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Regulation of the nuclear hormone receptor nur77 in muscle: Influence of exercise-activated pathways in vitro and obesity in vivo

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ABSTRACT

Regular physical exercise is well known to improve glucose and lipid metabolism in skeletal muscle. However, the transcription factors regulating these adaptive changes are not well-characterised. Recently the nuclear orphan receptor nur77 was shown to be induced by exercise and linked to regulation of metabolic gene expression in skeletal muscle. In this study we investigated the regulation of nur77 in muscle by different exercise-activated pathways. Nur77 expression was found to be responsive to adrenergic stimulation and calcium influx, but not to activation of the AMP dependent kinase. These results identify the adrenergic-cyclic AMP-PKA pathway to be the most potent activator of nur77 expression in muscle and therefore the likely cause of increased expression after exercise. We also identified nur77 expression to be reduced in the muscle of obese/insulin resistant rats after high fat feeding. Furthermore exposure to fatty acids, insulin or inflammation was not the cause of decreased nur77 expression in insulin resistant muscle. This suggests a reduced responsiveness to adrenergic stimulation as the likely cause of diminished nur77 expression in muscle of high fat fed rats, which has been observed in obese/insulin resistant individuals. Our results suggest adrenergic stimulation as the most important stimulus for nur77 expression and point to a significant role for this transcription factor in adaptive changes in muscle after exercise and in insulin resistant states.

1. Introduction

Skeletal muscle represents approximately 40% of body mass [1] and accounts for 20–30% of total resting oxygen uptake [2,3] which can increase to 90% during maximal physical activity [4]. Muscle is the major site of glucose and lipid metabolism and dysregulation of either of these metabolic pathways can contribute to the development of metabolic diseases such as type 2 diabetes and cardiovascular complications [5].

It is well established that regular physical exercise improves glucose homeostasis and increases capacity for lipid oxidation in skeletal muscle [6–9]. The beneficial consequences of exercise include acute effects, as well as chronic adaptations in skeletal muscle which both impinge on insulin action. Whereas the acute benefits of exercise most likely revolve around utilising excess energy stores, the chronic adaptations involve coordinated regulation of specific gene expression [10]. Therefore exercise-induced changes in transcriptional regulators in skeletal muscle are of great interest in helping to understand the long term beneficial effects of exercise.

Recently the nuclear orphan receptor Nr4a1, also known as nur77, was reported to be induced after exercise bouts in human skeletal muscle [11]. Interestingly, nur77 was also shown to be a transcriptional regulator of genes linked to glucose as well as lipid metabolism in muscle [12,13].

The Nr4a subfamily of nuclear receptors consists of three members: Nr4a1 (nur77), Nr4a2 (nurr1) and Nr4a3 (nor1). All three are immediate early genes reported to be induced in various tissues by a variety of stimuli, including growth factors, cytokines, hormones, fatty acids and physical stress [14–23]. Nr4a receptors are involved in diverse tissue specific functions such as T-cell apoptosis [24], inflammatory gene expression in macrophages [25], gluconeogenesis in the liver [26] and regulation of thermogenic capacity in brown adipose tissue [18,27]. However the regulation of nur77 expression in skeletal muscle is not well understood.

In this study we have investigated the regulation of nur77 expression in muscle cells by exercise-activated signalling pathways (adrenergic, calcium, and AMP dependent kinase (AMPK)). Furthermore we have examined regulation of nur77 expression in muscle of animals stimulated in vivo with a high fat diet, acute

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lipid infusion, insulin infusion or infusion of the AMPK activator AICAR.

2. Materials and methods

2.1. Reagents

Isoproterenol, palmitate, oleate, linoleate, LPS and insulin were purchased from Sigma-Aldrich (Carlsbad, USA). AICAR was obtained from Toronto Research Chemicals (Canada) and ionomycin, and protein kinase A inhibitor 6-22 amide (PKAi) were from Calbiochem (San Diego, USA).

2.2. Cell culture

L6 cells were maintained in alpha-MEM (Invitrogen, Carlsbad, USA) supplemented with antibiotics and 10% FBS (Sigma-Aldrich, Carlsbad, USA). At 70–80% confluence medium was changed to alpha-MEM supplemented with 2% FBS to initiate differentiation. At 7 days post-differentiation cells were incubated with the respective substance for the indicated time. Fatty acids were first coupled to BSA (10%) and then further diluted in differentiation medium resulting in a final BSA concentration of 2%.

