Up-regulation of uncoupling proteins by β-adrenergic stimulation in L6 myotubes

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Received 9 March 2001; accepted 15 March 2001

First published online 26 March 2001

Edited by Vladimir Skulachev

Abstract Catecholamine-induced and β-adrenergic receptor (β-AR)-mediated thermogenesis in skeletal muscle is a significant component of whole-body energy expenditure. Skeletal muscle expresses uncoupling protein (UCP) 2 and UCP3, which can dissipate the transmitochondrial electrochemical gradient and thereby may be involved in regulation of energy metabolism. We investigated the effects of β-AR stimulation on UCP2 and UCP3 expression in L6 myotubes. Stimulation of the cells with epinephrine increased the UCP3 mRNA level transiently at 6 h, and also the UCP2 mRNA level at 6–24 h. The stimulatory effects of epinephrine were also observed in the presence of carbacyclin and 9-*cis* retinoic acid, and mimicked by isoproterenol and salbutamol (β2-AR agonists), but abolished by propranolol and ICI-118,551 (β2-AR antagonists). Pharmacological and mRNA analyses revealed the existence of β2-AR, but not β1- and β3-ARs, in L6 myotubes. These results suggested that catecholamines up-regulate UCP2 and UCP3 expression through direct action on the β2-AR in skeletal muscle. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Uncoupling protein; β-Adrenergic receptor; L6 myotube; Skeletal muscle

1. Introduction

β-Adrenergic stimulation increases regulatory energy expenditure (thermogenesis) in brown adipose tissue (BAT) and skeletal muscle in rodents. In BAT, norepinephrine released from sympathetic nerve endings increases lipolysis and thermogenesis through stimulation of the β-adrenergic receptor (β-AR) of brown adipocytes [1,2]. A key molecule for BAT thermogenesis is uncoupling protein (UCP) 1, which is present exclusively in BAT and dissipates the transmembrane proton gradient of mitochondria as heat [3]. In contrast to BAT, the mechanism of regulatory thermogenesis in skeletal muscle is poorly understood, though it is recognized to contribute to whole-body energy expenditure more than that of BAT. Recently, two isoforms of UCP, UCP2 and UCP3, were found to be expressed in skeletal muscle [4–7]. It has been shown that mRNA expression of muscle UCP2 and UCP3 alters under various physiological and pathological conditions related to energy metabolism [8]. Moreover, Clapham et al. reported that mice overexpressing UCP3 in skeletal muscle are hyperphagic and lean [9]. Collectively, it has been proposed, but not generally accepted, that these new UCP members are involved in the regulation of energy metabolism in skeletal muscle [10].

Apparently conflicting results have been reported concerning the effects of β-adrenergic stimulation on UCP mRNA expression in skeletal muscle. For example, cold exposure, which is the most effective physiological stimulus to activate the β-AR and to induce BAT UCP1 expression, decreases [11], does not influence [12], or increases [13] muscle UCP3 expression. Administration of agonists of β3-AR decreases, increases, or does not change the mRNA levels of UCP2 and UCP3 in skeletal muscle [14–17]. These discrepancies might be due to the differences in rodent species used, duration of the treatment, and agonists used. In fact, Lin et al. [13] reported that cold exposure results in a transient increase in muscle UCP3 mRNA between 6–24 h but a substantial decrease after 3–6 days. Moreover, in these in vivo studies, it was difficult to discriminate the direct action of the β-AR of muscle from some indirect effects. In fact, cold exposure and β3-AR agonists cause lipolysis in adipose tissue and elevation of plasma-free fatty acids, which in turn act as potent stimulators of muscle UCP3 expression [18]. It is thus obvious that some in vitro system using cultured myotubes and/or isolated muscle tissue would be useful to examine the direct effects of adrenergic stimulation, but no such study has been reported thus far.

