Evidence for altered sensitivity of the nitric oxide/cGMP signalling cascade in insulin-resistant skeletal muscle

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Nitric oxide activates guanylate cyclase to form cGMP, comprising a signalling system that is believed to be a distinct mechanism for increasing glucose transport and metabolism in skeletal muscle. The effects of a selective cGMP phosphodiesterase inhibitor, zaprinast, on basal glucose utilization was investigated in incubated rat soleus muscle preparations isolated from both insulin-sensitive (lean Zucker; *Fa/?*) and insulin-resistant (obese Zucker; *fa/fa*) rats. Zaprinast at 27 μ M significantly increased cGMP levels in incubated soleus muscle isolated from lean, but not obese, Zucker rats. Muscles were incubated with ¹⁴C-labelled glucose and various concentrations of zaprinast (3, 27 and 243 μ M). Zaprinast (at 27 and 243 μ M) significantly increased rates of net and ¹⁴C-labelled lactate release and of glycogen synthesis in lean Zucker rat soleus muscle; glucose oxidation was also increased by 27 μ M zaprinast. In addition, regardless of concentration, the phosphodiesterase inhibitor failed to increase any aspect of ¹⁴C-labelled glucose utilization in

soleus muscles isolated from obese Zucker rats. The maximal activity of nitric oxide synthase (NOS) was significantly decreased in insulin-resistant obese Zucker muscles. Thus the lack of effect of zaprinast in insulin-resistant skeletal muscle is consistent with decreased NOS activity. To test whether there is a defect in insulin-resistant skeletal muscle for endogenous activation of guanylate cyclase, soleus muscles were isolated from both insulin-sensitive and insulin-resistant Zucker rats and incubated with various concentrations of the NO donor sodium nitroprusside (SNP; 0.1, 1, 5 and 15 mM). SNP significantly increased rates of net and ¹⁴C-labelled lactate release, as well as glucose oxidation in muscles isolated from both insulin-sensitive and insulin-resistant rats. A decreased response to SNP was observed in the dose-dependent generation of cGMP within isolated soleus muscles from insulin-resistant rats. A possible link between impaired NO/cGMP signalling and abnormal glucose utilization by skeletal muscle is discussed.

INTRODUCTION

Two distinct signalling cascades operate in skeletal muscle to stimulate glucose utilization, namely the insulin- and contraction-mediated pathways [1,2]. Neither cascade has been fully characterized at the molecular level. One of the underlying features of non-insulin-dependent diabetes mellitus is an inability to respond to physiological concentrations of insulin, so-called insulin resistance [3,4]. Although the response of glucose metabolism to insulin is poor in insulin-resistant skeletal muscle, it will respond normally to contraction [5]. Perhaps this is a partial explanation of why exercise is believed to be beneficial for people with non-insulin-dependent diabetes mellitus [6]. Indeed there is growing interest in the utility of activation of the contraction apparatus as a means for stimulation of glucose utilization in insulin-resistant skeletal muscle [5,7]. Thus, once the mechanism by which contraction stimulates glucose utilization in skeletal muscle has been fully elucidated, specific targets for future pharmaceutical compounds as anti-diabetic agents could be developed.

We have recently shown that nitric oxide (NO) stimulates glucose transport and the rates of lactate release and glucose oxidation in isolated incubated rat skeletal muscle preparations [8]. The capacity for NO formation in skeletal muscle is well established because there exists the neuronal form of nitric oxide synthase (nNOS), which is primarily located at the sarcolemmal protein dystrophin complex [9,10]. The effector mechanism(s) used by NO to stimulate glucose metabolism in skeletal muscle have not been characterized. It is suggested that NO interacts with the haem group bound to soluble guanylate cyclase, leading to activation and elevation of cGMP levels [11]. Indeed, a nitric oxide donor, sodium nitroprusside (SNP), stimulates the gen-

eration of cGMP in isolated rat skeletal muscle as well as stimulating the rate of glucose utilization [8]. Thus, if cGMP levels in skeletal muscle could be increased by a mechanism other than high exogenous levels of NO, and if this elevation corresponded to higher rates of glucose utilization, then this would provide evidence that the NO/cGMP system has a role in regulating rates of glucose utilization in this tissue.

