

## Quantitative Analysis of Orphan Nuclear Receptors in Insulin-Resistant C2C12 Skeletal Muscle Cells

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### Abstract

Orphan nuclear receptors (ONRs) are members of the nuclear receptor (NR) super family of transcription factors that have been known to play a major role in lipid and glucose metabolism in skeletal muscle. Recently, pharmacological evidence supports the view that stimulation of NR alleviates Type 2 Diabetes (T2D). However the ligands and physiological functions of ONRs still remain unknown. To date, no systematic studies have been carried out to screen for ONRs expressed in insulin resistant skeletal muscle cells. Therefore, in this study, we have established a model for insulin resistance (IR) by treating C2C12 skeletal muscle cells with insulin (10nM) for 48 hours. Western Blot analysis of phosphorylated AKT confirmed IR. By quantitative PCR, we identified that a number of the ONRs respond significantly during the progression of cellular insulin resistance which included Coup-tf1, Coup-tf2, Ppar $\beta$ , NR4As, Reverba, and Rora, some of which have been associated with fatty acid oxidation regulation and glucose homeostasis and therefore could play a role in the aetiology of this disorder. Highlighted were observed increased mRNA expression levels of other ONRs in insulin resistant C2C12 skeletal muscle cells, indicated that these ONRs could potentially play a pivotal regulatory role of insulin secretion in lipid metabolism. Taken together, this study has successfully contributed to the analysis of ONRs in IR, and has filled in an important void in the study of these receptors as potential targets in the pharmacological treatment of this disorder.

**Keywords:** Orphan nuclear receptors; Transcription factors; Type 2 Diabetes; Insulin resistance

### Introduction

Orphan nuclear receptors (ONRs) are members of the nuclear receptor superfamily of transcriptional factors that are implicated in a number of metabolic processes including lipid and glucose metabolism and energy expenditure [1]. Unlike the steroid hormone receptors and other transcription factors of the nuclear receptor superfamily, the ligands for the ONRs are unknown and suggests that there is a host of other signalling pathways that are regulated by undiscovered ligands [2]. This may have wide-reaching implications in many disease states and processes. In particular, the ONRs play a critical role in glucose and lipid metabolism, and energy expenditure [1,3,4] and thus, are currently under investigation for their role in metabolic disease. Accordingly, several ONRs have been examined for their roles in insulin resistance and type 2 diabetes. For example, the liver X receptor (now an adopted ONR due to the identification of a ligand) global knock-out mouse model shows improved muscle, hepatic and adipose tissue insulin sensitivity [5]. Moreover, the PPARs (another class of adopted ONRs) have received much attention as potential pharmacological targets for combating obesity and diabetes due to their important role in cell metabolism (specifically lipid metabolism) regulation [6] and amelioration of insulin resistance in skeletal muscle and liver [7-9].

Although there have been a number of studies on the role of ONRs in insulin resistant mouse models, there is no information, to our knowledge, on the regulatory response of the ONRs in a progressive insulin-resistance skeletal muscle cell system. Accordingly, in this study we aimed to address the regulation of several ONRs in an insulin-resistant mouse C2C12 skeletal muscle. We identified that a number of the ONRs respond significantly during the progression of cellular insulin resistance and therefore could play a role in the aetiology of this disorder.

### Experimental

#### Cell culture and the creation of an insulin-resistant skeletal muscle cell system

Skeletal muscle C2C12 cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (HI-FBS) in the presence of 1 mM sodium pyruvate, 0.1 mM non-essential amino acid and 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The cells were maintained at 37°C in an incubator of 5% (v/v) CO<sub>2</sub> atmosphere. The cell culture medium was replaced every three days.

