Morris, P. G. and Dohm, G. L. (1995) *Diabetes* **44**, 695–698
- Stamler, J. S., Singel, D. J. and Loscalzo, J. (1992) *Science* **258**, 1898–1902
- Haugaard, N. and Haugaard, E. S. (1970) *Biochim. Biophys. Acta* **222**, 583–586