



## PAPER

# Transcriptional regulation of uncoupling protein-2 gene expression in L<sub>6</sub> myotubes

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**OBJECTIVE:** To increase the understanding of the transcriptional regulation of UCP2 gene expression in skeletal muscle cells, we examined the effect of all-*trans*-retinoic acid (tRA), a ligand (after the conversion to 9-*cis*-RA) of the retinoid X receptor (RXR), and linolenic acid, a polyunsaturated fatty acid and peroxisome proliferator-activated receptors (PPARs) ligand, on the expression of UCP2 mRNA in cultured L<sub>6</sub> myotubes.

**RESEARCH METHODS AND PROCEDURES:** UCP2 gene expression in L<sub>6</sub> myotubes was confirmed by Northern blot analysis. The time- and concentration-dependency of tRA and linolenic acid on UCP2 gene expression was assessed by dot blot quantification. The mRNA levels of PPAR subtypes ( $\alpha$ ,  $\gamma$  and  $\delta$ ) were determined by RT-PCR.

**RESULTS:** tRA induced UCP2 gene expression in a time- and concentration-dependent manner. Similar to tRA, UCP2 mRNA was markedly increased by 0.5 mM linolenic acid. In L<sub>6</sub> myotubes, PPAR $\delta$  mRNA was abundant, whereas PPAR $\alpha$  mRNA was lower and PPAR $\gamma$  mRNA was minimal.

**CONCLUSIONS:** UCP2 mRNA expression in L<sub>6</sub> myotubes is up-regulated by tRA and linolenic acid, possibly through a mechanism involving PPAR and RXRs.

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**Keywords:** PPAR; RXR; myotubes; retinoic acid; polyunsaturated fatty acid

## Introduction

Uncoupling proteins (UCPs) are members of the mitochondrial anion carrier superfamily. They are capable of dissipating the transmembrane proton gradient circumventing ATP synthesis and resulting in thermogenesis. Of these, UCP1, UCP2 and UCP3 have been extensively studied in relation to the regulation of energy expenditure, body weight homeostasis and thermoregulation. Like UCP1, UCP2 and UCP3 have recently been shown to mediate proton transport across bilipid layers.<sup>1–3</sup> However, factors regulating the expressions of these UCP molecules appear to be different. It is well established that UCP1 is regulated by catecholamines, thyroids, retinoids and cold, whereas UCP2 and UCP3 expressions are influenced by fatty acids, feeding

conditions and acute exercise.<sup>4–10</sup> These findings suggest that UCP2 and UCP3 may have a role in fatty acid metabolism, possibly by facilitating the oxidation of fatty acids. Both adipose tissue and skeletal muscle have been reported to express UCP2 and UCP3 mRNA.<sup>5,11–13</sup>

It is well established that brown adipose tissue (BAT) is an important tissue for thermogenesis in rodents. However, in humans, in whom brown fat is less abundant, skeletal muscle may be more important for thermogenesis and  $\beta$ -oxidation. Actually, skeletal muscle constitutes the largest tissue mass and is considered to be a major contributor to overall metabolic rate<sup>14</sup> and presumably thermogenesis in humans. In adult humans, UCP2 is expressed in a large number of tissues, including white adipose tissue (WAT) and skeletal muscle, whereas UCP3 appears to express mostly in skeletal muscle. Recently, UCP3 in L<sub>6</sub> myotubes has been shown to be up-regulated by thyroid hormone, ligands of peroxisome proliferator-activated receptor (PPAR) and retinoic acid.<sup>15</sup> Despite the increasing number of reports on UCP2 and UCP3, the molecular and/or cellular mechan-

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isms regulating UCP2 gene expression in skeletal muscle are still poorly understood.