Recently, we examined UCP3 mRNA expression in L6 myotubes and demonstrated that UCP3 expression was up-regulated by triiodothyronine, ligands of peroxisome proliferator-activated receptor (PPAR) and retinoid X receptor (RXR) and free fatty acids in vitro [19], consistent with previously reported in vivo results. Thus, in the present study, we investigated the effects of β-adrenergic stimulation on UCP2 and UCP3 mRNA expression in the presence or absence of PPAR and/or RXR ligands using L6 myotubes in culture. Our results showed that adrenergic stimulation directly up-regulated UCP2 and UCP3 mRNA expression through β2-AR.

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Abbreviations: BAT, brown adipose tissue; AR, adrenergic receptor; UCP, uncoupling protein; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGC-1, PPAR gamma coactivator-1; CRE, cAMP response element; PKA, cAMP-dependent protein kinase

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PII: S0014-5793(01)02341-9

2. Materials and methods

2.1. Cell culture

Rat L6 myoblasts (clone L6Y) were kindly provided by Dr. Amira Klip (Programme in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada). They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37°C, 5% CO_2. When the cells reached confluence (Day 0), they were differentiated in a medium containing 2% horse serum (differentiation medium). The fresh differentiation medium was substituted at Day 4 and then test substances dissolved in 0.1% dimethyl sulfoxide were added. L6 myotubes were cultured further for 3–24 h, and harvested for RNA extraction at Day 5.

2.2. RNA analysis

Total RNA was extracted by the guanidium-thiocyanate method [20] 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: 927-bp and 924-bp PCR products of rat UCP2 and UCP3 cDNAs, respectively [21], and a 452-bp PCR product of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. They were labeled with [α-^{32}P]dCTP using a multiprime DNA labeling kit (Amersham, Buckinghamshire, UK). The blots were hybridized 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 bio-imaging analyzer BAS 2000 (Fuji Film, Tokyo, Japan).

mRNA expression of the three β-AR subtypes was examined by RT-PCR analysis. 1 μg of total RNA was reverse-transcribed by using an Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA, USA) for 60 min at 42°C in a solution of 0.5 mM dNTP mixture, 20 pM oligo(dT)$_{18}$ primer, 1 unit/μl RNase inhibitor and 10 units/μl MMLV reverse transcriptase. PCR was carried out at 95°C for 45 s, 55°C for 45 s, 72°C for 2 min for 25 cycles on a GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems, Tokyo, Japan) in a solution containing 1×AmpliTaq PCR buffer, 0.2 mM each dNTP mix, 0.4 mM primers and 0.04 units/μl Amplitaq DNA polymerase. Oligonucleotide primers used were: β1-AR, 5′-411AGACGTGCTATGTGTGACGG-3′ and 5′-782CAGCTGTCGATCTTCTTCACC-3′; β2-AR, 5′-64CACGACATCACTCAGGAACG-3′ and 5′-351GACGCACAACACATCAATGG-3′; β3-AR, 5′-552AGAGTGTCACTCCAATCCGC-3′ and 5′-1079TAGCTGCAGAGAAGACGACG-3′.

2.3. cAMP assay

L6 myotubes were incubated in Hank's solution at 37°C for 10 min with various concentrations of β-agonists and 0.1 mM 3-isobutyl-1-methylxanthine. After the reaction was terminated by adding 0.1 N HCl, cAMP formed was assayed using an enzyme immunoassay kit (Amersham).

2.4. Chemicals

Epinephrine, isoproterenol, propranolol, carbacyclin, 9-*cis* retinoic acid, dibutyryl-cAMP and forskolin were purchased from Sigma (St. Louis, MO, USA). BRL 37344, CGP-20712A, ICI-118,551 were purchased from RBI (Natick, MA, USA). CL316,243 was provided by American Cyanamid Co. (Pearl River, NY, USA).

2.5. Data analysis

All experiments were repeated two times, and representative Northern blot data are shown in the figures. Values are expressed as means of two experiments. Data of cAMP accumulation are means ± S.E.M. for three independent experiments.