2.3. Animal studies

Male Wistar rats weighing ~250 g were purchased from the animal resources center (Perth, Australia). The animals were kept in a temperature-controlled room (22 ± 1 °C) on a 12-h light/dark cycle with free access to food and water. Rats were fed ad libitum for a period of 3 weeks with a standard lab diet (8% calories from fat, 21% calories from protein, and 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Specialty Stock Feeds, Yanderra, Australia) or with a high fat diet (45% of calories from fat, 20% calories from protein, and 35% calories from carbohydrates, 4.7 kcal/g, based on rodent diet no. D12451; Research Diets, New Brunswick, NJ). All experiments were carried out with the approval of the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee, following guidelines issued by the National Health and Medical Research Council of Australia. Lipid infusion, euglycemic hyperinsulinemic glucose clamps and AICAR infusions were performed as described elsewhere [28–30].

2.4. RNA isolation and quantitative real-time PCR

After stimulation cells were harvested directly in TRI-reagent (Sigma-Aldrich, Carlsbad, USA) and RNA was isolated according to the manufacturer's instructions. For the animal experiments tissue samples were snap frozen in liquid nitrogen immediately after dissection and 50 mg tissue was used for RNA extraction with TRI-Reagent. RNA integrity was evaluated on agarose gels and concentration was spectrophotometrically determined using a Nanodrop spectrophotometer. RNA was transcribed into cDNA with random primers (New England Biolabs, Ipswich, USA) using the cDNA synthesis kit from Qiagen (Mississauga, ON). Taqman probes (Rn00577766_m1; Applied Biosystems) were employed to quantify nur77 expression and normalized to 18 s-rRNA expression. PCR runs were performed on an ABI 9600HT cycler using Taqman Gene Expression Mastermix (Applied Biosystems, Foster City, USA) with the standard PCR protocol as recommended by the manufacturer. A standard curve, consisting of serial dilutions of pooled cDNA samples, was measured with every PCR run to quantify nur77 transcript.

2.5. Statistical analysis

Values are given as means ± standard error from at least three independent experiments, which were performed in duplicate or triplicate. Significance was tested for by Student's *t*-test or two-way ANOVA followed by Holm–Sidak post-hoc test and *p*-values lower than 0.05 were considered to be significant.

3. Results

3.1. Nur77 expression in response to increases in cAMP or intracellular calcium levels and activation of AMPK

Exercise involves stimulation of the adrenergic-cyclic AMP-PKA (protein kinase A), calcium signalling and AMPK pathways. Therefore we applied agonists of these pathways to differentiated L6 myotubes and examined the effect on nur77 gene expression. L6 myotubes were treated with the beta-adrenergic pan-agonist isoproterenol, the beta-2 agonist salbutamol and the activator of adenylyl cyclase forskolin for up to 24 h and nur77 mRNA expression was measured. There was a rapid but transient increase in nur77 expression following all treatments with a peak of expression (~200-fold) after 1 h returning to basal levels after 4–6 h (Fig. 1). This confirmed similar measurements in C2C12 cells [12,13] and gave an indication of the time course of nur77 expression in L6 myotubes. Because these agents were added to the cells with a change of media, the effect of a media change on nur77 expression was also examined. As previously observed in adipocytes [31] changing the media of cultured muscle cells also increased the expression of nur77 by several fold, however this increase was much less than that brought about by addition of media containing agonists of the adrenergic signalling pathway (Fig. 1). In all subsequent experiments in muscle cells isoproterenol treatment (100 nM) was included as a positive control and the effect of a media change (from here on referred to as control) was used for comparison to decide if any effects were specific for the added agonist.

To examine the effects of an increase in calcium flux on nur77 expression, L6 myotubes were treated with the ionophore ionomycin and nur77 transcript measured for 4 h after stimulation. Nur77 expression was significantly increased following ionomycin treatment at 1 h and was still above basal levels after 4 h of stimulation (Fig. 2). Compared to the effect of isoproterenol however, only a very mild induction of nur77 expression was observed. To exclude activation of PKA by ionomycin [32] as a possible cause for increased nur77 expression we used a peptide inhibitor of PKA (PKAi) [33]. Inhibition of PKA had no effect on the induction of nur77 by ionomycin (Fig. 2).