The hydrolysis of cGMP is catalysed by a selective cGMP phosphodiesterase [12]. Three isoenzymes of phosphodiesterase might be involved in the degradation of cGMP, namely phosphodiesterases I, II and V. Zaprinast is considered a selective inhibitor of cGMP type V phosphodiesterase [13]. Therefore zaprinast should cause the elevation of cGMP by inhibiting endogenous cGMP phosphodiesterase activity [13]. Indeed, concentration-dependent elevations in intracellular cGMP levels in response to zaprinast incubation in isolated Wistar rat soleus muscle have been observed (M. E. Young and B. Leighton, unpublished work). Consequently we have determined the effects of zaprinast on cGMP levels in soleus muscles isolated from both insulin-sensitive and insulin-resistant Zucker rats (see [5] and [14] for insulin resistance in Zucker rat skeletal muscle).

Activation of the NO/cGMP system probably occurs during the contraction of skeletal muscle [8]. Because the rate of release of NO is increased by prior contraction of skeletal muscle [15,16] and NO stimulates glucose utilization [8], it is important to measure the activity of the NO/cGMP system in insulin-resistant muscle. In addition, the expression of NOS increases in skeletal muscles isolated from exercise-trained rats [17]. In consideration of any potential relationship between the NO/cGMP system and insulin-resistant skeletal muscle it is important that the activity (and subcellular distribution) of NOS is measured in a number of

Abbreviations used: EDL, extensor digitorum longus; NOS, nitric oxide synthase; SNP, sodium nitroprusside.

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skeletal muscles isolated from lean (insulin-sensitive) and obese (insulin-resistant) Zucker rats. We also determined whether the incubation of soleus muscles (from both obese and lean Zucker rats) with zaprinast was associated with altered rates of net and ^{14}C -labelled lactate release, glycogen synthesis and glucose oxidation. Finally, the effects of incubation of soleus muscles from both insulin-sensitive and insulin-resistant rats with SNP on rates of glucose utilization and cGMP generation were determined, to investigate further any possible defect in NO/cGMP signalling downstream of generation of endogenous NO.

MATERIALS AND METHODS

Animals

Female lean (*Fa/?*) and obese (*fa/fa*) Zucker rats 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 listed previously [8,18–20] except for Zaprinast (Sigma, Poole, Dorset, U.K.) and reagents for the NOS assay, such as [^{14}C]arginine from Amersham (Little Chalfont, Bucks., U.K.), calmodulin, flavin mononucleotide, PMSF and L-arginine (Sigma). Leupeptin was purchased from Boehringer Mannheim (Germany).

Incubation procedures and analysis

Rats were killed by cervical dislocation; stripped solei were prepared as described previously [18]. The tendons of the muscles were ligated, rapidly weighed and tied at the resting length *in situ* on stainless steel clips, and 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 and 10 $\mu\text{units/ml}$ insulin. Flasks were sealed and aerated continuously with O_2/CO_2 (19:1). In addition, when the effects of zaprinast (always dissolved in DMSO) were investigated, this compound (at the appropriate concentration) was also present in the preincubation medium. The same concentration of DMSO (1 %, v/v) was always added to the control medium (i.e. that lacking zaprinast). SNP was only ever present in the incubation medium. After preincubation of muscles for 45 min at 37°C in an oscillating water bath (100 rev./min), the muscle strips were transferred to similar flasks containing identical medium with added [^{14}C]glucose (0.5 $\mu\text{Ci/ml}$); when cGMP levels were to be determined, the [^{14}C]glucose was omitted. The flasks were sealed and re-gassed for the initial 15 min period in a 1 h incubation (only 15 min incubation when cGMP levels were determined). At the end of the incubation period, muscles were blotted and rapidly frozen in liquid N_2 . The concentration of lactate in the incubation medium was determined spectrophotometrically [18,21]. In addition, the rate of release of ^{14}C -labelled lactate into the medium was measured by the method described previously [22,23]. This ^{14}C -labelled lactate is derived only from extracellular glucose and has been shown in previous studies to be a good indication of the rate of glucose transport [18]. Furthermore any difference between the rates of net and ^{14}C -labelled lactate release probably indicates that glycogenolysis is activated in the isolated incubated soleus muscle. The rate of [^{14}C]glucose incorporation into glycogen was used to follow

glycogen synthesis as described previously [18–20,24]. In other experiments the incubation medium was acidified with 6 % (w/v) HClO_4 before the collection of $^{14}\text{CO}_2$ in plastic centre wells containing 0.2 ml of phenylethylamine/methanol (1:1, v/v) [19]. The amount of $^{14}\text{CO}_2$ formed was calculated from the specific radioactivity of extracellular glucose.

