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Differential Regulation of Mouse Uncoupling Proteins among Brown Adipose Tissue, White Adipose Tissue, and Skeletal Muscle in Chronic β_3 Adrenergic Receptor Agonist Treatment

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Uncoupling proteins (UCPs) are inner mitochondrial membrane transporters that dissipate the proton gradient, releasing stored energy as heat, without coupling to other energy-consuming processes. Therefore, the UCPs are thought to be important determinants of the metabolic efficiency. To elucidate relationships between the UCPs expressions and insulin sensitivity improvement, we treated KK-A^y mice with β_3 adrenergic receptor agonist for 21 days and examined the changes of the UCPs mRNA expressions in various tissues. Chronic treatment of a specific β_3 adrenergic receptor agonist, CL316,243 (0.2mg/kg body weight/day s.c.) markedly increased the expressions of uncoupling protein 1 (UCP1), uncoupling protein 2 (UCP2), and uncoupling protein 3 (UCP3) by 14-fold, 6-fold, and 16-fold, respectively, in the brown adipose tissue (BAT). The UCP1 and UCP3 mRNA expressions in the white adipose tissue (WAT) were also increased by 12-fold and 9-fold, respectively, but the UCP2 mRNA expression was not changed in this tissue. Interestingly, the UCP2 and UCP3 mRNA expressions were strikingly decreased in the skeletal muscle and heart. Particularly, the UCP3 mRNA expression level in the skeletal muscle was dropped to 10% of that of the saline-treated control mice, indicating that the UCPs mRNA expressions are regulated in tissue-specific ways. The concentrations of plasma insulin and circulating free fatty acid (FFA) were significantly decreased, suggesting that they correlate with the reductions of the UCP2 and UCP3 mRNA expressions in the skeletal muscle and heart. It has been thought that the UCP1 and UCP3 mRNA expressions in the BAT and WAT are mainly controlled by the hypothalamus via the sympathetic nervous system, while the levels of insulin, FFA or both may play important roles in the control of the UCP2 and UCP3 mRNA expressions in the skeletal muscle and heart.

The biochemical mechanisms responsible for the regulations of energy expenditure and the efficiency of energy usage are poorly understood. To increase the energy expenditure, there are possible ways including physical activity increment and energy dissipation as heat. Nonshivering thermogenesis is a major component of the energy expenditure to control body weight and metabolism. It is the major function of the brown adipose tissue (BAT), found in small mammals such as mice and the young of large mammals (1), which contains uncoupling protein 1 (UCP1), the first uncoupling protein to be identified (2–4). The UCP1 mRNA expression in the BAT is controlled mainly by the hypothalamus via the sympathetic nervous system (5). Norepinephrine binds to β_1 and β_3 adrenergic receptors to initiate a cyclic adenosine 3′, 5′-monophosphate (cAMP) signal transduction pathway to induce the UCP1 mRNA expression (6–9). Indeed, Kozak *et al.* identified several cAMP response elements in the promoter of the mouse UCP1 gene (10), and showed that a 211-bp enhancer called "activator element" located upstream in the UCP1 gene is most important for tissue-specific and β adrenergic regulation of the UCP1 (11). Chronic treatment of β_3 adrenergic receptor agonist decreases weight gain, and improves insulin sensitivity in obese and diabetic animals (12). It suggests that the UCP1 contributes to improvement of insulin resistance, obesity and diabetes.

Recently, uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) were discovered as members of the UCP family (13–16). Being similar to the UCP1, the UCP2 and UCP3 seem to function as uncouplers of oxidative phosphorylation (13, 17), but their tissue distribution is obviously different from that of the UCP1.

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In rodents, the UCP2 is expressed in many tissues including sites not thought to mediate adaptive thermogenesis, such as the spleen, lymph node, thymus, and gastrointestinal tract. On the other hand, the UCP3 is expressed abundantly and preferentially in the skeletal muscle in humans, and in the BAT and skeletal muscle in rodents (13–19). The skeletal muscle and BAT are regarded as important sites for regulated energy expenditure in humans and rodents (20, 21), respectively. Although the UCP2 and UCP3 expressions are modulated by food intake and obesity (17, 22, 23), the roles and regulation mechanisms of these gene in controlling energy balance remain uncertain.

In this study, we administered β_3 adrenergic receptor agonist to KK-A^y mice, which were chosen as a model for obesity and diabetes, to examine the changes of the UCPs mRNA expressions accompanied with the improvement of insulin resistance. We here report the different patterns of the UCPs mRNA expressions among the organs, suggesting that the UCPs mRNA expression are regulated in tissue-specific manners.