3. Results

3.1. Time-dependent effects of epinephrine on UCP2 and UCP3 mRNA levels in L6 myotubes

Lin et al. [13] reported that the mRNA levels of UCP2 and UCP3 in skeletal muscle increased transiently after rats were exposed to cold: that is, the UCP3 mRNA level increased 3-fold between 6 and 24 h and decreased to 50% of the control value after 6 days in the cold, whereas the UCP2 mRNA level doubled on Day 3 and returned to normal after 6 days. To determine whether similar time-dependent expression of UCPs is also mimicked by epinephrine in vitro, first, we measured the UCP2 and UCP3 mRNA levels in L6 myotubes stimulated by epinephrine for 3–24 h. The UCP2 mRNA level began to increase at 3 h and reached rather steady levels at 9–24 h (Fig. 1). The UCP3 mRNA level was also increased at 6 h, but decreased to the basal level at 12–24 h. Thus, epinephrine stimulation of L6 myotubes induced mRNA expression of both UCP2 and UCP3 in 6–24 h. In the following experiments, L6 myotubes were stimulated by adrenergic agonists for either 6 or 24 h.

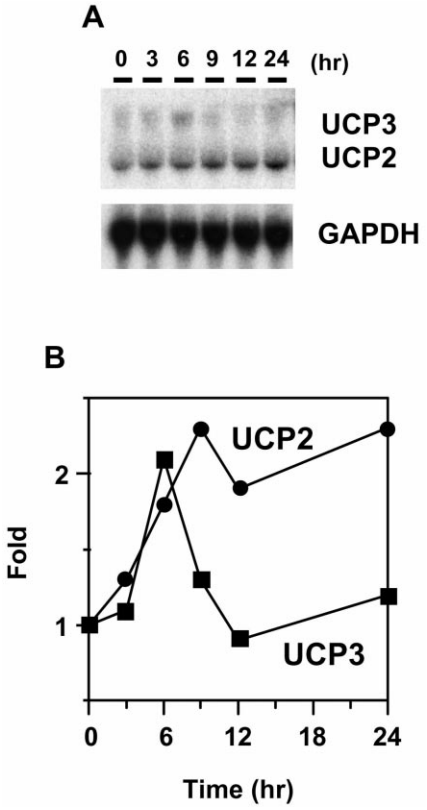

Fig. 1. Time-dependent effects of epinephrine on UCP2 and UCP3 mRNA expression in L6 myotubes. L6 myotubes were treated for 3–24 h with 1 μM epinephrine, and 30 μg of total RNA was used for Northern blot analysis. A: Representative blots. B: The UCP2 and UCP3 mRNA levels were normalized by the GAPDH mRNA level and represented as relative to 0 h. Values are means of two experiments.

3.2. Effects of β-AR stimulation on UCP2 and UCP3 mRNA expression in L6 myotubes

Since mRNA expression of UCP2 and UCP3 in L6 myotubes is known to be up-regulated by ligands of PPAR and RXR [19,22], we next examined the effects of epinephrine on the gene expression in the presence or absence of carbacyclin (a PPAR ligand) and 9-*cis* retinoic acid (an RXR ligand). When L6 myotubes were stimulated by epinephrine for 6 h, the UCP3 mRNA level was remarkably increased even in the presence of carbacyclin and 9-*cis* retinoic acid (Fig. 2A). Similarly, the UCP2 mRNA level was also increased by 6- and 24-