The content of cGMP was measured with a cGMP enzyme immunoassay system (Amersham), in accordance with the manufacturer's instructions for the assay of acetylated cGMP, with previously described modifications for skeletal muscle samples [8]. NOS activity was determined by measuring the formation of [^{14}C]citrulline from [^{14}C]arginine, essentially as described previously [25]. Some changes were made to the published method so that maximal catalytic activity was determined: the concentration of arginine present was 100 μM and the reaction time was 10 min. [^{14}C]Citrulline was separated from [^{14}C]arginine by use of Dowex ion-exchange chromatography.

The subcellular NOS activities were determined for gastrocnemius muscles isolated from lean and obese Zucker rats. Briefly, gastrocnemius muscles were isolated, frozen rapidly in liquid N_2 , pulverized and then homogenized in 20 mM Tris/HCl (pH 7.4)/0.5 mM EDTA/0.5 mM EGTA/1 mM dithiothreitol/1 mM leupeptin/0.2 mM PMSF before differential centrifugation. This involved centrifugation of the muscle homogenates (1 ml) at 20000 g for 10 min. This supernatant (referred to as supernatant) was removed and kept. The first pellet was extracted in the same buffer containing 200 mM KCl. Washing with KCl is known to remove NOS bound to cytoskeletal structures. The KCl lysate was further centrifuged at 100000 g for 20 min and the supernatant (referred to as the particulate fraction) and the final pellet were retained. NOS activity was determined in the supernatant, the particulate fraction and the redissolved final pellet.

RESULTS

NOS in Zucker rat skeletal muscle

Table 1 shows the maximal NOS activity measured in soleus, diaphragm, gastrocnemius and extensor digitorum longus (EDL) muscles isolated from lean and obese Zucker rats. The descending order of maximal NOS activity in skeletal muscles isolated from lean rats was $\text{EDL} > \text{gastrocnemius} > \text{diaphragm} > \text{soleus}$ (Table 1). In addition, NOS activity in muscle homogenates prepared from EDL, gastrocnemius and diaphragm isolated from obese Zucker rats was significantly decreased compared with that in the lean muscle homogenates (Table 1). The maximal NOS activity was not significantly different in soleus muscle preparations isolated from lean and obese Zucker rats (Table 1). The percentage decreases in the maximal catalytic activity of

Table 1 Maximal activity of NOS in various skeletal muscles isolated from insulin-sensitive lean and insulin-resistant obese Zucker rats

Results are presented as means $\pm$ S.E.M. for eight separate observations. The activity of NOS was obtained from measuring the rate of conversion of [^{14}C]arginine to [^{14}C]citrulline as described in the Materials and methods section. Statistically significant differences from the results obtained from the lean rats are indicated by * $P < 0.05$.

Rat type	NOS activity (pmol/min per mg of protein)			
	Soleus	Diaphragm	Gastrocnemius	EDL
Lean	3.05 ± 0.33	5.08 ± 0.28	6.33 ± 0.89	16.07 ± 2.43
Obese	2.83 ± 0.28	$3.59 \pm 0.45^*$	$3.78 \pm 0.59^*$	$10.20 \pm 1.49^*$

Table 2 Distribution of NOS activity in various skeletal muscle fractions prepared by differential centrifugation of gastrocnemius homogenates

Results are presented as means $\pm$ S.E.M. for six separate observations. The activity of NOS was obtained from measuring the rate of conversion of [14 C]arginine to [14 C]citrulline as described in the Materials and methods section. The various fractions correspond to the following: supernatant, the first supernatant fraction; pellet, whole cell debris; particulate, supernatant of pellet previously washed with 200 mM KCl. Statistically significant differences from the results obtained from the lean rats are indicated by * P < 0.05 and ** P < 0.01.