Shimokawa *et al*<sup>16</sup> have reported that the PPAR $\gamma$  ligand pioglitazone induced an *in vivo* stimulation of UCP2 mRNA expression in skeletal muscle, especially soleus muscle. In contrast, UCP3 mRNA level is decreased by this ligand, indicating differential *in vivo* gene regulation. These authors suggested that the insulin-sensitizing effect of thiazolidinediones in skeletal muscle might be due to increased energy expenditure through UCP2 gene expression, but not UCP3.

To better understand the transcriptional regulation of UCP2 gene expression in skeletal muscle cells, we examined the effect of all-*trans*-retinoic acid (tRA), a ligand (after the conversion to 9-*cis*-RA) of retinoid X receptors (RXRs), and linolenic acid (polyunsaturated fatty acid and PPAR  $\alpha/\delta$  ligand) on the gene expression of UCP2. To avoid the question of indirect effects and other potential confounding variables in whole animals, we performed the present studies in L<sub>6</sub> cells, a cell line derived from rat skeletal muscle.

## Methods

### Cell culture

L<sub>6</sub> cells, a rat skeletal muscle cell line, obtained from ATCC were plated at a density of  $2 \times 10^5$  cells per well in six-well plates and grown in Eagle's minimum essential medium- $\alpha$  ( $\alpha$ -MEM, Life Technologies) in the presence of 2% fetal bovine serum (Life Technologies) and 1% antibiotic-antimycotic solution (final concentrations 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B; Life Technologies), at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air. Media were changed every other day. Cells were maintained in this medium until confluence was reached (usually 5 days). Cells were cultured for a few days beyond confluence to obtain fully differentiated conditions. Cells were further incubated with the serum-depleted (0.1%) medium for 24 h, and subjected to various treatments. tRA (Sigma, St Louis, MO) was prepared in 0.01% dimethylsulfoxide (DMSO) at the concentrations indicated. Linolenic acid (Sigma) and bovine serum albumin (BSA) complex was prepared according to Roche *et al*.<sup>17</sup> To obtain a 5 mM fatty acid stock solution, linolenic acid was dissolved at 37°C for 8 h under nitrogen atmosphere in Krebs-Ringer bicarbonate buffer containing 5% fatty acid-free BSA. After the incubation, the pH of the fatty acid solution was adjusted to 7.4 and the stock solution was filtered through a 0.2  $\mu$ m filter. This stock solution was stored at -20°C under nitrogen to prevent oxidation. Stock solution was diluted in culture medium to obtain various concentrations of linolenic acid at a fixed concentration of 0.5% BSA.

### RNA preparation and Northern blot analysis

Total cellular RNA was extracted using a modification of the method Chomczynski and Sacchi.<sup>18</sup> The integrity of the

isolated RNA was verified using 1% (w/v) agarose gels stained with ethidium bromide (Sigma). The RNA was quantified by spectrophotometric absorption at 260 nm using multiple dilutions of each sample.

Northern blot analysis using full-length mouse UCP2 cDNA provided by Dr Craig Warden<sup>1</sup> was performed as described previously.<sup>19</sup> A portion of RNA (20  $\mu$ g per lane) was denatured with formaldehyde and formamide and analyzed by electrophoresis in 1.2% (w/v) agarose gels. After transfer to nylon membranes (NEN, Boston, MA) and UV crosslinking, RNA blots were hybridized overnight at 42°C with UCP2 cDNA which had been labeled with [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol, NEN) by random prime labeling using Prime-a-Gene (Promega, Madison, WI).

