Expression of Uncoupling Protein in Skeletal Muscle and White Fat of Obese Mice Treated with Thermogenic β3-Adrenergic Agonist

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Abstract

The mitochondrial uncoupling protein (UCP) is usually expressed only in brown adipose tissue (BAT) and a key molecule for metabolic thermogenesis. The effects of a highly selective β3-adrenergic agonist, CL316,243 (CL), on UCP expression in skeletal muscle and adipose tissues were examined in mice. Daily injection of CL (0.1 mg/kg, sc) to obese yellow KK mice for two weeks caused a significant reduction of body weight, associated with a marked decrease of white fat pad weight and hypertrophy of the interscapular BAT with a sixfold increase in UCP content. Clear signals of UCP protein and mRNA were detected by Western and Northern blot analyses in inguinal, mesenteric and retroperitoneal white fat pads, and also in gastrocnemius and quadriceps muscles, whereas no signal in saline-treated mice. The presence of UCP mRNA in muscle tissues was also confirmed by reverse transcription-PCR analysis. Weaker UCP signals were also detected in control C57BL mice treated with CL, but only in inguinal and retroperitoneal fat pads. Immunohistochemical examinations revealed that UCP stains in the white fat pads were localized on multilocular cells quite similar to typical brown adipocyte, and those in the muscle tissues on myocytes. The mitochondrial localization of UCP in myocytes was confirmed by immunoelectron microscopy. In addition to UCP protein, UCP mRNA was also detected in myocytes by in situ hybridization analysis. Thus, chronic stimulation of the β3-adrenergic receptor induces ectopic expression of UCP in adipose tissues conventionally considered as white fat and even in skeletal muscle, which probably contributes to the potent anti-obesity effect of the β3-adrenergic agonist. (*J. Clin. Invest.* 1996. 97:2898–2904.) Key words: brown adipose tissue • immunohistochemistry • obesity • yellow KK mice

Introduction

Heat production in brown adipose tissue (BAT)[1] is a significant component of whole body energy expenditure, at least in small rodents, and its dysfunction contributes to the development of obesity (1). BAT thermogenesis is directly controlled by sympathetic nerves distributed to this tissue principally through the β-adrenergic action of norepinephrine. It is now established that there are three isoforms of β-adrenergic receptor (AR) in brown adipocytes, β1-, β2-, and β3-ARs (2). Among them, recent interests have been concentrated mostly on the β3-AR, because this isoform is expressed primarily, but not exclusively, in brown and white adipocytes. The presence of β3-AR, different from the β1- and β2-ARs, was proposed initially in pharmacological studies of lipolysis in white and brown adipocytes (3). The recent cDNA cloning of this receptor from human, mouse and rat sources (4–6) has further promoted the development of agonists specific to the β3-AR, which might be a selective stimulant of lipolysis and BAT thermogenesis and be useful as an anti-obesity drug.

We previously (7) examined the effects of a highly selective β3-AR agonist, CL316,243 (CL), on energy metabolism and adiposity in one of the genetically obese and diabetic animal models, yellow KK mice, showing that chronic administration of CL enhanced oxygen consumption, reduced adiposity, improved glucose tolerance, and increased GDP-binding to BAT mitochondria, an index of the thermogenic activity of BAT. Similar thermogenic and anti-obesity effects of this drug were also reported in rats (8). In contrast to rodents, humans have only minute amounts of BAT, and thereby the contribution of BAT to the regulation of energy balance has been claimed to be much less or negligible in humans. However, this would not always be the case, if the expression of uncoupling protein (UCP), a key molecule for BAT thermogenesis, can be activated in tissues other than BAT by some means. In this context, we have explored the possible expression of UCP in white fat and also in skeletal muscle. We report here, contradicting the well-accepted view of exclusive expression of UCP in BAT (9, 10), that chronic treatment of yellow KK obese mice with CL induces UCP in various fat pads usually considered as white adipose tissue and even in skeletal muscle.

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1. *Abbreviations used in this paper:* AR, adrenergic receptor; BAT, brown adipose tissue; CL, CL316,243; UCP, uncoupling protein; WAT, white adipose tissue.

Methods

Chemicals. CL316,243, disodium (R,R)-5([2-{3-chlorophenyl}-2-hydroxyethyl]-aminoproppyl)-1,3-benzodioxole-2,2-dicarboxylate (11), was provided from American Cyanamid Co. (Pearl River, NY).

Animals. Female yellow KK and C57BL mice (Charles River Japan, Osaka) were obtained at the age of 7 wk and housed in plastic cages at 22±2°C with 12 h light-dark cycle, and given free access to laboratory chow (CE-2; Clea Japan, Tokyo) and tap water. They were given a subcutaneous injection of either CL 316,243 (0.1 mg/kg) or saline once a day. After 2 wk, mice were killed by cervical dislocation, and skeletal muscle (gastrocnemius), interscapular BAT, and subcutaneous (inguinal) WAT were rapidly removed in their entirety and frozen in liquid nitrogen for Western blot and RNA analyses. The animal care and procedure were approved by the Animal Care and Use Committee of Hokkaido University.