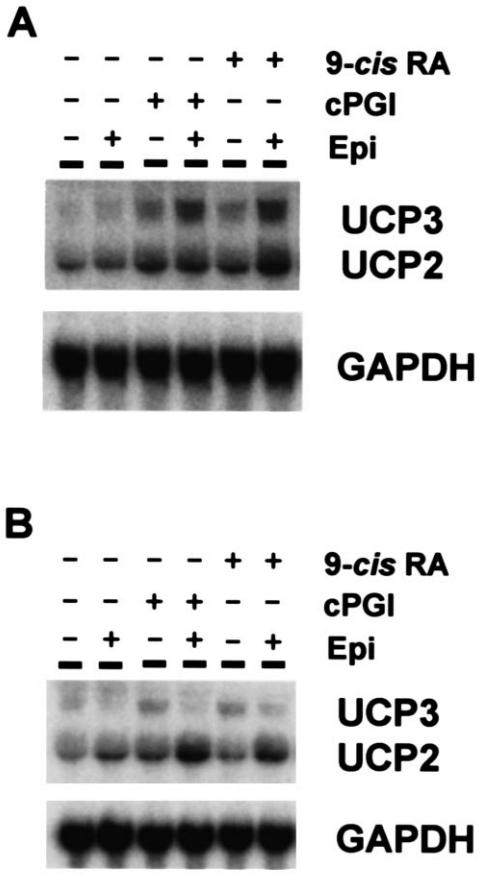

Fig. 2. Effects of epinephrine on UCP2 and UCP3 mRNA expression in the presence or absence of carbacyclin and 9-*cis* retinoic acid. L6 myotubes were treated for either 6 h (A) or 24 h (B) with 1 μM epinephrine (Epi), and for 24 h with 1 μM carbacyclin (cPGI) or 9-*cis* retinoic acid (9-*cis* RA).

h stimulation with epinephrine (Fig. 2A,B). In contrast, in the presence of carbacyclin and 9-*cis* retinoic acid, the 24-h epinephrine stimulation tended to decrease the UCP3 mRNA level, although the changes were not statistically significant in four independent experiments (Fig. 2B).

3.3. *Effects of various β-AR agonists and antagonists on UCP2 and UCP3 mRNA expression in L6 myotubes*

It has been shown that β2-AR is the major subtype responsible for various physiological and pharmacological responses to catecholamines in skeletal muscle [23]. To determine the β-AR subtype(s) participating in the up-regulation of UCP2 and UCP3 in L6 myotubes, we examined the effects of various agonists or antagonists selective for individual β-ARs. As shown in Fig. 3A, the UCP2 mRNA level was increased by a non-selective β-AR agonist (isoproterenol), a selective β2-AR agonist (salbutamol), and also by a β3-AR agonist (BRL 37344) in the presence or absence of carbacyclin. However, it was not changed by an agonist highly specific to β3-AR (CL316,243). The isoproterenol-induced increase in the UCP2 mRNA level was attenuated and completely prevented by a non-selective β-AR antagonist (propranolol) and a β2-AR selective antagonist (ICI-118,551), respectively, but not by a β1-AR selective antagonist (CGP-20712A) (Fig. 4A). Similar but less clear effects of β-AR agonists (Fig. 3B) and antagonists (Fig. 4B) were also observed on the UCP3 mRNA level. The UCP2 and UCP3 mRNA levels were also increased when the L6 myotubes were treated with forskolin and dibutyryl-cAMP for 24 h and 6 h, respectively, in the presence of carbacyclin or 9-*cis* retinoic acid (data not shown). These results suggested that the epinephrine-induced UCP2 and

