Fuel oxidation in skeletal muscle is increased by nitric oxide/cGMP – evidence for involvement of cGMP-dependent protein kinase

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Abstract The cyclic guanosine-3′,5′-monophosphate (cGMP) analogue, 8-bromo-cGMP (1 mM), increased glucose oxidation in isolated soleus muscle. The nitric oxide (NO) donor, sodium nitroprusside (SNP) (15 mM), increased glucose, pyruvate, palmitate and leucine oxidation. Removal of extracellular Ca^{2+} did not affect SNP-stimulated glucose oxidation (or other glucose utilization parameters), thus eliminating the influx of Ca^{2+} as a mechanism for the increases. The guanylate cyclase inhibitor, LY-83583 (10 μM), inhibited SNP-stimulated palmitate oxidation and activation of cGMP-dependent protein kinase (PKG). Activation of PKG might supersede any inhibitory effects of NO on respiration to stimulate metabolic fuel oxidation in skeletal muscle.

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Key words: Cyclic guanosine-3′,5′-monophosphate-dependent protein kinase; Skeletal muscle; Nitric oxide; Fuel utilization; Contraction; Cyclic guanosine-3′,5′-monophosphate

1. Introduction

The neuronal (nNOS), endothelial and inducible (iNOS) forms of nitric oxide synthase (NOS) are all found, under different conditions, within skeletal muscle cells [1–3] and they generate nitric oxide (NO) [4]. NOSs are located at the sarcolemmal membrane [1] and within mitochondria [2]. NO binds to the haem group of skeletal muscle guanylate cyclase, increasing cyclic guanosine-3′,5′-monophosphate (cGMP) levels [5]. Very few proteins or processes in skeletal muscle have been identified as physiologically relevant targets for cGMP dependent protein kinase (PKG) [6,7].

The current common perception is that NO inhibits respiration [8], by inactivating mitochondrial enzymes, like cytochrome oxidase and aconitase [8]. Much of this work has been carried out on isolated incubated mitochondria [8]. Indeed, few studies have monitored the effects of NO on respiration or fuel oxidation in quantitatively the most significant tissue, skeletal muscle.

A key hypothesis is that NO is a mediator of contraction-stimulated glucose utilization [5]. The NO donor, sodium nitroprusside (SNP), stimulates rates of glucose oxidation, in association with increased rates of glucose transport and glycolysis, and decreases rates of insulin-mediated glycogen synthesis in isolated soleus muscle preparations [5,9]. SNP-stimulated rates of glucose oxidation are inhibited by the guanylate cyclase inhibitor LY-83583 (which also decreases the generation of cGMP by SNP; [5]). cGMP levels in soleus muscles are also increased by inhibition of phosphodiesterase catalysed cGMP hydrolysis by zaprinast (a selective inhibitor of cGMP type V phosphodiesterase; [9,10]). Zaprinast, like NO donors, increases glucose transport and utilization and cGMP content in isolated incubated soleus muscle in vitro [9,10]. Furthermore, zaprinast increases the rate of glucose oxidation [9,10]. Thus, the case for a role of cGMP in the stimulation of skeletal muscle glucose oxidation by NO is compelling.

Activation of PKG is one possible mechanism of action of cGMP. Alternatively, NO and/or cGMP could increase the intracellular calcium (Ca^{2+}) concentration in skeletal muscle via an increase in the rate of Ca^{2+} influx, since NO/cGMP regulate the activity of Ca^{2+} channels in cells [11]. An increase in intracellular Ca^{2+} levels might increase rates of glucose transport [12], glycogenolysis [13], and in particular glucose oxidation [14], as well as decreasing insulin-mediated glycogen synthesis [15]. Physiological increases in extra mitochondrial Ca^{2+} concentrations cause activation of dehydrogenases associated with increases in both fuel oxidation and respiration [14]. We incubated soleus muscle preparations in the presence or absence of medium Ca^{2+} (by adding EGTA, to chelate any Ca^{2+}) and measured the effects of SNP on glucose metabolism.