Western blot analysis. Each tissue was homogenized in 5–10 volumes of a solution containing 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4) for 30 s with a Polytron. After centrifugation at 1,500 *g* for 5 min, the fat cake was discarded, and the infranant (fat-free extract) was used for Western blot analysis of UCP as described previously (12, 13). Briefly, the fat-free extract (10–40 μg protein) was solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose filter. After blocking the filter with 6% nonfat dry milk, it was incubated with a rabbit antiserum (×1,000 diluted) against rat UCP purified from interscapular BAT (14). Then, the filter was incubated with [^{125}I]protein A (ICN, Irvine, CA). The dry blot was exposed to x-ray film for autoradiography or an imaging plate of BAS1000 (Fuji Film, Tokyo, Japan).

RNA analysis. Total RNA was extracted from 0.1–1 grams of tissue using TRIzol (GIBCO BRL, Tokyo). For Northern blot analysis, 20 or 40 μg total RNA was electrophoresed on a 1.0% agarose/formaldehyde gel and transferred to and fix on a nylon membrane. A 488-bp cDNA probe corresponding to the coding region of rat UCP (15) was prepared by digesting whole UCP cDNA (a gift from Dr. D. Ricquier, CNRS, Meudon, France) by BamHI, and labeled by random priming with [α-^{32}P]dCTP (ICN). The blots were hybridized to the labeled probe at 42°C for 20 h in the presence of 500 μg/ml salmon sperm DNA, and exposed to x-ray film.

For reverse transcription-PCR analysis, total RNA was digested with DNaseI (Pharmacia, Uppsala, Sweden) in the presence of RNase inhibitor (Toyobo, Tokyo, Japan). After phenol/chloroform extraction and ethanol precipitation, RNA (1 μg) was reverse-transcribed with M-MLV RT (GIBCO BRL, Gaithersburg, MD) for 15 min at 37°C in a solution containing 500 nM reverse specific primer, 0.2 mM of each dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. cDNA were denatured for 5 min at 94°C and submitted to 30 cycles of amplification (1 cycle: 94°C, 1 min; 60°C, 2 min; 72°C, 2 min) in a solution containing 0.5 U of *Taq* polymerase (Wako, Osaka, Japan), 125 μM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$ and 1 μM sense and antisense oligonucleotides. The sense primer corresponded to the region 589–608 of UCP cDNA (5′-GTGAAGGTCAGAATGCAAGC-3′) and the antisense primer to the region 785–766 (5′-AGGGCCCCCTTCATGAGGTC-3′). The amplification product, whose expected size is 196 bp, was analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide.

Immunohistochemistry. Mice treated with CL or saline for two weeks were anesthetized with ethyl ether and perfused transcardially with 4% paraformaldehyde in PBS, and the fat pads and gastrocnemius muscle were removed and fixed in Bouin solution. The fixed tissues were dehydrated in ethanol, paraffin-embedded, and cut into 7-μm-thick sections. The dewaxed sections were incubated in 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity, and then with 10% normal goat serum, the rabbit antiserum against rat UCP (×500 diluted), goat anti–rabbit IgG (×400 diluted, Vector Laboratories, Burlingame, CA), and finally with avidin-biotin-peroxidase complex (Vector Laboratories) according to the conventional ABC method. The sections were also counter-stained with hematoxylin and examined by a light microscope.

For electron microscopic observations, specimens of gastrocnemius muscle fixed in 1% glutaraldehyde −0.1 M phosphate buffer (pH 7.4) were dehydrated in dimethylformamide, and embedded in Lowicry K4M (Chemische Werke Lowi, Waldkaiburg, Germany) in gelatin capsules polymerized by ultraviolet irradiation. Ultrathin sections were mounted on nickel grids, immersed in 2% normal goat serum in PBS for 1h, and treated with the rabbit antiserum against rat UCP (×100 diluted) in PBS containing 1% BSA overnight in a moist chamber at 4°C. After being well washed with PBS, the sections were treated with colloidal gold-labeled anti–rabbit IgG F(ab′)2 (10 nm; Bio Cell, Cardiff, UK) diluted at 1:15 in PBS containing 1% BSA for 2 h at room temperature, postfixed with 2.5% glutaraldehyde for 10 min, and stained with uranyl acetate for 15 min and lead citrate for 1 min. In controls, non-immune rabbit serum was substituted for the primary antiserum.

In situ hybridization. Tissue specimens obtained from mice transcardially perfused with 4% paraformaldehyde-PBS were postfixed in the same solution overnight, immersed in 20% sucrose–0.1 M phosphate buffer (pH 7.4), frozen with dry ice, and cut into 12-μm sections. Sections were digested with 20 μg/ml proteinase K (Boehringer Mannheim, Germany), acetylated with acetic anhydride, delipidized with chloroform, and dried with air. Digoxygenin (DIG)-labeled sense and antisense riboprobes for UCP mRNA were prepared by transcription of plasmids inserted with the 196-bp RT-PCR product (see *RNA analysis*) by using T7 or SP6 RNA polymerase as described in the manual of an RNA labeling kit (Boehringer Mannheim). The tissue sections were hybridized for 20 h at 55°C in a solution containing 50% formamide, 10% dextran sulfate, 10 mM Tris-HCl (pH 7.6), 1× Denhardt's solution, 0.6 M NaCl, 0.25% SDS, 1 mM EDTA, 200 μg/ml tRNA and 75 ng/50 μl antisense or sense DIG-labeled riboprobes. After the sections were washed with 2× SSC/50% formamide, they were treated with 40 μg/ml RNaseA (Sigma Chemical Co., St. Louis, MO), and incubated with alkaline phosphatase-conjugated anti-DIG serum, and then with nitroblue tetrazolium and 5-bromo-4chloro-3-indolyl phosphate for color development (Boehringer Mannheim). The sections were counterstained with hemtoxylin and observed by light microscopy.