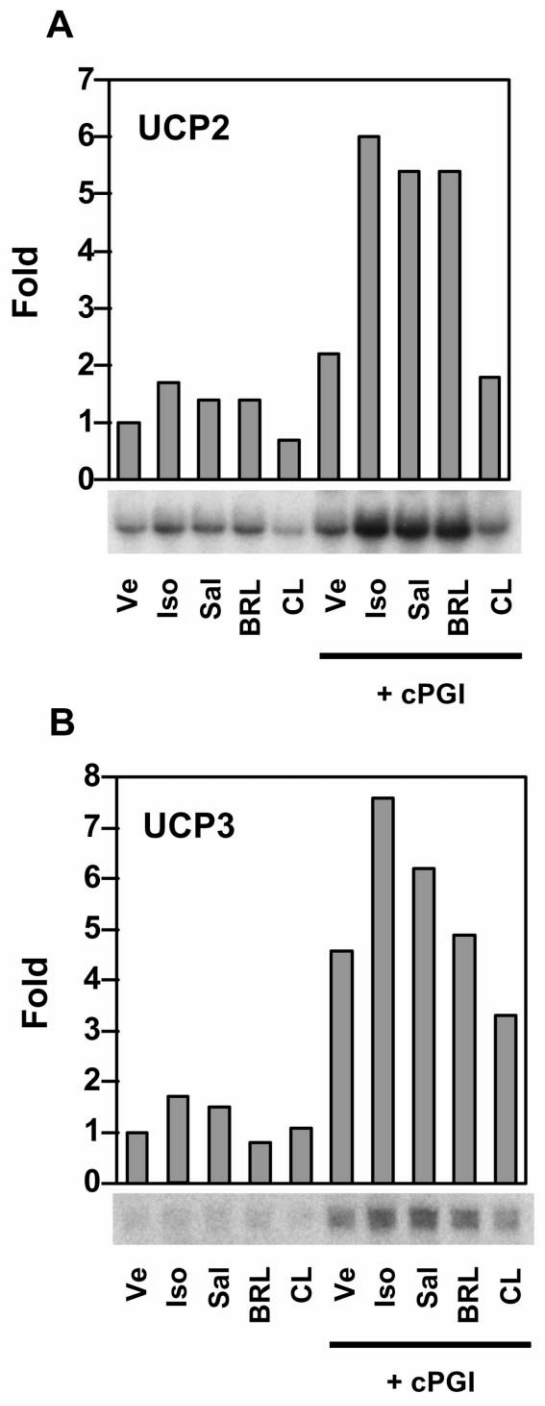

Fig. 3. Effects of various β-AR agonists on UCP2 and UCP3 mRNA expression in L6 myotubes. L6 myotubes were treated with 1 μM β-AR agonists for 24 h (A) or 6 h (B) in the 24-h presence or absence of 1 μM carbacyclin (cPGI). The UCP2 and UCP3 mRNA levels were normalized by the GAPDH mRNA level and represented as relative to untreated vehicle controls. Values are means of two experiments. Ve, vehicle; Iso, isoproterenol; Sal, salbutamol; BRL, BRL 37344; CL, CL316,243.

UCP3 mRNA expression was mediated largely through β2-AR.

3.4. β2-AR in L6 myotubes

To determine the subtypes of β-AR in L6 cells, mRNA expression of β1-, β2- and β3-AR in L6 myoblasts and myotubes was examined by RT-PCR analysis. As shown in Fig. 5A, β2-AR mRNA was detected in both myoblasts and myotubes, while β1- and β3-AR mRNAs were below detectable levels. To confirm the functional activity of β2-AR, we also measured the cAMP level in L6 myotubes after stimulation by various agonists (Fig. 5B). Salbutamol stimulated cAMP accumulation most effectively, showing a half-maximal dose (ED_{50}) of 1.1×10^{-6} M. BRL 37344 was much less effective, and CL316,243 was not effective at all even at higher doses.