The effects of a cGMP analogue, 8-bromo-cGMP, on rates of glucose oxidation were measured. The effects of SNP on the oxidation of carbohydrate (pyruvate), non-esterified fatty acid (palmitate) and amino acid (leucine) were also determined. For palmitate oxidation, these experiments were repeated in the presence of the guanylate cyclase inhibitor LY-83583, to determine the role of cGMP. Since PKG might be important, we measured the activation of the soluble form of PKG in soleus (type I fibres) and EDL (type II fibres) muscles by cGMP. The effects of SNP and LY-83583, either alone or in combination, on soluble PKG activity were also determined.

2. Materials and methods

2.1. Animals and materials

Male Wistar rats (70 and 140 g; Harlan-Olac, Bicester, Oxon., UK) were kept in the department's animal house under controlled conditions (23 ± 1°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. Enzymes, chemicals, biochemicals and radiochemicals were purchased from sources previously given [5,9,16–18], except for 3-isobutyl-1-methylxanthine (IBMX), kemptide, protein kinase inhibitor (PKI), cGMP, ATP, leucine, palmitate (Sigma), [1-^{14}C]leucine, [1-^{14}C]palmitate, [1-^{14}C]pyruvate, [γ-^{32}P]ATP (Amer-

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Abbreviations: cGMP, cyclic guanosine-3′,5′-monophosphate; EDL, extensor digitorum longus; NO, nitric oxide; NOS, nitric oxide synthase; PKG, cGMP-dependent protein kinase; SNP, sodium nitroprusside

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sham Life Science) and P81 phosphocellulose paper (Whatman International PLC).

2.2. Incubation procedures and analysis

Rats were killed by cervical dislocation, and stripped solei prepared as described previously [17]. Small rats (70 g) were initially sedated with Sagatal (100 mg/kg), prior to cervical dislocation and whole EDL and soleus muscles isolated for determination of PKG activity. The tendons were ligated and the muscles rapidly weighed and tied at 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/insulin (10 μUnits/ml). Flasks were sealed and aerated continuously with O_2/CO_2 (19:1). LY-83583 (10 μM), 8-bromo-cGMP (1 mM), pyruvate (1 mM), palmitate (0.5 mM) or leucine (1 mM) were all added to the pre-incubation and incubation medium. After pre-incubation of muscles for 30 min at 37°C in an oscillating water bath (100 rev./min), the muscles were transferred to similar medium with added radiolabelled substrate (0.5 μCi/ml) and various compounds (e.g. LY-83583 and/or SNP or 8-bromo-cGMP). In certain experiments, Ca^{2+} was omitted from the incubation medium, and 10 mM EGTA added to chelate extracellular Ca^{2+}. The flasks were sealed and regassed 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 incubation medium was acidified with perchloric acid (6%, w/v) before collection of $^{14}CO_2$ in plastic centre wells containing 0.2 ml of 1:1 (v/v) phenylethylamine/methanol [17]. The amount of $^{14}CO_2$ formed was calculated from the specific radioactivity of the extracellular substrate (i.e. glucose, pyruvate, palmitate or leucine). This calculation does not take into account any dilution of [^{14}C]substrate by endogenously available substrate. In certain experiments, the rates of net and ^{14}C-labelled lactate release and glycogen synthesis were determined, as previously described [16]. ^{14}C-labelled lactate is derived only from extracellular [^{14}C]glucose, and previous studies have shown that the rate of ^{14}C-labelled lactate release is a good indication of the rate of glucose transport [16]. Muscle glycogen synthesis is measured by the rate of [U-^{14}C]glucose incorporation into glycogen [16–18].

For measurement of PKG, muscles were initially incubated for 110 min with constant gassing, in the presence or absence of SNP (15 mM) and/or LY-83583 (10 μM), followed by a 10-min incubation to remove residual SNP and/or LY-83583. At the end of the second incubation, muscles were blotted and rapidly frozen in liquid N_2. Muscle PKG activity was determined by measuring the rate of phosphorylation of the substrate peptide, kemptide, essentially as described previously [19].