Data analysis. Values are expressed as means±SE for 5–10 mice. Biochemical and histological analyses of UCP protein and mRNA were repeated using tissue samples obtained from at least four mice, and the representative results are shown in figures.

Results

Obese yellow KK and control C57BL mice, whose respective body weights were about 30 and 17 grams, were given CL (0.1 mg/kg per day) or saline once a day. After 2 wk, yellow KK mice treated with CL, compared with those injected with saline, weighed less (30.4±0.6 vs. 36.9±0.8 grams) and had smaller white fat pads (2.70±0.17 vs. 4.98±0.19 grams of retroperitoneal, mesenteric and inguinal fat pads). The CL-treatment also produced a hypertrophy of the interscapular BAT with about 1.8- and 6-fold increases in protein and UCP contents, respectively, but had no effects on the tissue weight and protein content of gastrocnemius muscle. Similar effects of CL were also observed in C57BL mice, but to less extents (17.4±0.2 grams vs. 17.6±0.2 grams of body weight, and 0.98±0.14 grams vs. 1.12±0.05 grams of white fat pads). These results are well consistent with our previous observations (7), confirming the anti-obesity effect of CL.

There is a report (16) that β3-AR agonists not only produce hypertrophy of typical BAT associated with a preferential increase in UCP, but also evoke an apparent expression of

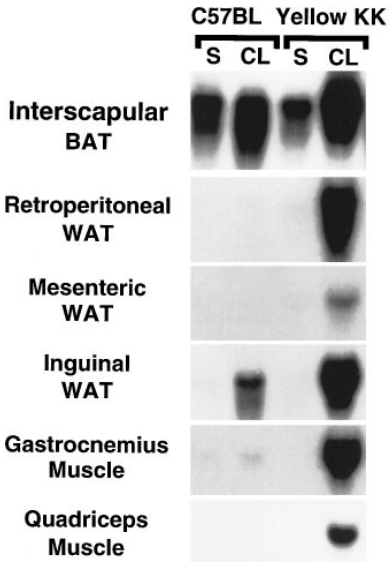

Figure 1. Northern blot analysis of UCP mRNA of fat pads and muscles. Yellow KK obese or C57BL control mice were given CL or saline (S) for 2 wk, and total RNA (20 μg from BAT, 40 μg from other tissues) were used for Northern blot analysis.

UCP in abdominal (periovarian) fat pads usually considered as white adipose tissue. To explore the possibility of such ectopic expression of UCP, mRNA of UCP was examined by Northern blot analysis of total RNA extracted from various regions of fat pad and also from skeletal muscle (Fig. 1). In mice given saline, no UCP signals was detected in all samples except BAT, confirming the well-accepted view that UCP is unique to brown adipocyte. In contrast, in yellow KK mice treated with CL, a clear band smaller than 18S ribosomal RNA was detected not only in BAT but also in retroperitoneal, mesenteric and inguinal fat pads. In addition, a similar band was also detected even in gastrocnemius and quadriceps muscles. The size of these bands was almost identical to that of BAT. To confirm the presence of UCP mRNA in the tissues other than BAT, the samples of total RNA were analyzed by reverse transcription-PCR. A clear PCR product with the expected size (196 bp) was detected in the samples from white fat pads, and also from gastrocnemius muscle of yellow KK mice treated with CL (Fig. 2). In contrast, no specific PCR product was detected in gastrocnemius muscle and white fat pad of saline-treated mice.

Western blot analysis of UCP protein. To determine whether the UCP protein is present not only in adipose tissues but also in skeletal muscle, a crude fat-free extract of the tissue was applied to Western blot analysis. As shown in Fig. 3, a clear protein band was detected at ~ 33 kD in white fat pads and skeletal muscle of Yellow KK mice treated with CL. The molecular

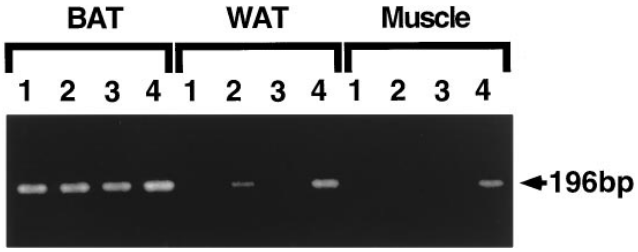

Figure 2. Reverse transcription-PCR analysis of UCP mRNA. Total RNA used in Fig. 1 was reverse transcribed and amplified. *Muscle*, gastrocnemius muscle. Lane *1*, saline-treated C57BL; lane *2*, CL-treated C57BL; lane *3*, saline-treated yellow KK; and lane *4*, CL-treated yellow KK.