Next the effect of stimulation of AMP dependent kinase (AMPK) (which is also activated during exercise), on the regulation of nur77

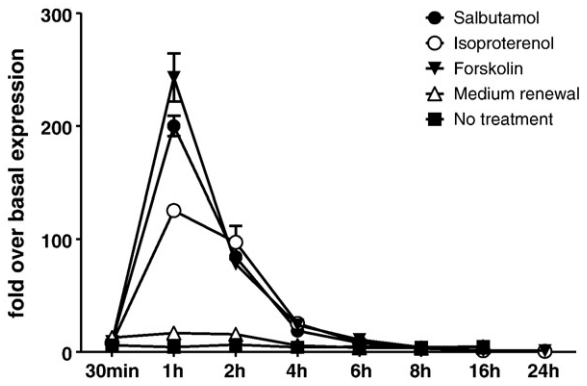

Fig. 1. Nur77 mRNA in L6 myotubes in response to adrenergic stimulation. L6 myotubes were stimulated with the beta-adrenergic pan-agonist isoproterenol (100 nM), the beta-2-adrenergic agonist salbutamol (200 nM) and the adenylate cyclase activator forskolin (10 μM) in a time course experiment for up to 24 h. The effect of medium renewal at time zero on nur77 gene expression was also investigated. Nur77 transcript is expressed as fold over basal expression. The results are mean values of three independent experiments ± standard error.

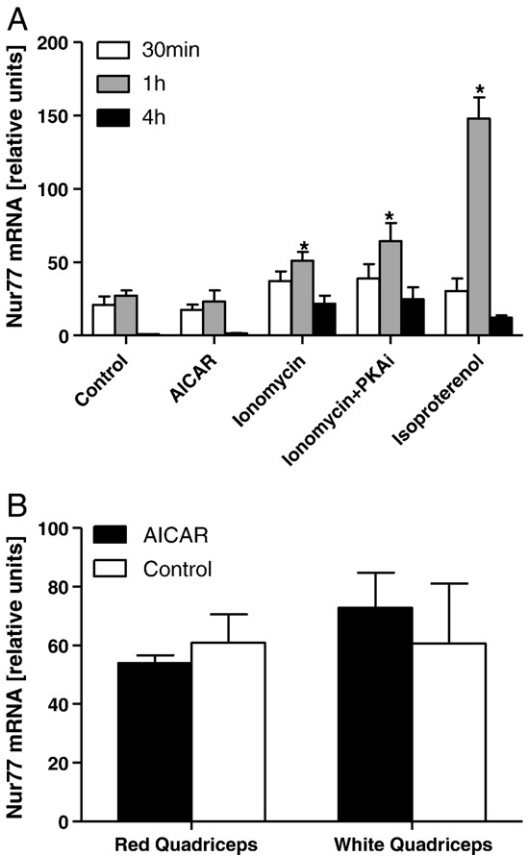

Fig. 2. Effect of AMPK activation and calcium influx on nur77 expression in L6 myotubes. (A) Nur77 gene expression in L6 myotubes after AICAR (2 mM) or ionomycin (1 μM) stimulation. STO-609 (10 μM) and PKA inhibitory peptide (PKAi;10 μM) were used in combination with ionomycin. Isoproterenol (100 nM) was used as positive control. Values are means of 5 independent experiments with the exception of the inhibitors which were analysed in three independent experiments ± standard error. (B) Red and white quadriceps muscles were analysed for nur77 expression after 2 h of AICAR or saline (control) infusion. Results are mean values of 6 (AICAR) or 4 (control) animals ± standard error. Statistical significance ($p<0.05$) was tested with two-way ANOVA and Holm–Sidak post-hoc test or Student's *t*-test.

gene expression was tested. L6 myotubes were incubated with the AMPK activator AICAR for up to 4 h. We observed no changes in nur77 expression above those caused by replacing the media in response to AMPK activation within 4 h (Fig. 2).

In order to compare our findings in L6 myotubes with the in vivo situation we infused rats with AICAR. After 2 h of infusion, rats were sacrificed and red and white quadriceps muscles were snap frozen. Quantification of nur77 transcript revealed no significant changes in gene expression within the red or white quadriceps muscle with AICAR infusion (Fig. 2B).

