Up-regulation of uncoupling protein 3 by thyroid hormone, peroxisome proliferator-activated receptor ligands and 9-*cis* retinoic acid in L6 myotubes

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Abstract Uncoupling protein 3 (UCP3), expressed abundantly in the skeletal muscle, is one of the carrier proteins dissipating the transmitochondrial electrochemical gradient as heat, and thereby has been implicated in the regulation of energy metabolism. We have investigated UCP3 mRNA expression in the widely used L6 myocyte cell line by Northern blot analysis. UCP3 mRNA was not detected in L6 myoblasts, but appeared after their differentiation to myotubes. The UCP3 mRNA level was increased when L6 myotubes were treated with increasing concentrations of triiodothyronine (T3), oleic acid, α-bromopalmitate and carbacyclin, a non-selective ligand of peroxisome proliferator-activated receptors (PPARs), whereas it was not influenced when treated with selective ligands of PPARα (WY 14643) and PPARγ (troglitazone). A ligand of retinoid X receptor (RXR), 9-*cis* retinoic acid, was also effective by itself and in combination with carbacyclin in stimulating UCP3 mRNA expression. The mRNA analysis of individual PPAR isoforms revealed that L6 cell expressed a significant level of PPARδ but undetectable levels of PPARα and PPARγ. These results suggest that UCP3 expression in myocytes is differentiation-dependent and regulated by the T3 receptor, RXR and PPARδ.

Key words: Uncoupling protein 3; L6 myotube; PPAR; Fatty acid; T3; Retinoic acid

1. Introduction

Uncoupling proteins (UCPs) are a family of mitochondrial transporters that dissipate the transmitochondrial proton gradient more as heat than via ATP synthesis. UCP1, a classical UCP, is present exclusively in brown adipose tissue, which is the major site of regulatory thermogenesis during cold exposure and voluntary hyperphagia in small rodents. It is well accepted that the activity and gene expression of UCP1 are controlled synergistically by triiodothyronine (T3) and norepinephrine released from sympathetic nerves [1,2]. Recently, two isoforms of UCP, UCP2 and UCP3, were identified in rodents and man, based on their similarity to UCP1 [3–5]. Since these new UCPs have been reported to reduce the mitochondrial potential when overexpressed in vitro, they may have uncoupling activity, and thereby plausibly be involved in the regulation of whole-body thermogenesis and energy expenditure. Particularly interesting is that UCP3 is expressed mainly in skeletal muscle, which is thought to be the major thermogenic organ in larger mammals, including humans. In fact, several reports showed that the gene expression of muscle UCP3 altered remarkably under various physiological and pathological conditions related to energy metabolism (for review, see [6]). For example, the UCP3 mRNA level in skeletal muscle is increased during fasting and decreased by refeeding [7–9]. T3 treatment also induces a marked rise in the UCP3 mRNA level [7,10]. However, cold exposure, which is the most effective physiological stimulus to induce UCP1 expression, does not influence UCP3 expression [4,8,10].

Despite the increasing number of reports on UCP3, the molecular and/or cellular mechanisms regulating UCP3 expression in skeletal muscle are still poorly understood. This is largely because most studies were carried out in vivo, not in vitro, thus making it difficult to identify stimulatory or inhibitory factors directly acting in muscle cells. In the present study, we examined UCP3 mRNA expression in a widely used myocyte cell line L6, and demonstrated that UCP3 expression in myocytes was up-regulated by T3, ligands of PPAR, and retinoid X receptor (RXR).

2. Materials and methods

2.1. Cell culture

Rat muscle L6 cells (clone L6Y) were kindly provided by Dr. Amira Klip (Programme in Cell Biology, The Hospital for Sick Children, Toronto, Ont., Canada). They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 units/ml penicillin and 50 μg/ml streptomycin. When the cells reached confluence (Day 0), a medium containing 2% horse serum, 50 units/ml penicillin and 50 μg/ml streptomycin (differentiation medium) was used for cell differentiation. The fresh differentiation medium was substituted at Day 4, and after 2–3 h test substances dissolved in 0.1% DMSO were added. After a 24-h culture, the cells were harvested for RNA extraction.