Rat type	NOS activity (pmol/min per mg of protein)		
	Supernatant	Particulate	Pellet
Lean	5.51 $\pm$ 0.66	32.89 $\pm$ 6.31	4.06 $\pm$ 0.57
Obese	3.57 $\pm$ 0.29**	20.19 $\pm$ 3.94*	3.93 $\pm$ 0.60

Figure 1 Concentration-dependent effects of zaprinast on the rate of net lactate release in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of zaprinast are indicated by * P < 0.05 and *** P < 0.001.

NOS in diaphragm (29%), gastrocnemius (40%) and EDL (36%) were similar.

The subcellular distribution of NOS activity in lean and obese Zucker gastrocnemius muscles was determined (Table 2). Most NOS activity was associated with the particulate fraction (Table 2). NOS activity was significantly decreased in supernatant and particulate fractions prepared from gastrocnemius muscles isolated from obese Zucker rats; no significant difference was seen in the pellet activity (Table 2). The largest decrease in NOS activity was in the particulate fraction (Table 2).

Zaprinast effects on Zucker rat soleus muscles

Both lean and obese Zucker rat solei were isolated and the effects of zaprinast on the rates of net and 14 C-labelled lactate release, glycogen synthesis and glucose oxidation were determined. The effect of zaprinast incubation on muscle cGMP content was also determined. All incubations were in the presence of a basal concentration of insulin (10 μ -units/ml). In the absence of zaprinast, no significant difference in the basal rate of net lactate release was observed between the lean and obese Zucker rat solei (Figure 1). In contrast, the rate of 14 C-labelled lactate release in

Figure 2 Effects of various concentrations of zaprinast on the rate of 14 C-labelled lactate release in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of zaprinast are indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001.

the absence of zaprinast was significantly higher in the lean Zucker than in the obese Zucker solei (P < 0.001; Figure 2). Zaprinast, at 27 and 243 μ M, significantly increased rates of both net and 14 C-labelled lactate release by a similar magnitude from solei isolated from lean Zucker rats (Figures 1 and 2). In the case of solei isolated from obese Zucker rats, zaprinast, only at the highest concentration (243 μ M), caused a small but significant increase in the rates of net and 14 C-labelled lactate release (Figures 1 and 2).

The basal rate (i.e. in the absence of zaprinast) of glycogen synthesis was significantly higher in soleus muscles isolated from lean Zucker rats (P < 0.001; Figure 3) than in obese Zucker rat

Figure 3 Effects of various concentrations of zaprinast on the rate of glycogen synthesis in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of zaprinast are indicated by * P < 0.05 and *** P < 0.001.

Figure 4 Effects of various concentrations of zaprinast on the rate of glucose oxidation in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of zaprinast are indicated by ** P < 0.01 and *** P < 0.001.

Table 3 Effects of zaprinast and SNP incubation on cGMP generation in muscles isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml. insulin. Results are presented as the percentage increase in muscle cGMP content, after incubation with either zaprinast or SNP, compared with muscle incubated in the absence of both zaprinast and SNP. Values are shown as means $\pm$ S.E.M. for at least three separate observations. Statistically significant differences between lean and obese muscle percentage increases are indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001. Control soleus muscle cGMP concentrations for lean and obese Zucker rats were 8.621 ± 1.522 and 11.826 ± 1.627 pmol/g wet wt. (n = 6) respectively.

Incubation	Increase in muscle cGMP (%)	
	Lean	Obese
Zaprinast (27 μ M)	91.9 $\pm$ 25.8	0.0 $\pm$ 29.1*
SNP (0.1 mM)	126.9 $\pm$ 28.2	33.8 $\pm$ 20.3*
SNP (1 mM)	228.2 $\pm$ 22.4	108.7 $\pm$ 15.6**
SNP (15 mM)	485.6 $\pm$ 29.2	141.2 $\pm$ 38.9***

solei. Zaprinast, at 27 or 243 μ M, increased the rate of glycogen synthesis in the lean Zucker solei (Figure 3). In contrast, at all concentrations investigated, zaprinast had no effect on obese Zucker soleus muscle rates of glycogen synthesis (Figure 3).