2.3. Statistical analysis

All results are presented as mean ± S.E.M. Student's *t*-test was used to establish the significance of differences between means.

3. Results

3.1. Effects of SNP on soleus muscle glucose utilization in the presence and absence of calcium, and the effects of 8-bromo-cGMP on soleus muscle glucose oxidation

The effects of SNP (15 mM) on rat soleus muscle glucose utilization in the presence and absence of medium Ca^{2+} are given in Table 1. SNP (15 mM) significantly increased rates of net lactate release, ^{14}C-labelled lactate release and glucose oxidation in the presence of medium Ca^{2+}, but significantly decreased the rate of glycogen synthesis (Table 1). When Ca^{2+} was removed from the medium, the rates of net lactate release, ^{14}C-labelled lactate release and glucose oxidation all decreased, with no significant effect on the rate of glycogen synthesis (Table 1). The absence of Ca^{2+} from the medium did not affect the stimulation of rates of net and ^{14}C-labelled lactate release, or the inhibition of glycogen synthesis, by SNP (Table 1). Importantly, SNP significantly increased the rate of glucose oxidation, in the absence or presence of Ca^{2+} (Table 1). Percentage changes in the rates of glucose utilization by SNP were the same either in the absence or presence of Ca^{2+} (Table 1). The cGMP analogue, 8-bromo-cGMP, significantly increased the rate of soleus muscle glucose oxidation (0.20 ± 0.03 vs. 0.34 ± 0.04 μmol/h/g wet wt., for control vs. 8-bromo-cGMP ($n = 11$); $P < 0.01$).

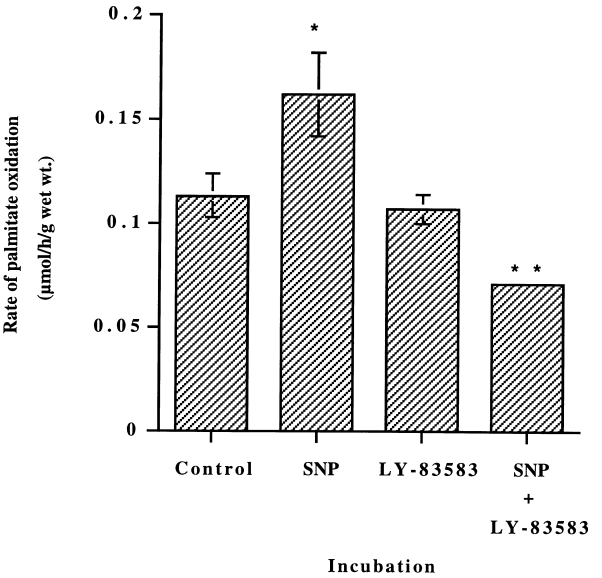

Fig. 1. Effects of the NO donor, SNP (15 mM), and/or the guanylate cyclase inhibitor, LY-83583 (10 μM), on rates of oxidation of palmitate (0.5 mM) in isolated incubated soleus muscle preparations. Results are presented as means ± S.E.M. for five separate incubations. Statistically significant differences between muscles incubated in the absence of both SNP and LY-83583 (control) compared with muscles incubated in the presence of SNP and/or LY-83583 are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.2. Effects of SNP on pyruvate, palmitate and leucine oxidation, and the effects of LY-83583 (± SNP) on palmitate oxidation

SNP (15 mM), significantly increased the rates of oxidation of pyruvate (3.23 ± 0.26 vs. 4.24 ± 0.2 μmol/h/g wet wt., for control vs. SNP ($n = 5$); $P < 0.01$), palmitate (0.11 ± 0.01 vs. 0.16 ± 0.02 μmol/h/g wet wt., for control vs. SNP ($n = 5$); $P < 0.05$) and leucine (0.58 ± 0.03 vs. 0.79 ± 0.06 μmol/h/g wet wt., control vs. SNP ($n = 5$); $P < 0.01$). The magnitude of the increase in oxidation by SNP was about the same for all substrates (pyruvate, 32%, palmitate, 43%, and leucine, 36%). The guanylate cyclase inhibitor LY-83583 (10 μM) alone had no significant effect on palmitate oxidation (Fig. 1). Incubation of soleus muscle in the presence of both SNP (15 mM) and LY-83583 (10 μM) caused a significant decrease in isolated incubated soleus muscle rates of palmitate oxidation, in comparison to control rates (Fig. 1).