3.2. Nur77 expression in skeletal muscle of high fat fed rats

Our previous experiments suggested that nur77 expression is most responsive to beta-adrenergic stimulation and thereby to activation of the cAMP-PKA pathway. It is reported that the response to adrenergic stimulation is blunted in the muscle of obese/insulin resistant individuals [34–37]. Therefore nur77 expression in skeletal muscle of rats after high fat feeding, a well-characterised model of fat accumulation and insulin resistance was investigated [38].

After 3 weeks of feeding a high fat diet rats were sacrificed and tibialis anterior, red and white quadriceps muscles were dissected and snap frozen. In high fat fed rats the expression of nur77 was significantly reduced compared to chow fed controls (~50%). This effect was of a similar extent in all three muscle groups analysed (Fig. 3).

3.3. Effect of insulin, fatty acids and lipopolysaccharide (LPS) on nur77 expression in L6 myotubes

Insulin, fatty acids and inflammation (LPS) all have an impact on metabolic regulation of skeletal muscle and are increased in obese/insulin resistant individuals. These agents also induce nur77 expression in adipocytes [39], pancreatic beta-cells [23] and macrophages [21], respectively. Therefore we treated L6 myotubes with these substances to investigate any effect they may have on nur77 expression in muscle cells.

L6 myotubes were treated with 100 nM insulin and RNA was isolated from samples taken after 30 min, 1 h and 4 h. Isoproterenol treatment was included as positive control. Analysis revealed no significant induction of Nur77 mRNA in insulin-treated cells above the levels of control cells (Fig. 4A).

Next the effect of the fatty acids oleate, palmitate and linoleate on nur77 expression in L6 myotubes was investigated. Stimulation of up to 16 h did not result in any changes in nur77 transcript above the levels of control cells (Fig. 4B). Stimulation of muscle cells with the pro-inflammatory agonist LPS over the same time course also failed to raise nur77 expression above control cells although stimulation with isoproterenol as a positive control rapidly increased nur77 expression as expected (Fig. 4B).

Furthermore we investigated if fatty acids might prevent isoproterenol induced nur77 in L6 myotubes and therefore stimulated cells with isoproterenol in combination with oleate, palmitate or linoleate. None of the tested fatty acids did alter isoproterenol induced nur77 expression (Fig. 4C).

3.4. Effect of insulin and lipid infusion on nur77 expression in skeletal muscle of rats

Unstimulated L6 myotubes have very low levels of nur77 mRNA compared to skeletal muscle, most likely because cultured myotubes lack innervation. This makes it difficult to use myotubes to test if various stimuli reduce basal expression of nur77. Therefore we also looked for possible effects of insulin and fatty acids on nur77 expression in vivo. We used rats infused with insulin or lipids to investigate the effect on nur77 expression in skeletal muscle.

First we analysed tissue from chow or high fat fed rats that were stimulated with insulin during a euglycemic hyperinsulinemic clamp. After approximately 2 h of insulin infusion under euglycemic conditions the rats were sacrificed and nur77 expression in the tibialis anterior muscle was analysed. In line with our findings in L6

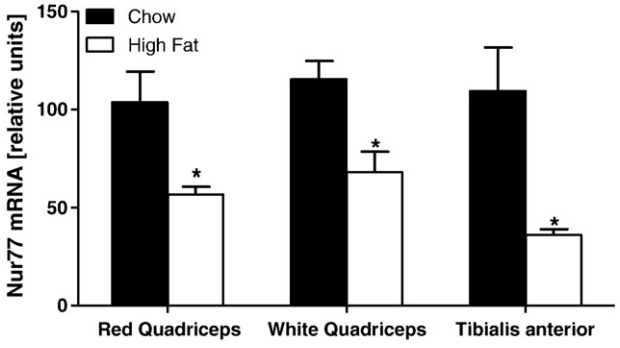

Fig. 3. Effect of high fat diet feeding on nur77 expression in skeletal muscle of rats. After 3 weeks of high fat diet feeding nur77 expression was analysed in the red and white quadriceps and the tibialis anterior muscle. Results are mean values of 6 animals (tibialis $n=3$) ± standard error. Statistical significance $p<0.05$ was tested with Student's *t*-test.