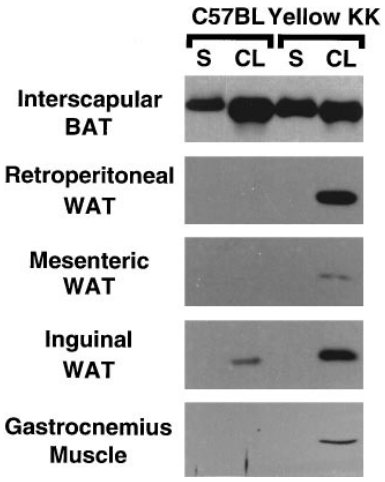

Figure 3. Western blot analysis of UCP. Mice were treated as in Fig. 1, and fat-free extracts containing 10 μg protein of BAT, 20 μg of WAT, and 40 μg of muscle were used for Western blot analysis.

size of this protein was identical to a strong UCP signal present in BAT. In contrast, in Yellow KK mice treated with saline, no band was detected in all tissues except BAT. The UCP protein in skeletal muscle was not detected in C57BL mice regardless of whether they were treated with CL or not.

Histological localization of UCP protein and mRNA. The localization of UCP protein was examined immunohis-

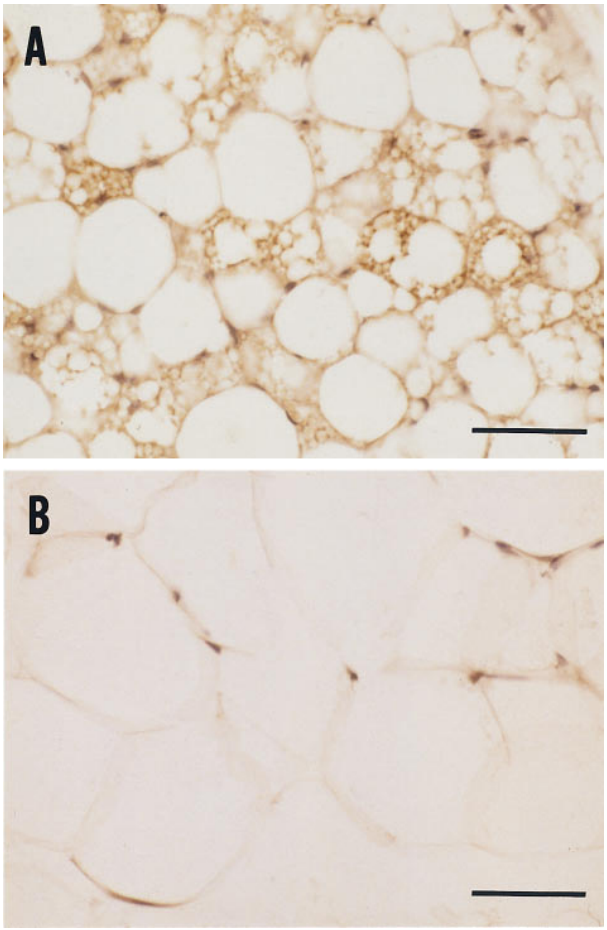

Figure 4. Immunohistochemical detection of UCP in white fat pad. Sections of inguinal fat pad of a yellow KK mouse treated with CL (*A*) or saline (*B*) were stained by a rabbit antiserum against rat UCP. Bar, 50 μm.