C2C12 myoblast cells were seeded into 6-well plates and allowed to grow until the cells reached 70% confluence before differentiation into skeletal muscle cells with 2% horse serum for 5 days. Prior to treatment with insulin, the cells were washed twice with PBS and pre-incubated for 4 hr in medium containing 0.5% (v/v) HI-FBS. The medium was then removed and replaced with fresh medium with 0.5% (v/v) HI-FCS in the presence of IL-6 and incubated at 37°C in a humid atmosphere of air containing 5% (v/v) CO<sub>2</sub> for the requisite time. For the development of insulin-resistant skeletal muscle phenotype, C2C12 cells were exposed to chronic insulin (10 nM) treatments over the series of 0 h, 0.5 h, 2 h, 4 h, 8 h, 24 h, and 48 h (12). Media was refreshed every eight

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hours to avoid depleting media components and insulin. For protein phosphorylation detection, 10 nM insulin was added for 30 min before cell lysates harvest at each time point [10]. For control samples, fresh medium with 0.5% (v/v) HI-FCS, without insulin, was used. Following completion of the incubation period, the cells were harvested for RNA isolation and protein extract.

### Isolation of total cellular RNA

Total cellular RNA was isolated from cells cultured in 6-well tissue culture plates using TRIzol Reagent (Ambion®) according to the manufacturer's instruction. Briefly, cells were first rinsed twice with PBS and then lysed on the addition of 1ml of Tri-Reagent. The homogenate was then incubated at room temperature for 5 min followed by vortexing in 100 µl of chloroform. The RNA was extracted by centrifugation at 12,000 x g for 15 min at 4°C followed by precipitation with isopropanol. The concentration and purity of the isolated RNA was determined by measuring the absorbance at 260 nm and 280 nm using NANODROP 2000 (Thermo Scientific®).

### Synthesis of cDNA and Real-Time PCR

Two µg of total RNA was reverse transcribed into cDNA with the High Transcriptase cDNA kit (Life Technology®). Quantitative Real-Time PCR was carried out using SYBR<sup>®</sup> Green (BioLine®) according to the manufacturer's instructions. The level of gene expression was then measured using Vii7 qPCR machine (Life Technologies®). Specifically, 25 ng of cDNA was added to 5 µl of SYBR<sup>®</sup> Green RT-PCR reaction mix (BioLine®), 10 mM each of the forward primer and reverse primer (Geneworks®) (Table 1), and 0.5 µl of nuclease free water in a

final volume of 10 µl. Quantitative Real-Time PCR was then performed on a ViiA7 qPCR machine (Life Technologies®) using the following method: PCR reaction protocol consisted 40 cycles of denaturation at 95°C for 20 sec; annealing at 60°C for 30 sec, and an extension at 72°C for 30 sec. This was followed by the melt curve analysis which was carried out at 95°C for 1 min, 55°C for 1 min and 40 cycles of 70°C for 10 sec, increasing by 0.5°C each cycle. PCR products were normalized against the housekeeping gene, eukaryotic elongation factor 2 (*Eef2*).

### Western blot analysis

Protein extracts used in Western blot analysis were extracted using RIPA buffer® (Ambion®) containing Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific®) according to the manufacturer's instructions. Protein samples (20 µg) were used in SDS-PAGE in order to determine the levels of protein content for phosphorylated, Tyr30 AKT and total AKT, respectively. Subsequently, proteins were transferred to a polyvinylidene difluoride membrane (Milipore®) and incubated with blocking solution (1X PBS containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20) for 1 h at room temperature with shaking. The membrane was washed three times for 10 min each in washing solution (1X PBS and 0.1% (v/v) Tween-20) and incubated with primary antibodies (rabbit anti-mouse Tyr30 AKT, and total AKT), which was diluted 1/1000 in 1X PBS containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20, for 1 h at room temperature. The membrane was then washed and immersed in secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) diluted 1/2000 in 1X PBS containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20. Detection of membrane-bound antigen-antibody complexes as immunoreactive signals was detected