2.2. Northern blot and RT-PCR analyses

Total RNA was extracted by the guanidium-thiocyanate method [11] using ISOGEN (Nippon Gene, Toyama, Japan). For Northern blot analysis, 30 μg of total RNA was separated on a 1.2% agarose/formaldehyde gel and transferred to and fixed on a nylon membrane. DNA fragments used for hybridization probes were: a 924-bp PCR product of the rat UCP3 cDNA [12], and a 452-bp PCR product of the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. They were labeled with [α-^{32}P]dCTP using a multiprime DNA labeling kit (Amersham, Buckinghamshire, England). The blots were hy-

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bridized to the labeled cDNA probes in 0.5 M sodium phosphate (pH 6.8), 7% SDS, 1% BSA, 1 mM EDTA and 200 μg/ml heat-denatured salmon sperm DNA at 65°C for 12 h, washed with 2×SSC or 0.1×SSC at 65°C, and analyzed with a BAS 2000 bio-imaging analyzer (Fuji Film, Tokyo, Japan).

The mRNA level of PPAR isoforms was determined by real time quantitative RT-PCR analysis as described previously [13]. Oligonucleotide primers used were: PPARα, 5′-701GGCCTCAGGATACCACTATGG-3′ and 5′-761TCGCCGAAAGAAGCCC-3′; PPARγ, 5′-1182CAGTGGAGACCGCCCAG-3′ and 5′-1243GCAGGTTGTCTTGGATGTCC-3′; PPARδ, 5′-1278GCTGGTCGACAGTGATCTGG-3′ and 5′-1338TGGCCGGTCTCCGC-3′. The reaction mixture contained 1×TaqMan EZ buffer, 3 mM $Mn(OAc)_2$, 300 μM dNTP, 2.5 units of rTth DNA polymerase, 200 nM primers, and 50 ng of total RNA in 25 μl. RT was carried out at 55°C for 50 min, 60°C for 10 min, 95°C for 2 min for 1 cycle, and PCR at 95°C for 15 s, and 58°C for 1.5 min for 40 cycles on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Tokyo, Japan).

2.3. Chemicals

Sodium 3,5,3-triiodo-l-thyronine (T3), 9-*cis* retinoic acid, carbacyclin, and oleic acid were purchased from Sigma (St. Louis, MO, USA), WY 14643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid from Cayman Chemical Co. (Ann Arbor, MI, USA), and α-bromopalmitate (2-bromohexadecanoic acid) from Aldrich (Milwaukee, WI, USA). Troglitazone was a gift from Sankyo Pharmaceuticals Inc. (Tokyo, Japan).

3. Results

3.1. UCP3 mRNA expression in L6 cells

L6 myoblasts differentiated morphologically to fuse into myotubes when cultured in the presence of 2% horse serum 4–5 days after reaching confluence. mRNA expression of UCP3 was examined by Northern blot analysis at confluence and after differentiation. As shown in Fig. 1, no signal of UCP3 mRNA was detected at confluence (Day 0), but at 4 and 7 days after differentiation significant levels of UCP3 mRNA were observed, although they were much weaker than those of rat skeletal muscle.

3.2. Stimulatory effects of T3 and fatty acids on UCP3 mRNA expression

It has been reported that the tissue mRNA level of UCP3 is greatly increased in skeletal muscle by treating animals with T3 [7,10] or with triglyceride (Intralipid) and heparin [14], suggesting that T3 and fatty acids are potent inducers of muscle UCP3. To confirm this in vitro, the effects of T3 and fatty acids on UCP3 mRNA expression were examined in L6 myotubes. As shown in Fig. 2, the addition of T3 increased the UCP3 mRNA level dose-dependently. Oleic acid was also effective in stimulating UCP3 expression at a physiological concentration (100 μM). A similar stimulation