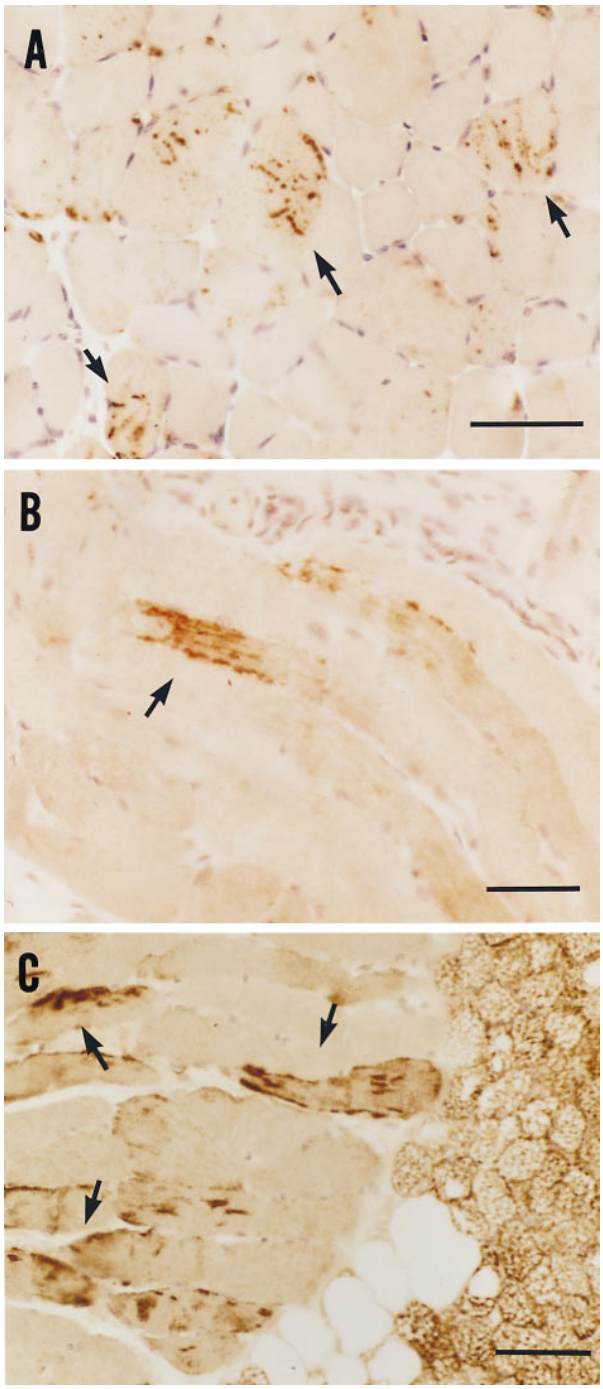

Figure 5. Immunohistochemical detection of UCP in skeletal muscle. A transverse section of gastrocnemius muscle (*A*), and longitudinal sections of gastrocnemius (*B*), and quadriceps (*C*) muscles of a yellow KK mouse treated with CL were stained by a rabbit antiserum against rat UCP. Sections of gastrocnemius muscle were also counterstained with hematoxylin. Arrows show typical UCP signals in myocytes. In a quadriceps muscle section (*C*), strong UCP signals were detected both in myocytes and adipocytes. Bar, 50 μm.

tochemically using the same anti-UCP serum used for Western blot analysis. Macroscopically, fat pads of CL-treated yellow KK mice looked less pale compared with the tissues of saline-treated mice. Histochemically, the tissue of saline-treated mice was composed of unilocular cells filled with a single large lipid droplet, and was always negative for UCP (Fig. 4). In contrast, many multilocular cells, which were positive for UCP, were found in the tissues of CL-treated mice. Unilocular cells were also present in the tissue but always negative for UCP, and their size was smaller than the typical cells found in saline-treated mice. Thus, CL-treated mice had lots of typical brown adipocytes in the fat pads usually considered as white adipose tissue.

Skeletal muscles were also examined immunohistochemically at both light and electron microscopic levels. As shown in Fig. 5, both longitudinal and transverse sections of the gastrocnemius muscle of CL-treated yellow KK mice were clearly stained with anti-UCP serum. The positive regions were in the myocytes, being not limited in the intermuscular adipocytes. No stains were detected when a preimmune serum or the anti-UCP serum preincubated with purified UCP was used. Sections from saline-treated yellow KK mice and C57BL mice were always negative for UCP (data not shown). Intracellular localization of UCP protein in myocytes was also determined by electron microscopy using a gold-labeled anti-UCP antiserum. As shown in Fig. 6, UCP-gold particles were detected predominantly over the mitochondria of the myocyte of CL-treated yellow KK mice. No gold particles were found in myocyte mitochondoria of saline-treated yellow KK mice and C57BL mice (data not shown).

Localization of UCP mRNA in skeletal muscles was examined by in situ hybridization using antisense and sense riboprobes for UCP mRNA. The longitudinal sections of the gastrocnemius muscle of Cl-treated yellow KK mice were densely stained by the antisense probe, but not by the sense probe (Fig. 7). No sections of saline-treated mice were stained even by the anitisense probe.

Discussion

The present results confirmed the previous observation (7) that the treatment with Cl, a highly selective β3-adrenergic agonist, ameliorated the excessive accumulation of body fat in genetically obese yellow KK mice. The major findings of the present study were, (*a*) The CL-treatment not only increased the expression of UCP in BAT but also evoked an ectopic expression of UCP in fat tissues conventionally considered as white adipose tissue and even in skeletal muscle, and (*b*) the effects of CL were more apparent in the obese mice than lean control C57CL mice.

It has been established that UCP is expressed exclusively in matured brown adipocyte, and thereby is a molecular marker of this specifically differentiated cell (9, 10). In fact, Northern and Western blot analyses of various tissues of mice treated with saline revealed that UCP mRNA and protein were found only in BAT. This was confirmed by the reverse transcription-PCR analysis, a much more sensitive method to detect UCP mRNA. However, in mice treated with CL, clear signals of UCP were detected not only in BAT but also in white adipose tissue and even in skeletal muscle. It is unlikely that the signals found in these tissues may be derived from some mRNA and protein other than UCP, because their molecular sizes were identical to those found in BAT. Ricquier et al. (17) reported the appearance of a false signal of UCP mRNA in rat liver when hybridized with the probes of whole UCP cDNA and its 3′ noncoding region. To avoid such uncertainty, we used a cDNA probe corresponding to the coding region of UCP