For dot blot analysis, multiple concentrations of the RNA were immobilized on nylon membranes using a dot blot apparatus (Biorad, Richmond, CA). The membranes were cross-linked by an UV-cross linker. The membranes were prehybridized using Ultrasensitive hybridization buffer (ULTRAhyb, Ambion, TX). After incubation for 0.5-1 h at 42°C, the membranes were hybridized with <sup>32</sup>P-labeled probes in the prehybridization buffer plus 50  $\mu$ g/ml denatured salmon sperm DNA (Sigma). After hybridization for 14-24 h at 42°C, the membranes were washed and exposed to a phosphor imaging screen for a few days. The latent image was scanned using a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA) and analyzed by Image Quant Software (Molecular Dynamics). To ensure that changes in mRNA levels were specific, nylon membranes were stripped and rehybridized with a GAPDH oligo probe. The probe to detect GAPDH mRNA was a 30-mer antisense oligonucleotide (Clontech, Palo Alto, CA) end-labeled using terminal deoxynucleotidyl transferase. Values for the relative amount of UCP2 mRNA were normalized to the signal obtained after stripping and rehybridizing the same membrane with labeled GAPDH probe.

### RT-PCR

The mRNA level of PPAR subtypes was determined by the reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from 1  $\mu$ g of total RNA. RT was performed at 25°C for 10 min, 48°C for 30 min, 95°C for 5 min for 1 cycle, and PCR at 95°C for 15 s, 60°C for 1 min for 30 cycles on an ABI PRISM 7700 Sequence detection system. Oligonucleotide primers used were: PPAR $\alpha$ , 5'-GGCCTCAGGATACCACTATGG-3' and 5'-TCGCCGAAAGAAGCCC-3'; PPAR $\gamma$ , 5'-CAGTGGAGACCGCCCAG-3' and 5'-GCAGGTTGTCTTGATGTCC-3'; PPAR $\delta$ , 5'-GCTGGTCGACAGTGATCTGG-3' and 5'-TGGCCGGTCTCCGC-3'.

### Statistical analyses

Data were expressed as means  $\pm$  s.e. Statistical analyses were performed by one-way analysis of variance (ANOVA) and the

statistical significance of the differences among groups was determined by Dunnett's multiple comparison test. Student's *t*-test was utilized for analyses between two groups. Values of *P* < 0.05 were regarded as significant.

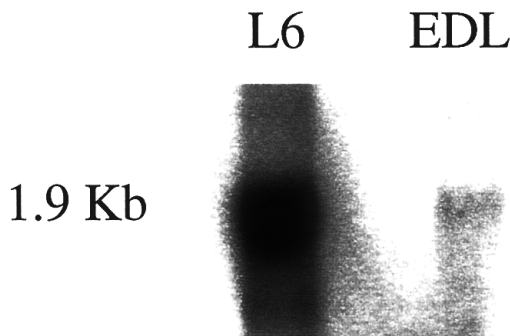
### Results

Expression of UCP2 mRNA in L<sub>6</sub> myotubes was identified by northern analysis of total RNA. Compared with rat extensor digitorum longus (EDL), UCP2 mRNA expression was more abundant in L<sub>6</sub> myotubes (Figure 1).

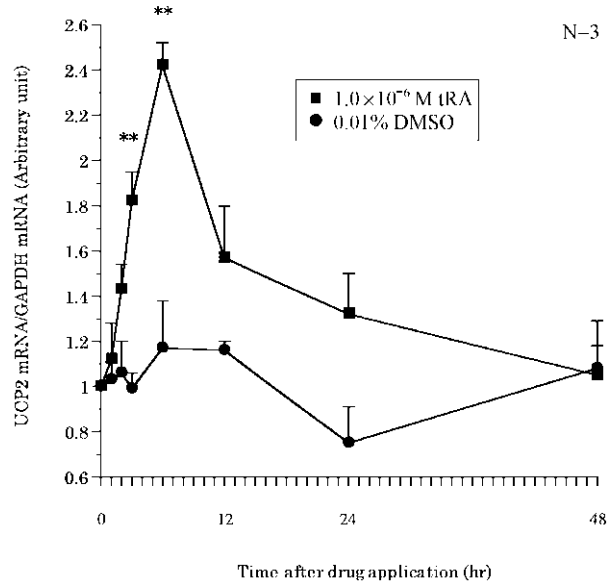
The time course of UCP2 mRNA expression in fully differentiated myotubes after treatment with tRA (10<sup>-6</sup> M) is displayed in Figure 2. UCP2 mRNA expression increased rapidly for 6 h after tRA addition followed by return to the basal level by 48 h. Vehicle-treated cells expressed a constant amount of UCP2 mRNA over the 48 h period (Figure 2). L<sub>6</sub> myotubes were treated for 6 h with various concentrations of tRA. The stimulatory effect of tRA on the expression of UCP2 mRNA was concentration-dependent with the maximum response observed at 10<sup>-6</sup> M of tRA (Figure 3).