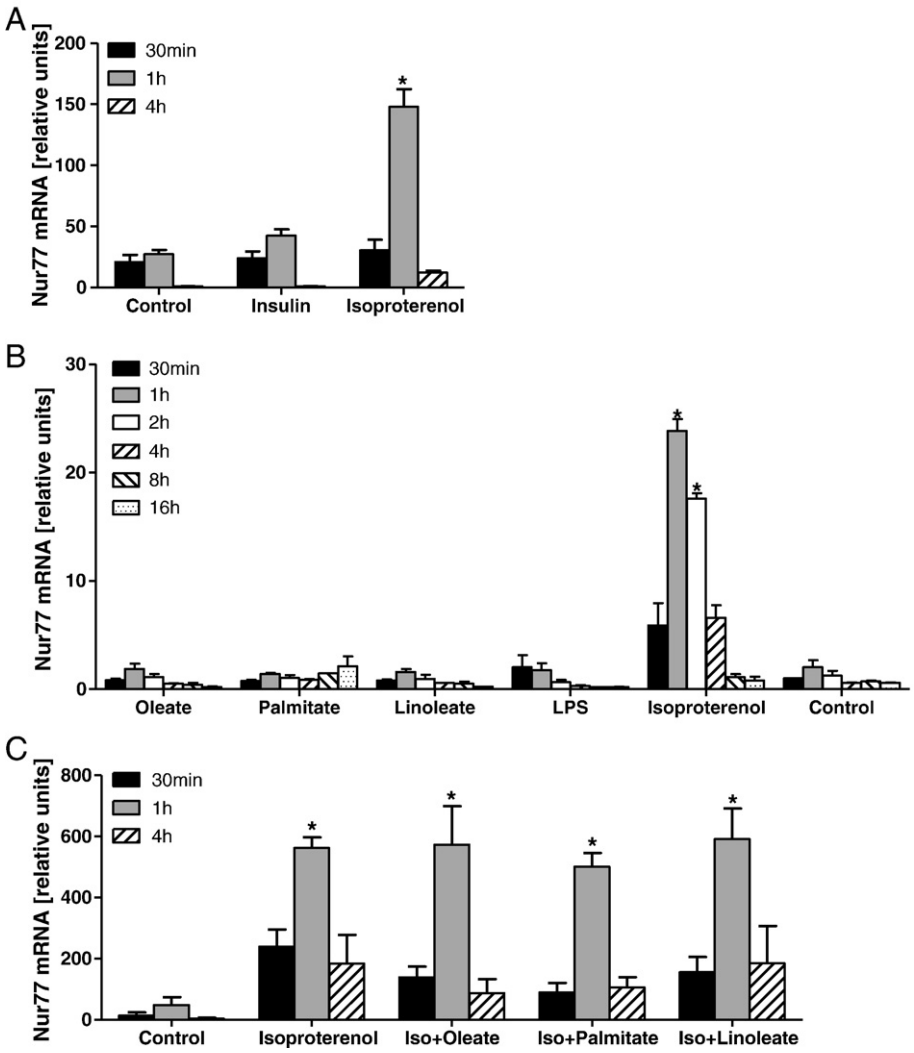

Fig. 4. Nur77 expression in L6 myotubes in response to insulin, fatty acids and LPS. L6 myotubes were stimulated for the indicated time and nur77 mRNA was quantified by qRT-PCR. (A) Stimulation with insulin (100 nM) for up to 4 h. ($n=4$; $p<0.05$ by two-way ANOVA and Holm–Sidak post-hoc test). (B) Stimulation with oleate (0.5 mM), palmitate (0.5 mM), linoleate (0.5 mM), LPS (1 μg/ml) or isoproterenol (100 nM) was performed for up to 16 h. (C) Effect of fatty acids on isoproterenol induced nur77 expression. Results are mean values of three independent experiments. $p<0.05$ by two-ANOVA and Holm–Sidak post-hoc test.

myotubes we could not detect differences in nur77 gene expression between insulin stimulated and control rats in the tibialis anterior muscle (Fig. 5A).

Finally we infused rats with intralipid/heparin to acutely raise fatty acids in the circulation and compared them with glycerol infused animals. After 3 h of intralipid/heparin or glycerol infusion the animals were sacrificed and the red quadriceps muscle was dissected and snap frozen. Analysis of nur77 expression revealed no significant differences between intralipid/heparin and glycerol infused rats (Fig. 5B).

4. Discussion

A wide variety of stimuli have been reported to increase nur77 expression in different non-muscle cell lines and tissues [14–23]. The only known stimulus to induce nur77 expression in muscle cells is beta-adrenergic stimulation or any other treatment which increases cAMP levels [12,13,40,41]. Physical exercise is the only physiological condition reported to induce nur77 in skeletal muscle [11].