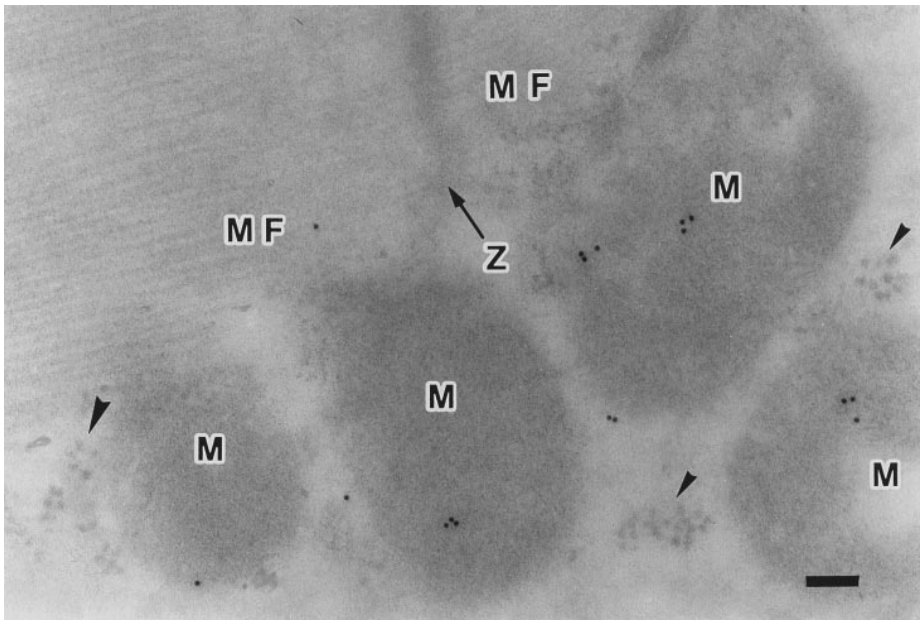

Figure 6. Immunoelectron microscopy of UCP in skeletal muscle. An ultrathin section of gastrocnemius muscle of a yellow KK mouse treated with CL was stained by a rabbit antiserum against rat UCP and colloidal gold-labeled anti-rabbit IgG F(ab′)2 (10 nm). *M*, mitochondrion; *MF*, myofibrill; *Z*, Z-band. Arrowheads indicate glycogen particles. Bar, 0.1 μm.

cDNA. A similar ectopic expression of UCP was reported in various fat pads other than typical BAT, particularly in periovarian fat depots, of rats exposed to cold (16). Immunocytochemical examinations showed that these fat pads, which had been considered conventionally as white fat, contained cells indistinguishable from typical brown adipocyte. Himms-Hagen et al. (8) also reported that the mesenteric and intraabdominal white fat depots contained numerous multilocular brown adipocyte-like cells in rats treated with CL. The reappearance of UCP in adipose depots was also demonstrated in adult dogs treated with another β3-AR agonist, ICI D7114 (18). Our results are quite consistent with these previous observations: that is, in mice treated with saline, the retroperitoneal, inguinal, and mesenteric fat depots were composed exclusively of typical unilocular white adipocytes and were always negative for UCP, whereas in mice treated with CL the fat depots contained numerous multilocular cells positive for UCP. Thus, β3-AR agonist treatment results in the appearance of well-differentiated brown adipocytes expressing UCP in adipose depots usually considered to be white fat. The origin of brown adipocytes in the white fat could be explained principally by either a new recruitment of preadipocytes to the tissue or a conversion of adipocyte phenotypes from white to brown. Although it is rather difficult to discriminate these two possibilities immediately, the results suggest that the classification of white and brown adipose tissues is a matter of expedience but not an absolute one. Actually, the conversion of BAT to white fat has often been observed in various types of obese animal (19, 20).

The most surprising finding of the present study was the appearance of UCP in skeletal muscle. Northern and Western blot analyses demonstrated a significant expression of UCP in gastrocnemius and quadriceps muscles of yellow KK mice treated with CL. Because the signals were detected only in yellow KK obese mice, it seems possible that the UCP signals are derived from adipocytes present in the muscle tissues. In fact, there were many UCP-positive brown adipocyte-like cells in the intermuscular fat pads, as in the fat pads of other regions, of the CL-treated obese mice. However, immunohistochemical examinations clearly revealed the UCP signals were not