3.3. cGMP-dependent protein kinase assay

Both soleus and EDL muscle preparations were incubated in the absence or presence of SNP and/or LY-83583. After incubation, PKG activities in muscle homogenate supernatants were determined in the absence or presence (1, 10 or 100 μM) of cGMP (Fig. 2a,b). Soleus muscles had a higher activity of PKG compared to the activity in EDL muscle (Fig. 2a,b). Incubation of either soleus or EDL muscles with either SNP (15 mM) or LY-83583 (10 μM) alone did not affect the activity of PKG in supernatants (Fig. 2a,b). However, incu-

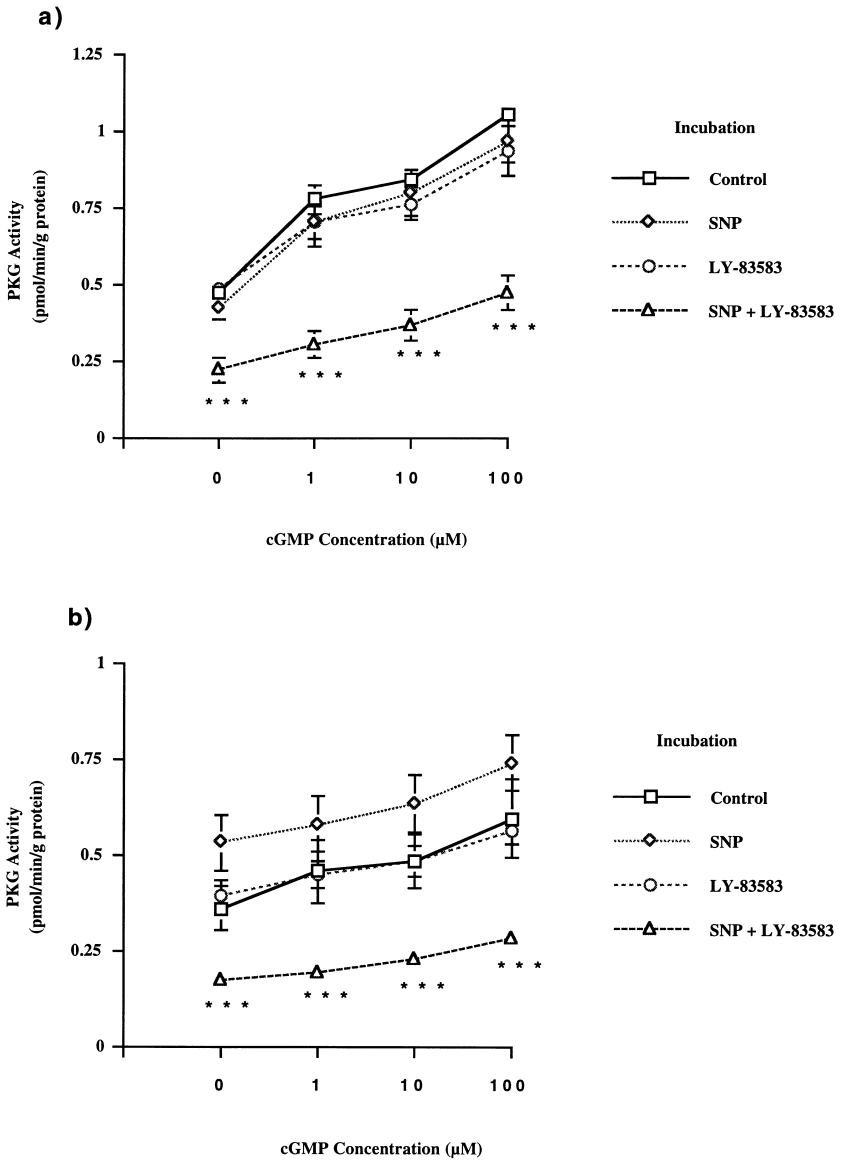

Fig. 2. Effects of incubation of isolated soleus (a) and EDL (b) muscles with the NO donor, SNP (15 mM), and/or the guanylate cyclase inhibitor, LY-83583 (10 μM), on subsequent activity of soluble cGMP-dependent protein kinase (PKG) activity, in the absence and presence (1, 10 or 100 μM) of cGMP in muscle homogenates. Results are presented as means ± S.E.M. for four separate observations. Statistically significant differences between muscles incubated in the absence of both SNP and LY-83583 (control) compared with muscles incubated in the presence of SNP and/or LY-83583, for the same assay cGMP concentration, are indicated by $^{***}P < 0.001$.

bation of muscle preparations with the combination of SNP (15 mM) and LY-83583 (10 μM) caused a significant decrease in the activity of PKG activity (both the absence and presence of cGMP; Fig. 2a,b).

Table 1
Effects of the NO donor, SNP (15 mM), on rates of glucose utilization in isolated incubated soleus muscle preparations, in the presence or absence of extracellular Ca^{2+}

Incubation	Rates of glucose utilization (μmol/h/g wet wt.; $n = 5$)			
	Net lactate release	^{14}C-Labelled lactate release	Glucose oxidation	Glycogen synthesis
−SNP, $+Ca^{2+}$	10.02 ± 1.03	5.47 ± 1.40	0.82 ± 0.06	2.23 ± 0.44
+SNP, $+Ca^{2+}$	20.81 ± 1.71***	20.27 ± 1.73***	1.91 ± 0.22***	1.36 ± 0.12*
−SNP, $-Ca^{2+}$	3.78 ± 0.54***	1.29 ± 0.80**	0.31 ± 0.03***	2.50 ± 0.21
+SNP, $-Ca^{2+}$	9.99 ± 1.18$^{\$\$\$}$	5.64 ± 0.59$^{\$\$\$}$	1.69 ± 0.10$^{***\$\$\$}$	1.77 ± 0.24$^{\$}$