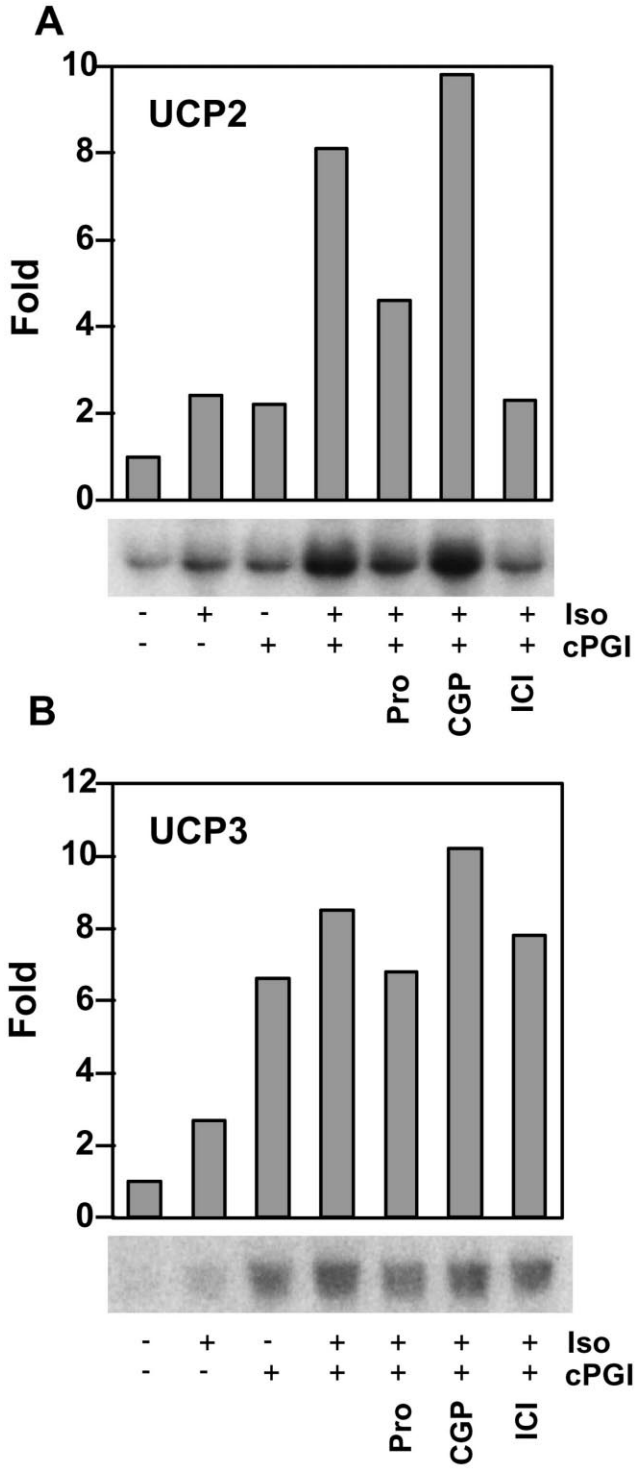

Fig. 4. Effects of various β-AR antagonists on UCP2 and UCP3 mRNA expression in L6 myotubes. L6 myotubes were treated with 1 μM isoproterenol (Iso) and 10 μM β-AR antagonists for 24 h (A) or 6 h (B) in the 24-h presence of 1 μM carbacyclin (cPGI). The UCP2 and UCP3 mRNA levels were normalized by the GAPDH mRNA level, and represented as relative to untreated controls. Pro, propranolol; CGP, CGP-20712A; ICI, ICI-118,551.