The induction of UCP2 mRNA by linolenic acid was also examined (Figure 3). Fatty acid-free BSA (0.5%) used as a vehicle for linolenic acid had little or no effect on UCP2 mRNA expression over a 48 h period. Similar to tRA, treatment of myotubes with 0.5 mM of linolenic acid increased UCP2 mRNA expression until 6 h after treatment followed by a decrease to the basal level thereafter (Figure 4). Linolenic acid induced a concentration-dependent increase in UCP2 mRNA levels 6 h after addition (Figure 5). Linolenic acid elicited a robust response of UCP2 mRNA at the dose range between 0.1 and 0.5 mM.

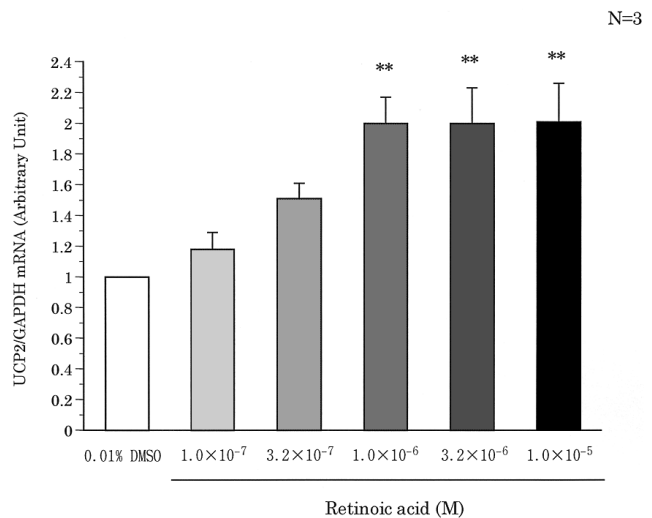
Three subtypes of PPAR gene expression were examined in L<sub>6</sub> myotubes by RT-PCR and for comparison, in rat adductor muscle. As shown in Figure 6, significant levels of PPAR $\delta$  were present in both L<sub>6</sub> myotubes and rat adductor muscle. PPAR $\alpha$  mRNA expression was less potent than PPAR $\delta$  and PPAR $\gamma$  gene expression was minimal in both L<sub>6</sub> myotubes and rat adductor muscle.



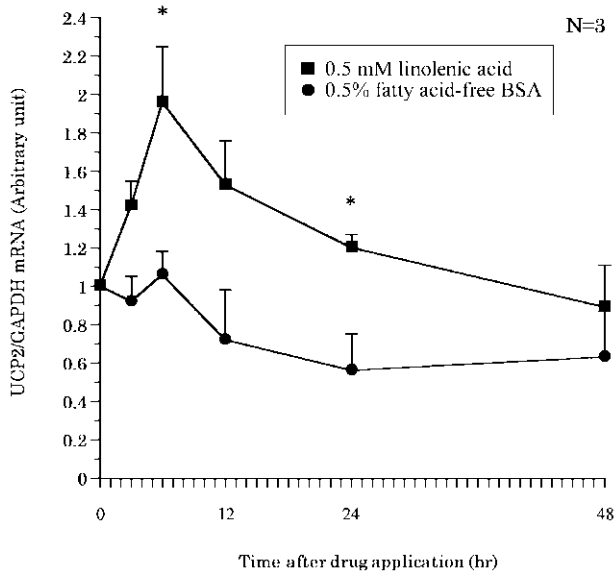
**Figure 1** Expression of UCP2 mRNA in L<sub>6</sub> myotubes and extensor digitorum longus (EDL). Total RNA (20  $\mu$ g) was extracted from L<sub>6</sub> myotubes and EDL and analyzed by Northern blot. The figure shows a representative blot.