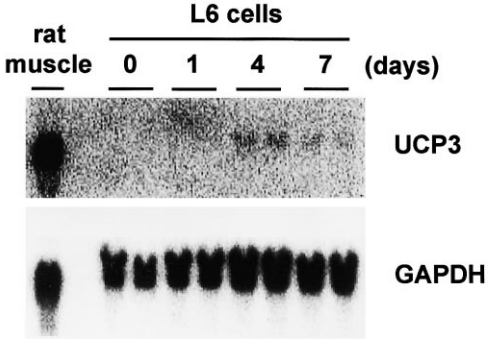

Fig. 1. Differentiation-dependent expression of UCP3 mRNA in L6 cells. L6 myoblasts were cultured in DMEM containing 10% FCS, and after reaching confluence (Day 0) they were differentiated to myotubes in a medium containing 2% horse serum for 1–7 days. Thirty μg of total RNA was used for Northern blot analysis. As a reference, 15 μg of total RNA of rat skeletal muscle was also analyzed.

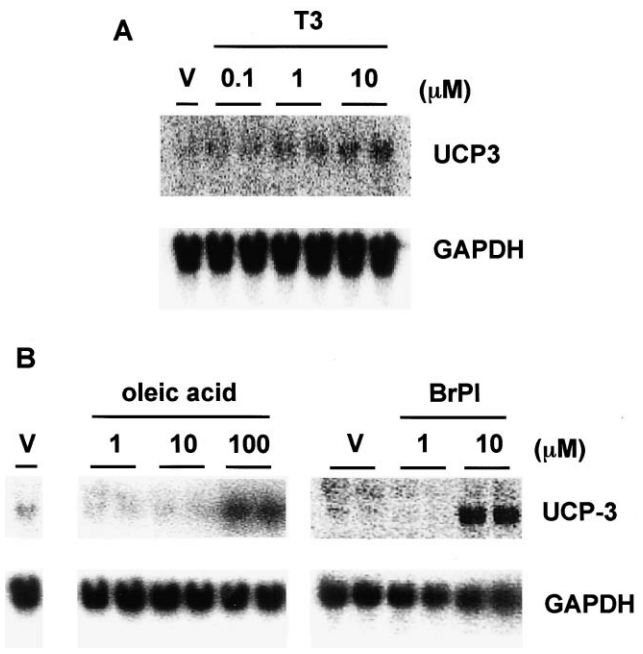

Fig. 2. Dose-dependent stimulation of UCP3 mRNA expression by T3 and fatty acids in L6 myotubes. L6 myotubes were treated for 24 h with the vehicle (V), 0.1–10 μM T3, 1–100 μM oleic acid or 1–10 μM α-bromopalmitate (BrPl).

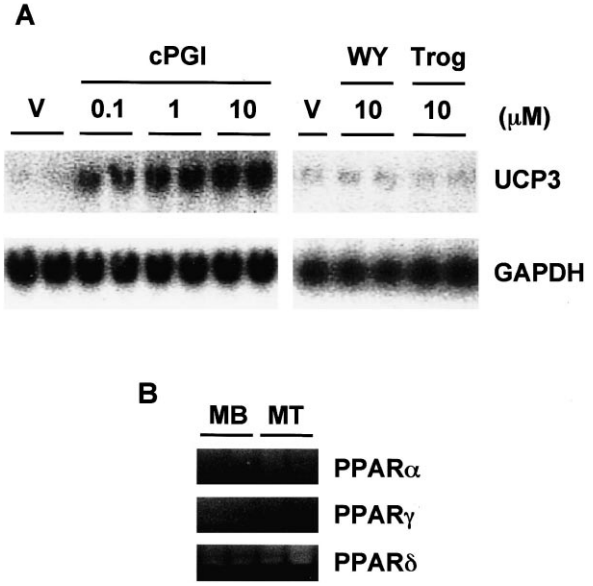

Fig. 3. Expression of PPAR isoforms and effects of their ligands on UCP3 mRNA expression in L6 myotubes. A: L6 myotubes were treated with various concentrations of carbacyclin (cPGI), WY 14643 (WY) or troglitazone (Trog) for 24 h. V, vehicle. B: mRNA expression of individual PPAR isoforms was analyzed by RT-PCR for L6 myoblasts (MB) and myotubes (MT).