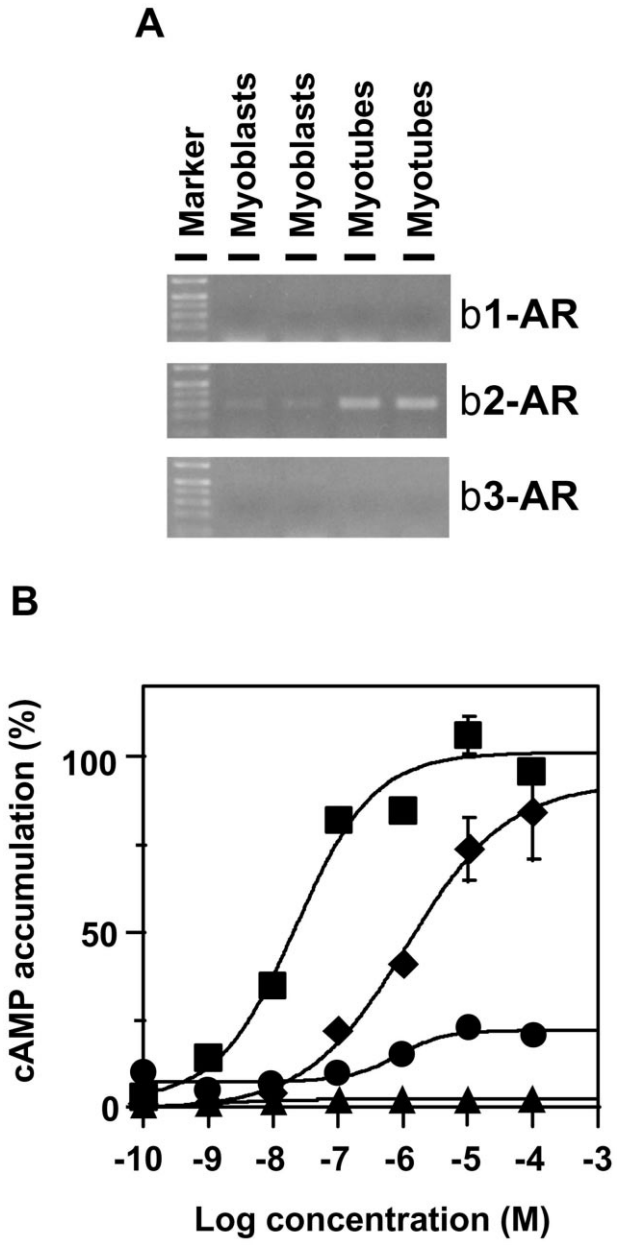

Fig. 5. β-Adrenergic receptors in L6 myotubes. A: mRNA expression of individual β-adrenergic receptor (β-AR) subtypes was analyzed by RT-PCR for L6 myoblasts and myotubes. B: Effects of various β-AR agonists on cAMP accumulation in L6 myotubes. ■ isoproterenol, ♦ salbutamol, ● BRL 37344, ▲ CL316,243. Values are means ± S.E.M. for three independent experiments.

4. Discussion

It has been reported that mRNA expression of UCP2 and UCP3 in myocyte cell lines such as L6 and C2C12 is up-regulated by ligands of PPAR and RXR [19,22,24]. This was confirmed in the present study: that is, the mRNA levels of UCP2 and UCP3 were increased when L6 myotubes were treated with carbacyclin (a PPAR ligand) and 9-*cis* retinoic acid (an RXR ligand). Furthermore, in this study, we demonstrated for the first time the direct effects of β-AR stimulation on UCP2 and UCP3 expression in vitro. The major findings were: (1) stimulation of the cells by epinephrine increased the UCP2 and UCP3 mRNA levels, regardless of the presence or absence of carbacyclin and 9-*cis* retinoic acid, and (2) the up-regulatory effects of catecholamines on UCP2 and UCP3 were mediated through the β2-AR pathway.

Our results clearly showed that UCP2 and UCP3 were induced in L6 myotubes stimulated by epinephrine, a physiological stimulator of β-AR in skeletal muscle. The effect of epinephrine was observed even in the absence of carbacyclin and 9-*cis* retinoic acid, indicating that β-adrenergic stimulation is able to induce UCP2 and UCP3 independently of the stimulatory action on nuclear receptors such as PPARs and RXR. These in vitro findings suggested that the effects of cold exposure and β-AR agonist administration on muscle UCP2 and UCP3 so far reported in vivo are attributable, at least in part, to the direct action of catecholamines (and β-AR agonists) on skeletal muscle. However, it is possible that the UCP2 and UCP3 induction in vivo is not only due to the direct action on muscle cells but also is mediated indirectly through some stimulatory factors. Plasma-free fatty acids are the most likely candidates for these factors, because they have agonistic activity for PPARs and their plasma level rises in response to β-AR agonist administration and cold exposure. In fact, various types of fatty acids including non-metabolizable fatty acid derivatives have been reported to induce UCP2 and UCP3 in myocytes in vivo and in vitro [18,19,24].