In soleus muscle preparations isolated from obese Zucker rats, the rate of glucose oxidation is markedly lower than that observed with the lean muscle (P < 0.001; Figure 4). Zaprinast, at 27 μ M, increased the rate of glucose oxidation in the soleus muscle isolated from lean Zucker rats (Figure 4). Such an effect was not observed for the obese muscle (Figure 4). In contrast, zaprinast at a concentration of 243 μ M caused a significant decrease in the rate of glucose oxidation and this effect was observed in both the lean and obese solei (Figure 4).

Solei from both lean and obese Zucker rats were isolated and incubated in either the absence or presence of zaprinast (27 μ M), before determination of cGMP content. Zaprinast, at 27 μ M, stimulated a 1.9-fold increase in cGMP levels in the lean Zucker

Figure 5 Concentration-dependent effects of SNP on the rate of net lactate release in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of SNP are indicated by * P < 0.05 and ** P < 0.01.

Figure 6 Effects of various concentrations of SNP on the rate of ¹⁴C-labelled lactate release in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of SNP are indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001.

rat solei (P < 0.05; Table 3). Zaprinast did not increase cGMP levels in soleus muscles isolated from obese Zucker rats (Table 3).

SNP effects on Zucker rat soleus muscles

The effects of SNP on the rates of net and ¹⁴C-labelled lactate release, glycogen synthesis and glucose oxidation were determined in muscle preparations isolated from both lean and obese Zucker rats. The effect of SNP incubation on muscle cGMP content was also determined. All incubations were in the presence

Figure 7 Effects of various concentrations of SNP on the rate of glycogen synthesis in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments.

Figure 8 Effects of various concentrations of SNP on the rate of glucose oxidation in incubated rat soleus muscle preparations isolated from lean and obese Zucker rats

All muscle preparations were incubated in the presence of 10 μ -units/ml insulin. Results are presented as means $\pm$ S.E.M. for at least eight separate experiments. Statistically significant differences from results from the respective lean and obese muscle incubations in the absence of SNP are indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001.

of a basal concentration of insulin (10 μ -units/ml). Incubation with SNP, at all concentrations tested (1, 5 and 15 mM), caused significant increases in the rates of net lactate release in both the insulin-sensitive (lean) and insulin-resistant (obese) Zucker rat solei (Figure 5). This SNP-stimulated increase in net lactate release was paralleled between lean and obese Zucker soleus muscles (Figure 5).

SNP significantly increased the rates of 14 C-labelled lactate release in both lean and obese muscles, at all concentrations tested (Figure 6). The magnitude of the SNP-stimulated 14 C-labelled lactate release was greater in the lean Zucker muscle than in the obese muscle. However, the relative increase in this rate was similar (i.e. at 15 mM SNP the percentage stimulations

of rates of 14 C-labelled lactate release were 139% and 100% respectively).

At all concentrations investigated, SNP had no significant effect on the rate of glycogen synthesis in muscles isolated from either lean or obese Zucker rats (Figure 7). In contrast, SNP, at concentrations of 5 and 15 mM, dose-dependently increased the rates of glucose oxidation in both the lean and obese Zucker rat soleus muscles (Figure 8). However, the absolute rate of glucose oxidation stimulation was greater in the lean muscle than in the obese muscle (Figure 8).

The effect of SNP at various concentrations (0.1, 1 and 15 mM) on cGMP generation in isolated soleus muscles from both lean and obese Zucker rats was investigated. SNP incubation significantly increased the cGMP content in soleus muscles isolated from both lean and obese Zucker rats, in a concentration-dependent fashion (Table 3). However, this SNP-stimulated increase in cGMP content was significantly less in the obese muscle than in the lean muscle (Table 3).