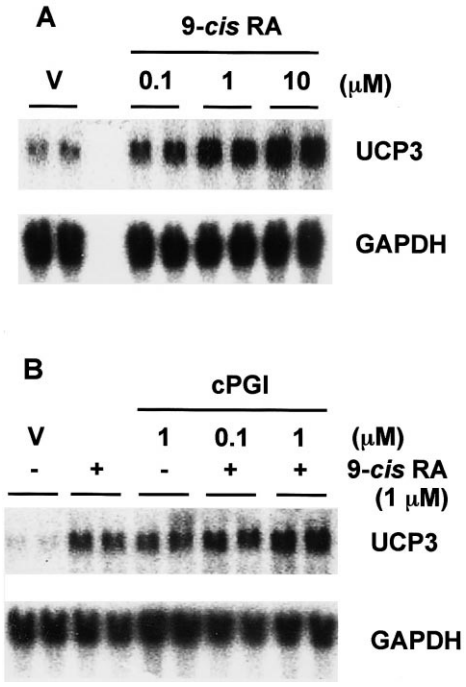

Fig. 4. Effects of 9-*cis* retinoic acid on UCP3 mRNA in L6 myotubes. L6 myotubes were treated for 24 h with: A: 0.1–10 μM 9-*cis* retinoic acid (9-*cis* RA); B: 0.1 and 1 μM carbacyclin (cPGI) in the presence or absence of 1 μM 9-*cis* retinoic acid. V, vehicle.

was seen with a much lower concentration (10 μM) of an unmetabolizable fatty acid derivative, α-bromopalmitate.

3.3. Effects of PPAR ligands on UCP3 expression

Fatty acids including α-bromopalmitate are known as ligands of the PPAR [15], which are involved in the induction of UCP1 and UCP2 in vivo and in vitro [2,16–18]. To confirm the role of PPARs and to determine which PPAR subtype is involved, we examined the effects of various PPAR ligands on UCP3 expression in L6 myotubes differentiated for 4 days. L6 myotubes were cultured for 24 h with carbacyclin (non-selective PPAR ligand), WY 14 643 (PPARα ligand) or troglitazone (PPARγ ligand). When the cells were treated with 0.1–10 μM carbacyclin, the UCP3 mRNA level was increased dose-dependently, whereas it was not influenced by WY 14 643 or troglitazone even at 10 μM (Fig. 3A). We also examined the effects of the PPAR ligands in L6 cells differentiated for 7 days, and obtained almost the same results (data not shown). To examine expression of individual PPAR isoforms in L6 cell, the RNA samples obtained from the cells before and 4 days after differentiation were analyzed by RT-PCR. As shown in Fig. 3B, significant levels of PPARδ mRNA were present in both L6 myoblasts and myotubes, while neither PPARα or PPARγ was detectable.

3.4. Effect of 9-cis retinoic acid on UCP3 mRNA expression

It is known that UCP1 expression in brown adipocytes is induced by activation of RXR, by forming heterodimers with PPARs [19]. We examined the effect of 9-*cis* retinoic acid, a ligand of RXR, on UCP3 expression in L6 myotubes. As shown in Fig. 4A, the UCP3 mRNA level was increased by increasing concentrations of 9-*cis* retinoic acid (0.1–10 μM). The effects of carbacyclin in the presence of 1 μM 9-*cis* retinoic acid were also examined (Fig. 4B). The UCP3 mRNA level was up-regulated by carbacyclin in the presence of 9-*cis* retinoic acid.