Nur77 was recently reported to be involved in the regulation of glucose and lipid metabolism in muscle [12,13]. Dysregulation of these two metabolic pathways is a hallmark of the cluster of clinical conditions referred to as the metabolic syndrome (insulin resistance, type 2 diabetes, dyslipidemia, hypertension, obesity) and interestingly all these conditions are improved by physical exercise [6–9]. In fact, lifestyle intervention consisting of increased physical exercise and moderate calorie intake is considered one of the most effective treatments for the metabolic syndrome [8].

We examined three different signalling cascades activated by exercise in skeletal muscle (adrenergic stimulation, calcium influx and AMPK activation). Beta-adrenergic stimulation or increases in cAMP levels mediated by substances such as forskolin were found to be very potent activators of nur77 expression in skeletal muscle cells. It is well documented that the PKA target cAMP response element binding protein (CREB) is critical for the induction of nur77 expression in different tissues [40,42,43].

Interestingly we found nur77 expression in muscle also to be responsive to increased intracellular calcium concentrations. Calcium influx is known to activate calmodulin dependent kinases (CamKs) as well as classical PKCs (protein kinase C). Both can phosphorylate and thereby activate CREB [44,45]. We excluded activation of PKA by ionomycin treatment as the cause for increased nur77 expression as PKA inhibition did not prevent ionomycin induced nur77 expression. This points towards CamK or PKC as activators of nur77 expression in muscle cells.

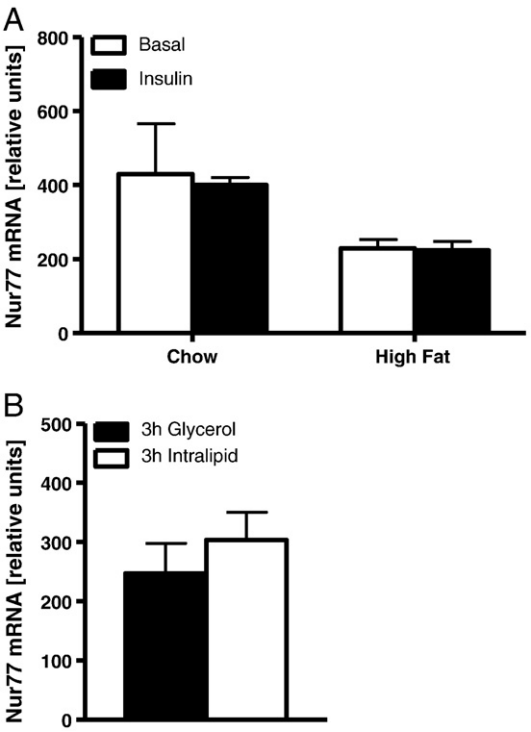

Fig. 5. Effect of insulin and lipid/heparin infusion on nur77 gene expression in rat skeletal muscle. (A) After 2 h of insulin infusion during an euglycemic hyperinsulinemic glucose clamp (insulin) nur77 expression in the tibialis anterior muscle was measured in chow or high fat fed rats and compared with control animals (basal). Results are mean values of four animals (6 for high fat) ± standard error; (B) Nur77 expression was analysed in the red quadriceps muscle of rats after 3 h of intralipid/heparin infusion or glycerol infused control rats. Results are mean values of 4 (intralipid) and 3 (glycerol) animals ± standard error.

Another exercise-activated pathway that could potentially influence nur77 expression in muscle is the AMPK pathway. AMPK has been reported to directly phosphorylate and activate CREB in liver and muscle cells [46]. Since CREB is the main transcription factor driving nur77 expression we were surprised that AMPK activation had no effect on nur77 expression. However, these in vitro results were replicated in AICAR-infused rats, which showed no change in nur77 expression in skeletal muscle compared to saline infused rats. Therefore it appears that AMPK activation is unlikely to be the stimulus for increased nur77 expression in skeletal muscle during exercise.