NR	Time						
	0	0.5	2	4	8	24	48
	Relative Expression against EEF2 ± SD						
<i>Coupt-1</i>	126.0 ± 5.5	85.8 ± 0.90	99.09 ± 5.90	88.07 ± 2.26	83.44 ± 4.70	<b>20.57 ± 2.90<sup>***</sup></b>	89.91 ± 4.27
<i>Coup-tf2</i>	564.12 ± 17.89	409.81 ± 19.57	597.64 ± 8.57	571.62 ± 11.71	434.74 ± 45.03	<b>111.52 ± 3.85<sup>***</sup></b>	293.08 ± 16.33
<i>Ppar α</i>	1.28 ± 0.066	1.61 ± 0.203	1.45 ± 0.070	1.30 ± 0.150	1.56 ± 0.110	2.20 ± 0.45	0.855 ± 0.03
<i>Ppar β</i>	635.60 ± 40.23	498.06 ± 40.11	746.39 ± 165.80	683.01 ± 32.40	549.11 ± 12.56	<b>1515.96 ± 38.22<sup>***</sup></b>	574.52 ± 28.47
<i>Ppar γ</i>	43.31 ± 3.80	27.10 ± 9.79	44.61 ± 2.78	36.33 ± 0.38	26.04 ± 1.57	34.52 ± 2.29	33.61 ± 2.45
<i>Nor-1</i>	13.98 ± 1.71	18.20 ± 0.83	<b>153.37 ± 7.89<sup>***</sup></b>	22.63 ± 1.50	61.82 ± 3.38	<b>487.34 ± 14.40<sup>***</sup></b>	74.32 ± 0.95
<i>Nurr1</i>	5.83 ± 0.53	14.15 ± 1.48	<b>67.63 ± 2.60</b>	10.19 ± 2.5	21.0 ± 2.38	<b>108.6 ± 3.11<sup>**</sup></b>	8.93 ± 1.24
<i>Nurr77</i>	44.12 ± 0.58	<b>574.41 ± 68.73<sup>***</sup></b>	<b>1084.83 ± 53.97<sup>***</sup></b>	26.08 ± 0.17	104.26 ± 4.18	<b>504.54 ± 8.01<sup>***</sup></b>	44.35 ± 3.0
<i>Reverb α</i>	160.69 ± 10.61	155.23 ± 3.35	151.85 ± 6.21	154.14 ± 1.95	<b>530.33 ± 5.18<sup>***</sup></b>	<b>1080.14 ± 48.62<sup>***</sup></b>	141.07 ± 6.21
<i>Reverb β</i>	0.25 ± 0.072	0.17 ± 0.04	0.17 ± 0.06	0.20 ± 0.11	0.32 ± 0.15	0.50 ± 0.20	0.35 ± 0.16
<i>Rar α</i>	148.43 ± 18.60	125.63 ± 15.35	104.27 ± 14.48	164.10 ± 16.14	183.88 ± 6.41	203.49 ± 3.80	127.75 ± 9.45
<i>Rar β</i>	4.40 ± 0.18	4.06 ± 0.57	3.96 ± 0.15	3.62 ± 0.53	8.05 ± 0.59	7.15 ± 0.74	8.8 ± 0.82
<i>Rar γ</i>	16.39 ± 2.5	9.35 ± 0.66	12.31 ± 1.74	16.79 ± 3.85	14.13 ± 1.30	<b>6.51 ± 1.02<sup>*</sup></b>	17.90 ± 0.32
<i>Err α</i>	132.65 ± 5.62	114.19 ± 5.08	111.96 ± 14.25	167.08 ± 1.65	193.07 ± 4.78	<b>395.98 ± 13.96<sup>***</sup></b>	163.63 ± 4.25
<i>Err β</i>	0.70 ± 0.35	0.38 ± 0.10	0.77 ± 0.07	0.92 ± 0.01	0.59 ± 0.08	0.76 ± 0.14	0.43 ± 0.05
<i>Lxr α</i>	88.43 ± 130.76	84.88 ± 16.73	168.43 ± 45.73	87.37 ± 20.62	152.99 ± 31.17	232.36 ± 15.76	165.51 ± 2.54
<i>Lxr β</i>	6.48 ± 2.35	6.37 ± 1.22	7.26 ± 1.58	7.26 ± 0.15	7.50 ± 1.01	12.06 ± 1.29	9.53 ± 0.32
<i>Rxr α</i>	631.83 ± 40.54	<b>321.42 ± 18.66<sup>**</sup></b>	414.05 ± 10.14	432.84 ± 19.63	525.97 ± 16.29	497.06 ± 36.49	612.73 ± 33.02
<i>Rxr β</i>	50.97 ± 7.01	39.87 ± 3.08	39.28 ± 1.73	50.38 ± 6.31	85.94 ± 0.50	57.64 ± 12.24	63.78 ± 3.84
<i>Rxr γ</i>	2.21 ± 2.31	1.57 ± 1.18	1.62 ± 0.99	1.59 ± 0.46	3.47 ± 3.6	4.57 ± 1.91	3.28 ± 0.30
<i>Ror α</i>	684.70 ± 77.43	386.92 ± 62.60	660.44 ± 59.14	457.74 ± 39.44	927.52 ± 109.80	<b>1900.72 ± 242.43<sup>***</sup></b>	852.56 ± 101.46
<i>Ror γ</i>	11.81 ± 2.25	8.70 ± 0.44	12.44 ± 4.38	6.62 ± 1.06	7.80 ± 2.09	<b>3.54 ± 0.53<sup>*</sup></b>	24.41 ± 6.19
<i>Hnf4 α</i>	2.19 ± 0.66	1.84 ± 0.73	2.36 ± 0.39	1.61 ± 0.29	1.45 ± 0.32	1.41 ± 0.24	2.15 ± 0.58
<i>Hnf4 γ</i>	33.79 ± 6.41	23.00 ± 1.40	37.19 ± 7.05	46.10 ± 3.02	27.27 ± 2.56	16.15 ± 2.14	34.48 ± 4.68
<i>Ear 2</i>	219.86 ± 42.03	186.17 ± 5.74	251.13 ± 16.12	250.40 ± 2.59	205.03 ± 7.82	77.06 ± 1.57	225.35 ± 15.61
<i>Shp</i>	0.16 ± 0.06	0.11 ± 0.01	0.23 ± 0.09	0.20 ± 0.12	0.06 ± 0.02	0.28 ± 0.03	0.29 ± 0.05