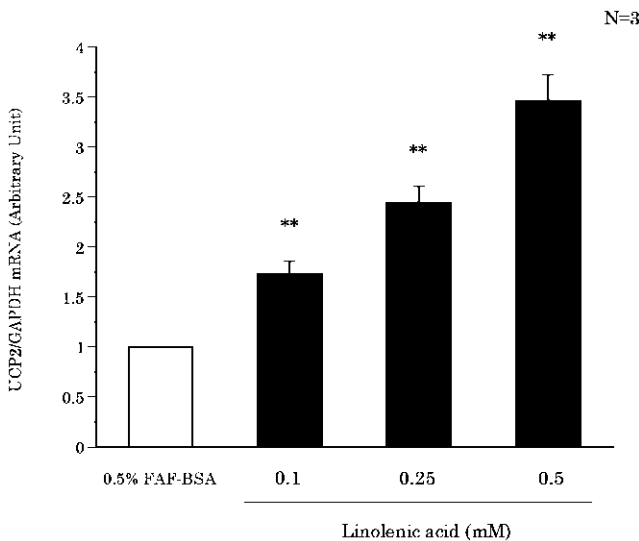
**Figure 2** Time course of expression of UCP2 mRNA from L<sub>6</sub> myotubes treated with all-*trans*-retinoic acid or vehicle, 0.01% dimethylsulfoxide. Cells were maintained in culture a few days past the confluence, and then serum was depleted for 24 h. Cells were harvested at the indicated times (0, 1, 2, 3, 6, 12, 24, 48 h) after addition of drugs. UCP2 mRNA levels are expressed in arbitrary units and the intensities of signals were normalized to those of GAPDH. Data are means  $\pm$  s.e. of values obtained in three independent experiments. \*\*Significantly different from vehicle-treated group at *P* < 0.01.



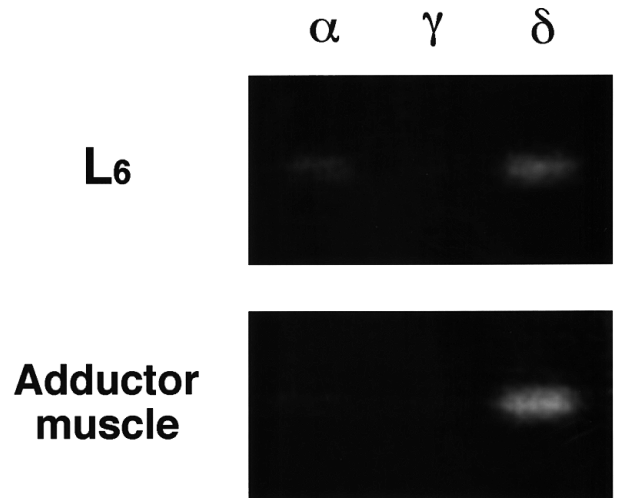
**Figure 3** Dose-response induction of UCP2 mRNA by all-*trans*-retinoic acid (tRA) in L<sub>6</sub> myotubes. Cells were treated at the indicated concentrations of tRA or vehicle, 0.01% dimethylsulfoxide (DMSO) for 6 h. UCP2 mRNA levels are expressed in arbitrary units and the intensities of signals were normalized to those of GAPDH. Data are means  $\pm$  s.e. of values obtained in three independent experiments. \*\*Significantly different from vehicle-treated group at *P* < 0.01.