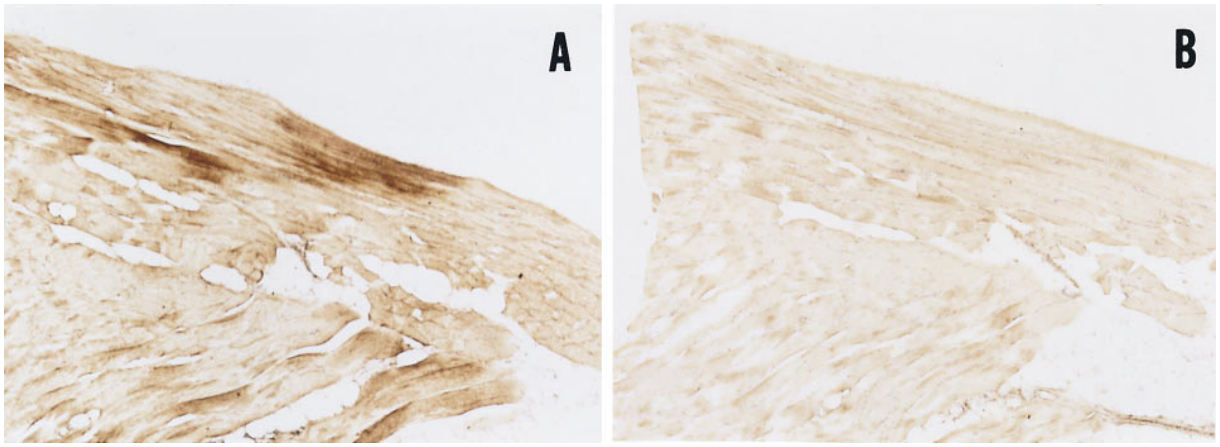

Figure 7. Detection of UCP mRNA in skeletal muscle by in situ hybridization. Longitudinal sections of gastrocnemius muscle of a yellow KK mouse treated with CL were hybridized by an antisense (*A*) and a sense (*B*) riboprobes.

limited in adipocytes but also in muscle cells. The expression of UCP in myocytes was also confirmed by in situ hybridization analysis. As far as we know, there has been no report of UCP expression in any cells other than adipocytes even after prolonged stimulation by, for example, cold exposure or treatment with adrenergic agonists. It should be noted, however, most studies so far reported used normal mice or rats. In fact, in the present study, we also confirmed the lack of UCP in skeletal muscle in control C57BL mice even after the treatment with CL. The agonist-induced expression of UCP in white fat depots and in BAT were also much more apparent in the obese mice. Moreover, in preliminary experiments, we could not detect UCP in skeletal muscle of rats treated with CL. Thus, our results indicate that obese mice, at least yellow KK mice, are much more sensitive to the adrenergic stimulation of UCP expression.

Concerning the cellular mechanism of CL-induced UCP expression in skeletal muscle, only fragmental and conflicting information is available at present. For example, using a human β3-AR cDNA probe, Emorine et al. (4) detected a hybridization signal of β3-AR mRNA in poly(A)$^+$ RNA from rat soleus muscle. However, when the probes for mouse and rat β3-AR were used, no hybridization signals were found in total RNA of skeletal muscle of these species (5, 21). Thus, although possible expression of β3-AR in skeletal muscle cannot be completely ruled out, it seems unlikely that CL acts directly on myocytes through the β3-AR. Alternatively, it is also possible that CL induces UCP expression in myocytes through some indirect action. In fact, it has been suggested that the insulinotropic and anorexic effects of β3-adrenergic agonist in vivo are not due to direct actions on the pancreas and brain (3, 22). Further studies are needed to clarify the relationship between β3-AR and UCP expression in skeletal muscle.

Although we have no evidence at present that UCP expressed in skeletal muscle is functionally active, the localization of UCP in myocyte mitochondria suggests that myocyte UCP is also capable of uncoupling between mitochondrial substrate oxidation and ATP formation in the same way as in brown adipocyte. We (7) demonstrated previously that CL-treatment increases the resting metabolic rate by 75% in C57BL mice whereas by more than 110% in yellow KK mice. In parallel with this, CL-treatment not only increased the total amount of UCP in BAT more in yellow KK mice but also evoked UCP expression in white fat pad and skeletal muscle almost selectively in yellow KK mice. It is thus likely that the ectopic expression of UCP contributes to the increased energy expenditure and the effectiveness of β3-adrenergic agonist in reducing body fat of obese animals.

In conclusion, chronic stimulation of β3-AR in mice can induce expression of UCP in white fat and skeletal muscle, which may contribute to the anti-obesity effect of β3-adrenergic agonists. Unlike in rodents, the contribution of BAT to whole body energy expenditure has been considered to be negligible in humans because of the apparent lack of BAT and UCP in adults. However, the present findings implies that the expression of UCP gene, which is usually completely suppressed in all cells except brown adipocyte, can be activated in white adipocytes and even in myocytes by β3-adrenergic stimulation. Although it remains to be examined whether this is also the case in humans, our results undoubtedly suggest that β3-adrenergic agonists may be hopeful for the treatment of human obesity, as for rodent obesity. This view is supported further by very recent reports demonstrating a significant role of β3-AR in human obesity (23, 24).

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References

1. Himms-Hagen, J. 1989. Brown adipose tissue thermogenesis and obesity. *Prog. Lipid. Res.* 28:67–115.

2. Lafontan, M., and M. Berlan. 1993. Fat cell adrenergic receptors and the control of white and brown fat cell function. *J. Lipid. Res.* 34:1057–1091.

3. Arch, J.R.S., and A.J. Kaumann. 1993. β_3 and atypical β-adrenoceptors. *Medic. Res . Rev.* 13:663–729.

4. Emorine, L.J., S. Marullo, M.M. Briend-Sutren, F. Patey, K. Tate, C. Delavier-Klutchko, and A.K. Strosberg. 1989. Molecular characterization of the human β_3-adrenergic receptor. *Science (Wash. DC).* 245:1118–1121.

5. Nahmias, C., N. Blin, J.M. Elalouf, M.G. Mattei, A.D. Strosberg, and L.J. Emorine. 1991. Molecular characterization of the mouse β_3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3721–3727.