Table 1: Quantitative real-time PCR of ONRs in C2C12 cells treated with 10 nM insulin over 48 h.

using chemiluminescence SuperSignal West Pico Substrate (Pierce®) and visualised using UVITEC Alliance digital imaging system (Thermo Fisher Scientific®).

## Statistics

Statistical analyses were performed using Microsoft Excel software and all data were analyzed using a Student's unpaired t-test  $\pm$  SD, where  $*p \leq 0.05$ ,  $**p \leq 0.01$ , and  $***p \leq 0.001$ .

## Results

### The effects of insulin on the phosphorylation of AKT and the production of insulin resistance model in C2C12 skeletal muscle cells

In order to create an insulin-resistant skeletal muscle cell line, we stimulated C2C12 cells with 10 nM of insulin over 0, 0.5, 2, 4, 8, 24, and 48 h replacing the media and insulin every 8 h. This method has been successfully used in a number of studies to create insulin-resistant C2C12 cells [11,12]. Total cellular protein was extracted using RIPA buffer and 20  $\mu$ g of total cellular protein was subjected to SDS-PAGE, and subsequently blotted onto Immobilon-PVDF membrane. The relative levels of pAKT was detected relative to total AKT (Figure 1). Following 30 min of insulin treatment there was an increase in the levels of pAKT that were further increased at 2 and 4 h of insulin treatment. The level of phosphorylation then rapidly decreased at 8 h and became almost undetectable at 24 and 48 h (Figure 1). Overall, there was no change in total AKT protein observed, suggesting that the C2C12 cells had obtained the insulin-resistant phenotype.