**Figure 4** Time course of expression of UCP2 mRNA from L<sub>6</sub> myotubes treated with linolenic acid or vehicle for 48 h. Cells were maintained in culture a few days past confluence, and then serum was depleted for 24 h. At the indicated times (0, 3, 6, 12, 24, 48 h) after addition of drugs, cells were harvested. UCP2 mRNA levels are expressed in arbitrary units and the intensities of signals were normalized to those of GAPDH. Data are means ± s.e. of values obtained in three independent experiments. \*Significantly different from vehicle-treated group at  $P < 0.05$ .



**Figure 5** Dose-response induction of UCP2 mRNA by linolenic acid in L<sub>6</sub> myotubes. Cells were treated at the indicated concentrations of linolenic acid or vehicle, 0.5% fatty acid-free bovine serum albumin (FAF-BSA), for 6 h. UCP2 mRNA levels are expressed in arbitrary units and the intensities of signals were normalized to those of GAPDH. Data are means ± s.e. of values obtained in three independent experiments. \*\*Significantly different from vehicle-treated group at  $P < 0.01$ .



**Figure 6** Expression of PPAR subtypes in L<sub>6</sub> myotubes and rat adductor muscle. mRNA levels of individual PPAR subtypes were analyzed by RT-PCR.

**Discussion**

In this study, we demonstrated for the first time that tRA and linolenic acid up-regulated UCP2 gene expression in L<sub>6</sub> myotubes in a time- and concentration-dependent manner.

Recent evidence suggests that reduced levels of UCP2 mRNA in skeletal muscle are associated with obesity. Nordfors *et al*<sup>20</sup> reported that UCP2 but not UCP3 expression was decreased in obese subjects. Moreover, trectadrine, a beta3-adrenergic agonist, was reported to decrease fat content and increase gastrocnemius muscle UCP2 gene expression in a diet-induced obesity model.<sup>21</sup> Whether there is a causal relationship between UCP2 and the etiology of obesity is unknown; however, a better understanding of the regulation of UCP2 gene expression in skeletal muscle may provide new strategies to treat obesity.

Regulation of UCP gene expression has been extensively studied in both brown and white adipose tissues. It is well known that BAT is the major organ where energy expenditure takes place. BAT is, however, negligible in adult humans, suggesting that there may be other major organs responsible for non-shivering thermogenesis. Skeletal muscle has been suggested to be the predominant site contributing to basal metabolic rate and thermogenesis in humans.<sup>22</sup> Our strategy to treat obesity entails enhancing thermogenesis in skeletal muscle, potentially by the up-regulation of UCP gene expression.

It has been demonstrated that PPARs are involved in the transcriptional regulation of UCP genes in skeletal muscle.<sup>10,15,16,23,24</sup> The mechanism by which PPAR ligands up-regulate UCP gene expression is complex. PPAR is a member of the nuclear receptor superfamily that includes receptors for the steroid, thyroid and retinoid hormones.<sup>25</sup> Three PPARs-related genes (PPAR $\alpha$ , PPAR $\gamma$  and PPAR $\delta$ ) have been identified in mammals. PPAR $\gamma$  is highly enriched in

adipocytes while the  $\delta$  subtype is ubiquitously expressed.<sup>26</sup> Like other members of this superfamily, PPARs contain a central DNA-binding domain that recognizes response elements in the promoters of their target genes. To recognize a PPAR response element (PPRE), PPARs must heterodimerize with the 9-*cis*-RA receptor (RXR). Once bound to a response element, PPARs activate transcription through a conserved C-terminal ligand binding domain.