DISCUSSION

Contraction of skeletal muscle is a powerful but poorly understood mechanism for increasing glucose utilization [5,7]. Prior contraction of isolated incubated skeletal muscle preparations causes an increased rate of NO release [15,16]. Recent studies *in vitro* with NO donors have provided evidence that this gaseous signalling molecule stimulates glucose transport, glycolysis and glucose oxidation in skeletal muscle [8]. Inhibition of NOS, which forms NO, decreases basal glucose transport *in vitro* [15] and causes insulin resistance *in vivo* [26], although this latter effect is disputed [27]. NO activates the soluble form of guanylate cyclase [11]. The activity of guanylate cyclase in skeletal muscle is significant [28]; this enzyme is currently being characterized [29]. Thus NO, once generated by the activation of NOS within muscle cells (for example during contraction), binds to the haem group of the soluble form of guanylate cyclase, to cause elevated levels of cGMP [11]. cGMP (which is regionalized solely around the sarcolemmal membrane [10]) might mediate the stimulation in glucose utilization.

We have shown that incubation of soleus muscle preparations isolated from insulin-sensitive Wistar rats with the selective cGMP phosphodiesterase inhibitor zaprinast causes an increase in cGMP content [30]. This increase in cGMP content demonstrates the existence of not only an endogenous cGMP phosphodiesterase (because of inhibition by zaprinast) but also an intrinsic guanylate cyclase, activated by endogenously generated NO. Others have reported maximal activities for both guanylate cyclase and cGMP phosphodiesterase in various skeletal muscles [28]. Zaprinast at 27 μ M, in addition to increasing cGMP content, also stimulates the rates of net lactate release, 14 C-labelled lactate release (which is a good indicator of the rate of glucose transport [18]), glucose oxidation and glycogen synthesis in isolated incubated Wistar solei [30]. An important comparison is that, quantitatively, the effects of zaprinast and the NO donor SNP on glucose metabolism are similar. Therefore, because both NO [8] and zaprinast [30] raise intracellular levels of cGMP, this strongly suggests that this second messenger has a key role in increasing the rates of both glucose transport and utilization in skeletal muscle. Little is known about the biochemical mechanism(s) by which cGMP can activate glucose transport, glycolysis or glucose oxidation. Information from studies in other cells contributes only partly to this understanding. Indeed, inhibition of cGMP phosphodiesterase activity in cardiomyocytes stimulates the rate of transport of non-hydrolysable analogues of glucose [31]. An increase in levels of cGMP or of cGMP analogues is associated

with increases in the rate of glucose oxidation and glycolytic flux [8,32]. Future studies should be concerned with identification of the molecular mechanism(s) by which cGMP increases the rate of glucose utilization (e.g. by studying the interaction between glucose transporter translocation and key enzymes of glucose metabolism, as well as the cGMP-dependent protein kinase).

Because the NO/cGMP system seems to activate both glucose transport and utilization [8], this raises the important question of whether this system operates in insulin-resistant skeletal muscle. The maximal activity of NOS was significantly decreased in obese insulin-resistant skeletal muscle compared with lean insulin-sensitive skeletal muscle (Table 1). NOS activity is markedly decreased in insulin-resistant dystrophic skeletal muscle; differential centrifugation studies show that the activity is redistributed from the sarcolemmal membrane to the cytosol, owing to a lack of the dystrophin complex (which is in low abundance in dystrophic muscle [9]). The decreased NOS activity in obese Zucker rat gastrocnemius muscle did not result in an elevation in NOS activity in the supernatant fraction (Table 2), suggesting that decreased activity might be caused by decreased expression rather than a change in the content of the dystrophin protein complex.

One novel finding in the present study was the lack of effect of zaprinast on both cGMP content and rates of glucose utilization in soleus muscle preparations isolated from obese Zucker rats. This lack of effect of zaprinast in the insulin-resistant muscle might be explained, at least in part, by a lack of stimulation of guanylate cyclase by endogenously generated NO. Thus the decreased NOS activity in insulin-resistant skeletal muscle might mean lower basal NO production, which in turn would result in lower cGMP generation, such that inhibition of the cGMP phosphodiesterase (by zaprinast) would have little effect on intracellular cGMP levels. Consequently, this might explain why zaprinast did not affect glucose utilization in the insulin-resistant muscle (Figures 1, 2, 3 and 4).