### The effects of insulin on the mRNA expression of orphan nuclear receptors in an insulin-resistant C2C12 skeletal muscle cell line

To determine if any of the ONRs responded to increases in insulin-resistance, we analysed a progressively insulin-resistant C2C12 skeletal muscle cell line. We analysed several ONRs by quantitative real-time PCR and observed several significant changes in a number of the ONRs as the cells became increasingly insulin resistant (Table 1). Of these, *Coup-tf1* and *Coup-tf2*, *Ror  $\gamma$*  and *Rar  $\gamma$*  were significantly decreased at 24 h of insulin treatment while *Ppar  $\beta$* , *Ppar  $\gamma$* , *Nor-1*, *Nurr1*, *Nur77*, *Reverb  $\alpha$* , *Rar  $\alpha$* , *Rar  $\beta$* , *Err  $\alpha$* , *Ror  $\alpha$*  and *Mr* were all significantly increased at 24 h of insulin treatment (Table 1). Other significant changes were observed for *Nor-1* (increased at 2 h), *Nur77* (increased at 0.5 and 2 h), *Reverb  $\alpha$*  (increased at 8 h), *Rxr  $\alpha$*  (decreased at 0.5 h), and *Vdr* (increased at 8 h) (Table 1). A representative figure (Figure 2) shows in graphical form a number of the orphan nuclear receptors that

were significantly changes in the presence of 10 nM insulin and the acquisition of the insulin-resistant skeletal muscle phenotype.

## Discussion

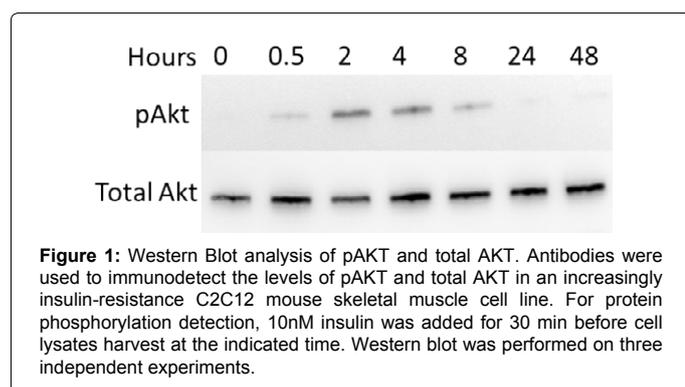
This study identified the regulatory response of several ONRs in response to skeletal muscle cells becoming increasingly resistant to insulin over time. We identified that 10 nM of insulin was sufficient to induce the phosphorylation of AKT after 30 min of treatment and that this phosphorylation was transient and rapidly declined post 2 h. This is supported by several studies that demonstrated that insulin resistant could be measured by the amount of pAKT relative to total AKT protein over time with chronic insulin treatment [12-15]. Thus, reduced pAKT levels are consistent with an increase in insulin resistance. Given that the ONRs regulate genes that are involved in many cellular mechanism, including metabolism, and have been linked to metabolic conditions including type 2 diabetes [16], it is reasonable to conclude that they may also be implicated in insulin resistance.