6. Muzzin, P., J.P. Revelli, F. Kuhne, J.D. Gocayne, W.R. McCombie, J.C. Venter, J.P. Giacobino, and C.M. Fraser. 1991. An adipose tissue-specific β-adrenergic receptor; Molecular cloning and down-regulation in obesity. *J. Biol. Chem.* 266:24053–24058.

7. Yoshida, T., N. Sakane, Y. Wakabayashi, T. Umekawa, and M. Kondo. 1994. Anti-obesity and anti-diabetic effects of CL316,243, a highly specific β_3-adrenoceptor agonist, in yellow KK mice. *Life Sci.* 54:491–498.

8. Himms-Hagen, J., J. Cui, E. Danforth, D.J. Taatjes, S.S. Lang, B.L. Waters, and T.H. Claus. 1994. Effect of CL-316,243, a thermogenic β_3-agonist, on energy balance and brown and white adipose tissues in rats. *Am. J. Physiol.* 266: R1371–R1382.

9. Klingenberg, M. 1990. Mechanism and evolution of the uncoupling protein of brown adipose tissue. *Trends Biochem. Sci.* 27:781–791.

10. Klaus, S., L. Casteilla, F. Bouillaud, and D. Ricquier. 1991. The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. *Int. J. Biochem.* 23: 791–801.

11. Bloom, J.D., M.D. Dutia, B.D. Johnson, A. Wissner, M.G. Burns, E.E. Largis, J.A. Dolan, and T.H. Claus. 1992. Disodium (R,R)-5{[2-(3-chlorophenyl)-2-hydroxyethyl]-aminoproppyl}-1,3-benzodioxole-2,2-dicarboxylate (CL316,243). A potent β-adrenergic agonist virtually specific for β_3 receptors. A promising antidiabetic and antiobesity agent. *J. Med. Chem.* 35:3081–3084.

12. Tsukazaki, K., H. Nikami, Y. Shimizu, T. Kawada, T. Yoshida, and M. Saito. 1995. Chronic administration of β-adrenergic agonists can mimic the stimulative effect of cold exposure on protein synthesis in rat brown adipose tissue. *J. Biochem.* 117:96–100.

13. Shimizu, Y., H. Nikami, K. Tsukazaki, U.F. Machado, H. Yano, Y. Seino, and M. Saito. 1993. Increased expression of glucose transporter GLUT-4 in brown adipose tissue of fasted rats after cold exposure. *Am. J. Physiol.* 264: E890–E895.

14. Lin, C.S., and M. Klingengerg. 1980. Isolation of the uncoupling protein from brown adipose tissue mitochondria. *FEBS Lett.* 113:299–303.

15. Bouillaud, F., D. Ricquier, J. Thibault, and J. Weissenbach. 1985. Molecular approach to thermogenesis in brown adipose tissue. cDNA cloning of the mitochondrial uncoupling protein. *Proc. Nat. Acad. Sci. USA.* 82:445–448.

16. Cousin, B., S. Cinti, M. Morroni, S. Raimbault, D. Ricquier, L. Penicaud, and L. Casteilla. 1992. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J. Cell. Sci.* 103:931–942.

17. Ricquier, D., S. Raimbault, O. Champigny, B. Miroux, and F. Bouillaud. 1992. The uncoupling protein is not expressed in rat liver. *FEBS Lett.* 303:103–106.

18. Champigny, O., D.Ricquier, O.Blondel, R.M. Mayers, M.G. Briscoe, and B.R. Holloway. 1991. β_3-Adrenergic receptor stimulation restores message and expression of brown-fat mitochondrial uncoupling protein in adult dogs. *Proc. Natl. Acad. Sci. USA.* 88:10774–10777.

19. Levin, B.E., R.A. Comai, R.A. O'Brien, and A.C. Sullivan. 1982. Abnormal brown adipose composition and β-adrenoreceptor binding in obese Zucker rats. *Am. J. Physiol.* 243:E217–E224.

20. Saito, M., Y. Minokoshi, and T. Shimazu. 1985. Brown adipose tissue after ventromedial hypothalamic lesions in rats. *Am. J. Physiol.* 248:E20–E25.

21. Granneman, J.G., K.N. Lahners, and A. Chaudhry. 1991. Molecular

cloning and expression of the rat β_3-adrenergic receptor. *Mol. Pharmacol.* 40: 895–899.

22. Susulic, V.S., R.C. Frederich, J. Laqitts, E. Tozzo, B.B. Kahn, M.E. Harper, J. Himms-Hagen, J.S. Flier, and B.B. Lowell. 1995. Targeted disruption of the β_3-adrenergic receptor gene. *J. Biol. Chem.* 270:29483–29492.

23. Lonnqvist, F., A. Torne, K. Nilsell, J. Hoffstedt, and P. Arner. 1995. A pathogenic role of visceral fat β_3-adrenoceptors in obesity. *J. Clin. Invest.* 95: 1109–1116.

24. Enocksson, S., M. Shimizu, F. Lonnqvist, J. Nordenstrom, and P. Arner. 1995. Demonstration of an in vivo functional β_3-adrenoceptors in man. *J. Clin. Invest.* 95:2239–2245.