Overall our findings suggest that activation of PKA via increases in cAMP is the most potent activator of nur77 expression in skeletal muscle. The main stimulus activating PKA in skeletal muscle in vivo is beta-adrenergic stimulation. The two sources of catecholamines in muscle are noradrenaline released from nerve endings and circulating adrenaline from the adrenal glands. Bilateral adrenalectomy is known to reduce the levels of adrenaline in skeletal muscle almost to zero [47]. Interestingly we have found that nur77 gene expression is downregulated approximately 3-fold in muscle of adrenalectomised rats as compared to sham operated animals (D. Proctor, T. Kanzleiter and G. Cooney unpublished observations). These findings support the notion that nur77 expression is mainly regulated by beta-adrenergic stimulation in skeletal muscle.

There is a growing body of literature suggesting that although in obese insulin resistant individuals the overall activity of the sympathetic nerve system (SNS) is increased, there is a blunted responsiveness to beta-adrenergic stimulation (presumably via a decrease in beta-adrenoreceptors [36]) in adipose tissue and skeletal muscle [34,35,48–50] (recently reviewed in [51]).

Therefore to determine if nur77 expression may be altered in association with insulin resistance we examined muscle from high fat fed rats. Basal nur77 expression was significantly reduced in muscles of high fat fed insulin resistant rats as compared to chow fed controls. Given the fact that high fat feeding of rats results in SNS hyperactivity, as evidenced by increased noradrenaline turnover and/or circulating levels [37,52,53], it is tempting to hypothesise that basal nur77 expression is reduced in insulin resistance due to decreased responsiveness to beta-adrenergic stimulation. Indeed denervation of skeletal muscle is known to induce insulin resistance [54,55] and was recently shown to diminish nur77 expression [13]. Furthermore in other rodent models of obesity and insulin resistance (*ob*/*ob* and *db*/*db* mice as well as Zucker rats) nur77 expression in muscle is reduced [39].

However, obesity and insulin resistance is accompanied by elevated levels of insulin and fatty acids, as well as subclinical chronic inflammation. Therefore we also examined a possible effect of these factors on nur77 expression, particularly as in previous studies nur77 was reported to be induced by insulin [56], by the pro-inflammatory agent LPS [21] and by fatty acids [23] in different cell types.

First we examined whether nur77 expression in skeletal muscle could be regulated by insulin as reported by Wu et al. [56]. In contrast to the results of Wu et al. we did not detect a change in nur77 expression in L6 myotubes stimulated with insulin within 4 h. In order to exclude any effects insulin might have on nur77 expression after prolonged exposure we extended our time course to 24 h but still could not observe any changes in nur77 expression (data not shown). To further confirm our results we measured nur77 expression in skeletal muscle of chow or high fat fed rats after a 2 h infusion with insulin during a hyperinsulinemic euglycemic clamp. Consistent with our in vitro results we could not find changes in nur77 expression in skeletal muscle in response to insulin stimulation.

Stimulation of L6 myotubes with the saturated fatty acid palmitate, the monounsaturated fatty acid oleate, the polyunsaturated fatty acid linoleate or the pro-inflammatory agonist LPS did not result in a change in nur77 expression. The fatty acids also failed to affect isoproterenol induced nur77 expression in myotubes. This observation in an in vitro system was also confirmed in vivo where no change in nur77 expression was seen in muscle from rats which had been infused for 3 h with intralipid/heparin to raise circulating fatty acids compared to glycerol infused rats as controls. Collectively our findings suggest that insulin, fatty acids and LPS have little direct effect on nur77 expression in vitro and in vivo and this supports the hypothesis that a blunted responsiveness to adrenergic stimulation may be the cause for diminished nur77 expression in skeletal muscle of high fat fed rats.

In summary, our results show nur77 expression in muscle cells to be markedly induced by stimuli activating the cAMP-PKA-CREB pathway and to a lesser extent by increases in intracellular calcium levels presumably via classical PKCs. Therefore we speculate that increases in nur77 expression in response to exercise are mainly mediated via activation of the adrenergic system. Furthermore we observed that basal nur77 expression was significantly reduced in skeletal muscle of high fat fed, insulin resistant, rats. We excluded several other potential factors (insulin, fatty acids and inflammation) as being responsible for the changes in nur77 expression and considering the findings from this study and others [13,39], we hypothesise that the decrease in nur77 observed in insulin resistant rats is probably due to decreased adrenergic responsiveness of the muscle. Given the recent reports linking nur77 to the regulation of glucose and lipid metabolism in skeletal muscle [12,13], further research into the physiological function and dysregulation of nur77 in muscle are warranted.

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