4. Discussion

In this study, we examined mRNA expression of UCP3 in vitro using the widely used myocyte cell line L6. The major findings were: (1) L6 cells expressed UCP3 mRNA at a low but significant level only after differentiation from myoblasts to myotubes, (2) UCP3 expression in L6 myotubes was increased by the treatments with T3, and some ligands of PPAR, and (3) UCP3 expression was enhanced further by simultaneous treatment with 9-*cis* retinoic acid, a ligand of RXR.

The present results clearly indicated that the expression of UCP3 in myocytes was differentiation-dependent. This seems compatible with the abundant expression of skeletal muscle in vivo. In fact, a recent analysis of a 7-kb 5′-flanking region of the UCP3 gene revealed that the binding motif of MyoD, a muscle-specific transcription factor family, was most abundant in this region [20]. Additionally, this region has one thyroid hormone and three peroxisome proliferator response elements. As expected, we demonstrated that T3 treatment increased UCP3 mRNA dose-dependently in L6 myotubes, confirming the stimulatory effect of T3 on muscle UCP3 expression repeatedly reported in vivo. These results, together with the effects of T3 on UCP1, suggest that the thermogenic and metabolic actions of T3 may be, at least in part, due to the increased gene expression of UCP1 in brown adipose tissue and UCP3 in skeletal muscle.

A possible role of fatty acids in muscle UCP3 expression was initially suggested from in vivo studies. For example, the UCP3 mRNA level in skeletal muscle is up-regulated when the plasma fatty acid level is elevated by either fasting [7–9,21,22] or co-infusion of triglyceride (Intralipid) and heparin [14] in rodents and humans. In the present study, we found a marked, dose-dependent increase in the UCP3 mRNA level in L6 myotubes treated with oleic acid and also with α-bromopalmitic acid. This is well consistent with a recent report that oleic acid induces UCP3 in another myocyte cell line, C2C12 [9]. Thus, fatty acids induce muscle UCP3 expression by acting directly on myocytes. It is known that fatty acids can serve as ligands of PPARs to transactivate some genes, including those of the fatty acid metabolism [15]. Considering the presence of the peroxisome proliferator response element in the 5′-flanking region of the UCP3 gene [20], it is quite likely that the stimulatory action of fatty acids is mediated by PPARs. Potential involvement of PPARs in UCP3 expression was further confirmed by the observation that carbacyclin, a potent non-selective PPAR ligand, greatly increased the UCP3 mRNA level. The mRNA analysis of individual PPAR isoforms revealed that L6 cell expressed a significant level of PPARδ but undetectable levels of PPARα and PPARγ. Although carbacyclin is an effective activator of all PPAR isoforms, it may be the most efficacious activator of PPARδ [23]. All these data suggest a predominant role of PPARδ in the activation of the UCP3 gene in L6 myocytes. In support of this idea, neither WY 14 643 (PPARα ligand) nor troglitazone (PPARγ ligand) was effective in UCP mRNA induction in L6 myotubes. The ineffectiveness of troglitazone appears to conflict with recent reports that UCP3 expression was in-

creased by PPARγ ligands in C2C12 myocytes [9] and primary cultured adipocytes [24]. The precise reason for such discrepancies is not clear at present, but it would be attributable to different expression levels of PPARγ among the cells.

It is well established that PPARs transactivate gene expression in the form of heterodimers with RXR. In the present study, a ligand of RXR 9-*cis* retinoic acid, by itself and also in combination with carbacyclin, increased the expression of UCP3 mRNA. The stimulatory action of RXR has been demonstrated for UCP1 expression in brown adipocytes, where the RXR/PPARγ heterodimer plays a predominant role [2]. Our results suggest that UCP3 expression in L6 myocytes is activated by the heterodimer of RXR with PPARδ, and probably also by the RXR homodimer. To confirm this, more detailed analysis of the regulatory mechanism of UCP3 gene expression in myocyte is required by using cells transfected with a reporter gene connected with the 5′-flanking region of the UCP3 gene.

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