Statistically significant differences between muscles incubated in the presence of Ca^{2+} and the absence of SNP (i.e. −SNP, $+Ca^{2+}$) compared with other incubations are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences between muscles incubated in the absence of Ca^{2+} and the absence of SNP (i.e. −SNP, $-Ca^{2+}$) compared with muscles incubated in the absence of Ca^{2+} and the presence of SNP (i.e. +SNP, $-Ca^{2+}$) are indicated by $^{\$}P < 0.05$ and $^{\$\$\$}P < 0.001$.

4. Discussion

Exercise causes marked elevations in skeletal muscle fuel utilization, not only during, but also for many hours after the cessation of exercise [20,21], e.g. elevated oxidative metabolism is sustained for 24 h after a marathon [22]. The biochemical mechanism(s) responsible for increased oxidative metabolism during and after exercise remain poorly understood. The present study provides novel results that further elucidate the roles of NO and cGMP in skeletal muscle fuel utilization [5,9], and in particular oxidation of metabolic fuels.

NO-stimulated skeletal muscle glucose utilization and oxidation is mediated, at least in part, by cGMP [5,9] (see also Section 1). We tested the hypothesis that NO-stimulated rates of skeletal muscle glucose utilization were mediated (either directly, or via cGMP) through increased influx of extracellular Ca^{2+}. Isolated soleus muscles were incubated in the absence or presence of Ca^{2+}, with or without the nitric oxide donor SNP. In the absence of Ca^{2+} the rates of net- and ^{14}C-labelled lactate release and glucose oxidation were decreased, with no significant effect on glycogen synthesis (Table 1). SNP (15 mM), in either the absence or presence of Ca^{2+}, markedly increased rates of net- and ^{14}C-labelled lactate release and glucose oxidation, and significantly decreased glycogen synthesis (Table 1). Since the magnitude of the effects of SNP is the same no matter what the conditions this suggests that extracellular Ca^{2+} is unimportant for stimulation of glucose oxidation (or glucose utilization) by NO.