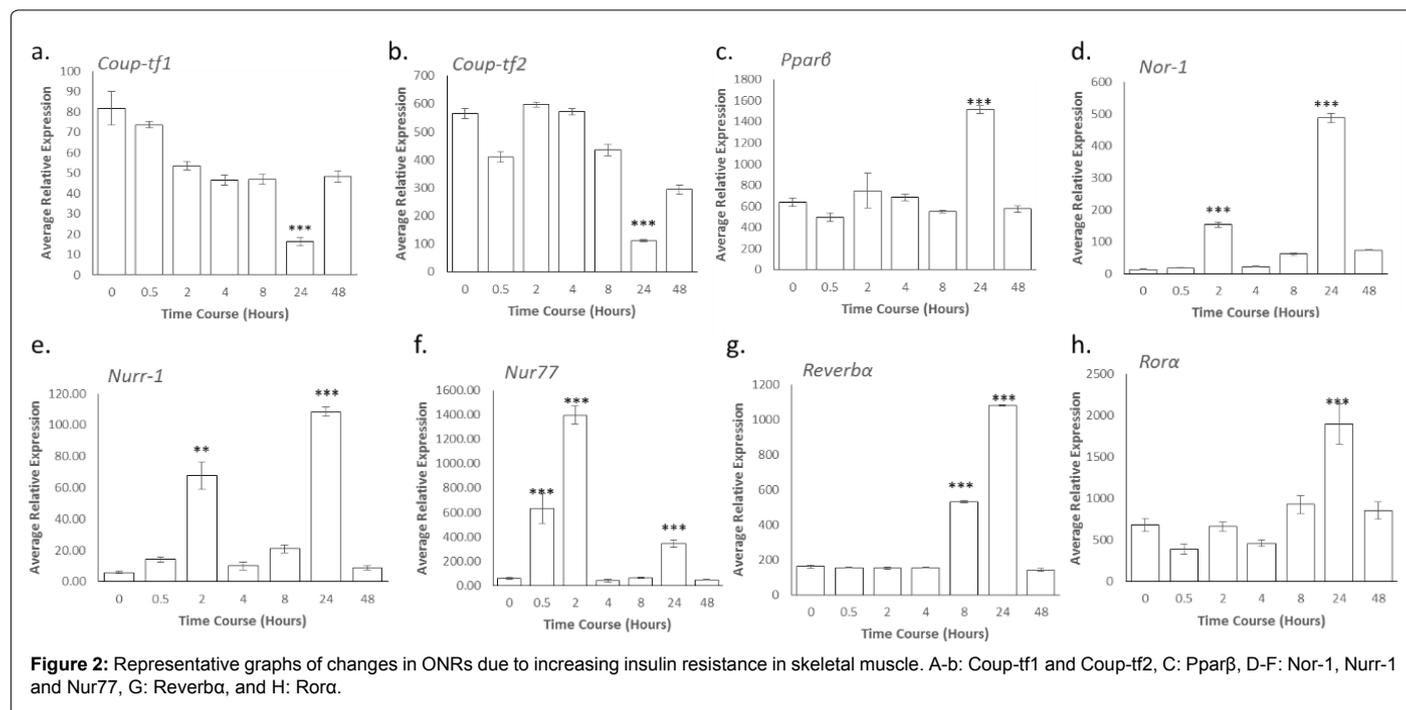
A number of ONRs were significantly regulated on the acquisition of the insulin resistant phenotype in C2C12 skeletal muscle cells including *Coup-tf1*, *Coup-tf2*, *Ppar $\beta$* , *Nor-1*, *Nurr1*, *Nur77*, *Reverb $\alpha$* , and *Rora* (Table 1). These ONRs have various roles in the context of metabolism and specifically in relation to carbohydrate and lipid metabolism and energy expenditure [1], and are discussed below.

*Coup-tf1* and *Coup-tf2* were both significantly down-regulated at 24 h of insulin treatment in the insulin-resistant skeletal muscle cell line. This is consistent with previous studies that identified that exogenous insulin reduced the levels of *Coup-tf2* mRNA and protein in INS-1  $\beta$ -cell lines and pancreatic islet  $\beta$ -cells [17]. Moreover, these authors found that C57BL/6J mouse hepatocytes that were cultured with 10 nM of insulin over 24 h had reduced levels of *Coup-tf2*. However, it is difficult to determine whether these effects in both our studies and the studies by Perilhou et al. [17] were due to insulin directly or indirectly targeting *Coup-tf2* expression or due to the cells (skeletal muscle and hepatocytes) undergoing changes in insulin resistance. From this study and others [12,18], chronic insulin treatment of 10 nM over 24 h is enough to reduce levels of pAkt and is indicative of the cells acquiring an insulin-resistant phenotype. *Coup-tf2* overexpression in C2C12 skeletal muscle cells also increased *Pgc1 $\alpha$*  and *Glut4* mRNA and protein and suggest that these cells may have improved insulin sensitivity and glucose uptake [19]. In fact, in mice that had a pancreatic  $\beta$ -cell specific *Coup-tf2* deficiency these animals had altered insulin secretion that was associated with peripheral insulin resistance and impaired glucose sensitivity [20].