The analyses of mRNA expression and cAMP accumulation in L6 myotubes revealed that β2-AR was the major AR subtype present in this cell line. Moreover, the epinephrine-induced UCP2 and UCP3 expression was mimicked by a β2-AR agonist, but prevented by a β2-AR antagonist. All these results indicate the pivotal involvement of β2-AR in the adrenergic stimulation of UCP2 and UCP3 expression in myotubes. In the present study, BRL 37344 was also effective in UCP2 induction and also in cAMP accumulation in L6 myotubes, although this compound is recognized as a β3-AR agonist. Considering that a highly specific β3-AR agonist, CL316,243, was not effective at all, it is likely that the effects of BRL 37344 may be mediated through β2-AR or other receptors such as a putative β4-AR. Possible involvement of an as yet unidentified β-AR was also suggested by Boss et al. [17], who observed that administration of BRL 37344 and CGP-12177 (a β1- and β2-AR antagonist and β3-AR agonist) increased mRNA expression of UCP2 and UCP3 in skeletal muscle even in β3-AR-deficient mice.

Lin et al. [13] reported in rats that cold exposure increased muscle UCP3 mRNA rapidly between 6 and 24 h, and UCP2 mRNA later between 1 and 3 days. Similar rapid and transient induction of UCP3 and delayed induction of UCP2 were also found in the present in vitro study. These observations suggest some different mechanisms and factors are involved in the regulation of mRNA expression of UCP2 and UCP3. Yoshitomi et al. [25] demonstrated the presence of the cAMP response element (CRE) in the 5′-flanking region of the mouse UCP2 gene, and actual induction of UCP2 by dibutyryl-cAMP and by 8-bromo-cAMP in 3T3-L1 adipocytes. In addition, Acin et al. [26] reported CRE-like elements in the 5′-flanking region of the human UCP3 gene. Thus, one of the possible mechanisms of UCP2 and UCP3 induction in L6 myotubes is serial activation of β2-AR-linked adenylate cyclase, cAMP-dependent protein kinase (PKA) and CRE binding protein, which binds directly to the CRE in the UCP2 and UCP3 gene promoter regions, although its presence has not been confirmed in the rat. Alternatively, it is also possible that some additional factors may be involved in UCP2 and UCP3 induction, as in the case of cAMP-induced UCP1 expression in brown adipocytes, where cAMP-dependently induced PPAR gamma coactivator-1 (PGC-1) acts as a coactivator of PPARs. In fact, it was reported that stimulation of brown adipocytes with agonists for β-AR and RXR increases PGC-1 expression [27], and that overexpression of PGC-1 induces UCP2 in C2C12 myotubes [28,29]. In preliminary experiments, to examine the possible involvement of PGC-1 in myocytes, we measured the PGC-1 mRNA level in L6 myotubes by RT-PCR analysis. However, unlike in brown adipocytes, in L6 myotubes the mRNA level of PGC-1 was not increased by stimulation with β-AR and RXR, suggesting a different role of PGC-1 in L6 myotubes. Since PGC-1 has a phosphorylated site by PKA [27], PKA may change the activity of PGC-1 in relation to other nuclear receptors such as PPARs. Further studies are needed to clarify the mechanisms in the β-AR-mediated UCP2 and UCP3 induction in myotubes.

In conclusion, β-AR stimulation, in addition to PPAR stimulation, up-regulates UCP2 and UCP3 expression in L6 myotubes. This may be related, at least in part, to thermogenesis in skeletal muscle induced by catecholamines in vivo.

Acknowledgements: We thank American Cyanamid Co. for providing CL316,243. We also wish to thank Dr. Kazuhiro Kimura (Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University) for his helpful discussion. This work was supported in part by PROBRAIN (Program for Promotion of Basic Research Activities for Innovative Biosciences).

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