The increase in cGMP and the rate of glucose oxidation caused by SNP is abolished by the guanylate cyclase inhibitor LY-83583 [5]. In addition, 8-bromo-cGMP increased the rate of glucose oxidation in soleus muscle in vitro (see Section 3). All this strongly supports the proposal that cGMP plays a vital role in one mechanism for stimulation of skeletal muscle glucose oxidation.

Skeletal muscle can oxidise a number of substrates, including fatty acids and amino acids. In the present study, SNP stimulated the rates of oxidation of pyruvate, palmitate and leucine in soleus muscles in vitro (see Section 3). The magnitude of stimulation by SNP was similar for all rates of substrate oxidation (i.e. 32%, 43% and 36% increases for rates of pyruvate, palmitate and leucine oxidation, respectively). SNP (15 mM) significantly increased rates of glucose oxidation by 133% (Table 1). The guanylate cyclase inhibitor LY-83583 (10 μM) alone had no effect on rates of palmitate oxidation (Fig. 1). However, LY-83583 inhibited SNP-stimulated rates of palmitate oxidation, to a value lower than control rates (Fig. 1). This is further evidence that NO-stimulated rates of skeletal muscle substrate oxidation are via cGMP since LY-83583 would inhibit guanylate cyclase activation by NO, thus preventing a rise in cGMP levels.

Septic shock, via cytokine production, causes increased expression iNOS in muscle [3]. iNOS expression is induced in cultured muscle cells by cytokines, and this is associated with increased rates of glucose transport [23]. Whether increased rates of leucine oxidation observed during sepsis [24] are mediated through increased NO generation in skeletal muscle (as observed in vitro in the present study) remains to be determined.

Activation of PKG may, at least in part, regulate fuel oxidation in skeletal muscle. Soleus contains more soluble PKG activity than EDL (Fig. 2a,b). Incubation of soleus or EDL muscle preparations with either SNP (15 mM) or LY-83583 (10 μM) did not affect soluble PKG activities (Fig. 2a,b). However, a combination of SNP (15 mM) and LY-83583 (10 μM) significantly decreased soluble PKG activity (in both the absence and presence of cGMP in the enzyme assay; Fig. 2a,b). These results suggest that an effect of NO on skeletal muscle with a low cGMP content (due to inhibition of guanylate cyclase by LY-83583) was marked inhibition of soluble PKG. Thus, cGMP appears to protect soluble PKG from inhibition by NO. Incubation of soleus muscles with both SNP (15 mM) and LY-83583 (10 μM) also decreased palmitate oxidation (Fig. 1) suggesting that a mechanism operates in the cell in which activation of PKG promotes skeletal muscle fuel oxidation.

This is the first study to provide evidence for a role for activation of soluble PKG, by cGMP, in the stimulation of oxidative metabolism in skeletal muscle. Glucose oxidation is activated by NO in smooth muscle [25]. NO increases rates of oxidation of glucose, pyruvate, palmitate and leucine (see Section 3), suggesting activation of a common mechanism for oxidation of all these substrates. Whilst a number of potential mechanisms exist, we suggest that mitochondrially located uncoupling protein-3 (UCP-3), which is highly expressed in skeletal muscle [26], becomes phosphorylated and activated by PKG. Uncoupling of respiration, through activation of UCP-3, would increase oxidative metabolism in mitochondria [27]. UCP-3 has a potential PKG phosphorylation site (not found in UCP-1 and UCP-2). The potential phosphorylation site (serine-160) has arginine and lysine residues upstream, characteristic of a PKG phosphorylation site [7], and is located in the middle of a hydrophilic region of UCP-3, suggesting it should be accessible to phosphorylation [26]. Whether this site is indeed phosphorylated by PKG in vivo, or whether such a phosphorylation would affect UCP-3 function, requires further work. Clearly much more research is required before the understanding of the NO/cGMP/PKG system in skeletal muscle fuel utilization is complete.

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