The present study has also shown that the NO donor SNP is able to stimulate basal glucose utilization (i.e. at 10 μ -units/ml insulin) in soleus muscle preparations isolated from both insulin-sensitive lean and insulin-resistant obese Zucker rats, as shown by increased rates of net lactate release, 14 C-labelled lactate release and glucose oxidation (Figures 5, 6 and 8 respectively). SNP had no effect on basal rates of glycogen synthesis (Figure 7). What is clear is that the magnitude of the absolute rate of 14 C-labelled lactate release and glucose oxidation in response to SNP was greater in lean than in obese muscle; however, the relative percentage increases were similar. In addition, the ability of SNP to stimulate cGMP formation was diminished in the obese muscle compared with the lean muscle (Table 3). This latter observation might be explained in one of two ways, namely altered sensitivity of the soluble guanylate cyclase to NO and/or nullification of NO before it is able to activate the soluble guanylate cyclase. What might cause an altered sensitivity of guanylate cyclase to NO is not known. Fatty acids decrease soluble guanylate cyclase responsiveness to NO [33], enabling speculation that altered sensitivity of guanylate cyclase to NO in obese Zucker rat skeletal muscle might be the result of enhanced fatty acid metabolism. Other animal models of insulin resistance exhibit lower skeletal muscle guanylate cyclase activity [34,35]. NO reacts with superoxide radicals, thus lowering the effective NO available; indeed, the superoxide scavenger superoxide dismutase increases the effectiveness of NO, and can, for example, promote vascular smooth muscle relaxation *in vitro* [36]. The diabetic state is often associated with excessive superoxide radical production, leading to endothelial dysfunction and macrovascular disease [37]. Indirect evidence suggesting excessive radical production

associated with insulin-resistant skeletal muscle includes treatment of obese Zucker rats with the antioxidant lipoic acid, and this causes an improvement in insulin sensitivity [38]. Whether the increased insulin sensitivity is a result of increased effectiveness of NO has not been investigated.

Exercise is well recognized as an effective means of improving impaired glucose tolerance in humans [6]. After exercise there is a greater sensitivity of glucose metabolism to insulin [39,40], suggesting that the molecular signals that occur during contraction are necessary for optimal insulin signalling. From this we hypothesize that the NO/cGMP pathway is required for optimal insulin signalling, without the NO/cGMP pathway necessarily lying on the same signalling pathway as insulin itself. If true, then decreased activity through this optimizing NO/cGMP system would lead to decreased sensitivity to insulin (e.g. deervation [41] or physical inactivity [42]); however, stimulation of this optimizing cascade would increase sensitivity to insulin (e.g. exercise [6,39,40]). Evidence that the NO/cGMP pathway might provide this role in optimizing the conditions under which insulin signalling occurs includes a study in which drug treatment of the insulin-resistant spontaneously hypertensive (SH) rat with Tarka (combinational treatment with the ACE inhibitor tran-dolapril and the Ca^{2+} antagonist verapamil) led to both improved insulin sensitivity [43] and increased NOS activity [44] within the skeletal muscle of treated animals. In addition, skeletal muscles isolated from exercise-trained rats have increased sensitivity to insulin [40] and increased expression of NOS [17]. Conversely, treatment of rats *in vivo* with the NOS inhibitor N^G -nitro-D-arginine methyl ester resulted in insulin resistance (and hypertension) [26]. Furthermore the present study has shown that insulin-resistant obese Zucker rat skeletal muscle seems to possess multiple defects in the NO/cGMP pathway (both NOS and activation of the soluble guanylate cyclase by NO seem impaired). The possibility still remains that other components of the NO/cGMP pathway in the insulin-resistant skeletal muscle are defective. Clearly, further research is required to elucidate fully the mechanism(s) by which NO/cGMP increases glucose utilization in both rodent and human skeletal muscle, and whether this cascade must be operational to maintain maximal insulin sensitivity. Once determined, the full therapeutic benefits of manipulation of the NO/cGMP cascade towards treatment of insulin resistance syndromes can be tested.

We thank Ulrika Andersson for help with the analysis of cGMP contents in Zucker rat solei, and we thank Professor G. K. Radda for his support. M.E.Y. is a Wellcome Trust Scholar. The Zucker rats were a gift from Zeneca Pharmaceuticals.

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