*Ppar $\beta$*  was significantly up-regulated at 24 h of insulin treatment in contrast to the other family members *Ppar $\alpha$*  and *Ppar $\gamma$*  who showed no response to the acquisition of the insulin resistant phenotype. *Ppar $\beta$*  plays an important role in controlling fatty acid metabolism in skeletal muscle cells [21]. Previous studies have shown that *Ppar $\beta$*  enhances insulin sensitivity [22] and prevents lipid-induced endoplasmic reticulum stress, and thus insulin resistance, in mouse and human skeletal muscle [23]. Accordingly, the increase in *Ppar $\beta$*  in the progressively insulin-resistant skeletal muscle cells may be a compensatory action to regulate insulin sensitivity. Actually, *Ppar $\beta$*  activation prevented palmitate-induced inflammation and insulin resistance in skeletal muscle cells by increasing fatty acid oxidation [23].

The NR4A family of ONRs (*Nor-1*, *Nurr1* and *Nur77*) were all induced at various times as the skeletal muscle cells became progressively





insulin resistant. There is little information available on the role of the NR4As in insulin resistance however, studies by Fu et al. [24] found that *Nur77* and *Nor-1* were induced with 10 nM insulin in 3T3-L1 adipocytes cells at 1 h and 2 h, respectively. This is in collaboration to our studies where we show both *Nur77* and *Nor-1* induced by 10 nM insulin at 30 min and 2 h (*Nur77*) and 2 h (*Nor-1*). We also observed significant induction of *Nurr1* during this 2 h time period, however in addition, we found that all three NR4As were significantly reduced to basal levels over 4 and 8 h, before being up-regulated at 24 h of insulin treatment. The increase at 24 h for all three NR4As is suggestive of the acquisition of the insulin-resistant phenotype due to the fact that the NR4As respond within 2 h of insulin treatment before falling to basal levels [25]. As C2C12 cells become insulin resistant, they undergo oxidative stress and impaired gene expression of the insulin signaling pathway [26]. It has been revealed that the NR4As are also induced under a host of functions including changes in metabolism, insulin sensitivity, and oxidative stress conditions [27].

*Reverba* was significantly induced at 8 and 24 h of insulin treatment and the acquisition of the insulin-resistant phenotype. This ORN plays a crucial role in the regulation of circadian rhythms and including those associated with food intake [28]. Mice that lack *Reverba* display changes in their daily energy homeostasis and predisposition to diet-induced obesity [29]. Moreover, the ablation of *Reverba* by siRNA in islet cells and MIN-6 pancreatic cells impaired glucose-induced insulin secretion, decreased genes implicated in lipid metabolism, and impaired  $\beta$ -cell function [30]. It was suggested by these authors that *Reverba* may therefore play a critical role in the daily dynamics of insulin secretion. Equally, *Erra* was significantly upregulated at 24 h following insulin treatment. Similar to *Reverba* there is little information on the role of this ORN in insulin resistance. The ERRs ( $\alpha$  and  $\beta$ ) are involved in transcriptional control of cellular energy metabolism and may also be implicated in the aetiology of metabolic disorders, such as type 2 diabetes and metabolic syndrome [31].

These studies show that some of the ORNs are sensitive to changes

in the skeletal muscle phenotype resulting from insulin-induced, skeletal muscle insulin-resistance and therefore may be amendable to therapeutic intervention to treat or better manage insulin resistance and disease progression. Further studies are required to delineate the role of these ORNs in insulin resistance progression and to establish a platform for drug therapy in skeletal muscle-associated insulin resistance and subsequent disease states.

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