A substantial body of evidence suggests that several factors are involved in the transcriptional regulation of UCP3 gene expression in skeletal muscle cells, including L<sub>6</sub> or C<sub>2</sub>C<sub>12</sub> myotubes. It has been reported that 9-*cis*-RA, carbacyclin (a non-selective PPAR ligand), oleic acid and BRL49653 (a PPAR $\gamma$  agonist) increased UCP3 gene expression,<sup>10,15</sup> whereas WY14643 (a PPAR $\alpha$  ligand) and troglitazone (a PPAR $\gamma$  agonist) failed to increase UCP3 gene expression.<sup>15</sup> This discrepancy in the effects of PPAR $\gamma$  agonists on UCP3 gene expression could be explained by which subtypes of PPAR are dominantly expressed in certain tissue. There is a controversy as to the involvement of PPAR $\gamma$  in the regulation of UCP gene expression. It has been shown that darglitazone up-regulates UCP2 gene expression in L<sub>6</sub> cells,<sup>23</sup> whereas Cabrero *et al*<sup>24</sup> recently reported that troglitazone down-regulated UCP3 gene expression in C<sub>2</sub>C<sub>12</sub> myotubes. In addition, Nagase *et al*<sup>15</sup> reported that PPAR $\delta$  is abundantly expressed in L<sub>6</sub> myotubes whereas neither PPAR $\alpha$  nor PPAR $\gamma$  is expressed. In the present study, however, we demonstrated that L<sub>6</sub> myotubes as well as rat adductor muscle expressed all three subtypes of PPAR ( $\alpha$ ,  $\gamma$  and  $\delta$ ), although PPAR $\gamma$  expression was at a low level. Our findings in rat adductor muscle are in accordance with the previous reports showing that PPAR $\gamma$  is expressed in skeletal muscle<sup>24,27</sup> whereas the discrepancy in PPAR subtype expression in L<sub>6</sub> cells between the present and previous report may reflect differences in cell clones. Taken together, PPAR $\gamma$  is also likely to be involved in the regulation of UCP gene expression in skeletal muscle.

Unlike UCP3, little is known about how UCP2 gene expression is regulated in skeletal muscle cells. The only finding reported up to now is that darglitazone, a PPAR $\gamma$  agonist, can increase UCP2 gene expression.<sup>23</sup> In the present study, we demonstrated that tRA and linolenic acid increase UCP2 gene expression in L<sub>6</sub> myotubes.

tRA is a natural vitamin A derivative known to have profound effects on the growth and differentiation of many mammalian cells.<sup>28</sup> tRA promotes cell differentiation and inhibits cell proliferation. RA receptors bind tRA as well as 9-*cis*-RA, whereas RXRs only show binding affinity for the 9-*cis* isomer.<sup>29-31</sup> tRA binds to RXR after conversion to the active ligand, 9-*cis*-RA.<sup>25</sup>

On the other hand, fatty acids have been reported to act as transcriptional regulators of the expression of lipid-related genes. PPAR $\alpha$  and PPAR $\delta$  regulate fatty acid oxidation and are transcriptionally activated by a variety of lipid-like compounds including polyunsaturated fatty acids. Polyunsaturated fatty acids promote the binding of PPAR $\alpha$ / $\delta$ -RXR heterodimers, and PPAR $\alpha$  and  $\delta$  may directly mediate some

of the beneficial effects of dietary polyunsaturated fatty acids.<sup>32</sup> The overall balance between fatty acid catabolism and storage may be determined by the relative levels of PPAR $\alpha$ / $\delta$  and PPAR $\gamma$  ligands. Therefore, it is likely that UCP2 gene expression in muscle cells is regulated via formation of PPAR/RXR heterodimers although the subtype(s) of PPAR involved in the regulation of UCP2 gene expression is unknown.

In summary, the present study demonstrates that tRA and linolenic acid induce a time- and concentration-dependent increase in UCP2 gene expression in L<sub>6</sub> myotubes. These data coupled with the previous report demonstrating that UCP3 gene expression in L<sub>6</sub> cells is up-regulated by RA and polyunsaturated fatty acids suggest that there are overlapping patterns of transcriptional regulation between UCP2 and UCP3 in myotubes, possibly through PPAR and RXR.

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