MATERIALS AND METHODS

Chemical. A β_3 adrenergic receptor agonist, CL316,243 (disodium (R,R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino] propyl]-1,3-benzodioxole-2,2-dicarboxylate) was synthesized in Tsukuba Research Laboratories of Eisai Co., Ltd., Japan.

Animal treatment. Male, five-week-old KK-A^y/TaJcl mice were purchased from Clea Japan (Tokyo, Japan), and individually caged under conventional conditions. They were provided with commercial diet (F, Oriental Yeast, Tokyo, Japan) and water with free access under the controlled temperature, humidity, and lighting conditions (2 ± 2°C, 55 ± 5 % and a 12-hr light/dark cycle with light on at 07:00). All procedures were conducted according to Eisai Animal Care Committee's guideline.

At 12 weeks of age, the mice were divided into two groups: CL316,243-treated group, n=5 and control group, n=5. The mice were given CL316,243 dissolved in non-pyrogenic saline at a dose of 0.2mg/kg body weight s.c. or the same volume of saline daily. Body weight and food intake were recorded everyday from the start of the treatment, and blood was collected from the caudal vein at Days 0, 3, 7, 14, and 21 in the fed state. Blood glucose, triglyceride (TG), free fatty acid (FFA), lactate and plasma insulin levels were measured with Glu CII-test (Wako, Osaka, Japan), Triglycerid E-test (Wako), NEFA C-test (Wako), Lactate reagent (Sigma, St. Louis, MO), and mouse insulin ELISA (Shibayagi, Shibukawa, Japan), respectively.

Northern blot analysis. At Day 21, two mice of each group were anesthetized by ether and killed by cardiac puncture, and the heart, interscapular BAT, epididymal white adipose tissue (WAT), femoral muscle, and liver were removed. They were immediately soaked in RNA zol B solution (TEL-TEST, Friendwood, TX) and stored at −80°C prior to use. Total RNA was prepared by homogenizing the tissues in 20 ml of the RNA zol B, and its procedures were performed as described by the manufacturer. Fifteen micrograms of total RNA per lane were loaded on a 1.2 % agarose gel containing 2.2 M formaldehyde, and electrophoresis was performed. It was confirmed that equal RNA was loaded for all lanes by ethidium bromide staining. RNA was transferred to nylon membranes (GenePlus, Du Pond, Boston, MA) and crosslinked by ultraviolet irradiation. Hybridization was carried out at 68°C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA), and the blots were washed twice with 0.1SSC/0.1% SDS at 55°C. The probes of the mouse UCPs, leptin, and tumor necrosis factor α (TNFα) were constructed by PCR method, and labeled with random primers in presence of [α-^{32}P]-dCTP. The mouse UCP1, UCP2, UCP3, leptin and TNFα probes correspond to bases 232-1155, 296-1225, 198-1124, 57-560, and 144-851 in Genbank accession numbers U63419, U69135, AB010742, U18812, and M11731, respectively. The filters were exposed to a film at −80°C for 18 to 30 hrs, and band intensity was quantified by a Bioimaging analyzer, BAS2000 system (Fuji Photofilm, Tokyo, Japan).

RT-PCR analysis. In order to reconfirm that mouse UCP2 and UCP3 mRNA expression were down-regulated in the muscle and heart by chronic CL316,243 treatment, we analyzed their expression patterns by RT-PCR method. Total RNAs from the muscle and heart of the animals treated with CL316,243 or saline were reverse-transcripted with Superscript preamplification system (GIBCO BRL, Rockville, MD). The following primers were used to amplify the UCP2 and UCP3 cDNA fragments: mUCP2.p-F (5′-AGT CAA GGG CTA GTG CGC ACC-3′, sense, nt +139 to +159, relative to the first nucleotide of the translation initiation codon) and mUCP2.p-R (5′-CTC CTT CCG GAG CAT GGT AAG-3′, antisense, nt +772 to +792) for the UCP2 cDNA fragment; mUCP3.p-F (5′-AGA ACC CAG GGG CTC AGA GCG-3′, sense, nt +137 to +157) and mUCP3.p-R (5′-TTC CTC CCT GGC GAT GGT TCT-3′, antisense, nt +487 to +507) for the UCP3 cDNA fragment. Mouse β-actin was used for an internal control. PCR amplification conditions were 33 cycles of 30 sec at 94°C, 1 min at 60°C and 1 min at 72°C with a final extension for 10 min at 72°C for the UCP2, and 27 cycles of 30 sec at 94°C, 1 min at 60 and 1 min at 72°C with a final extension for 10 min at 72°C for the UCP3. Amplified fragments were applied on 2.0 % agarose gel and electrophoresed.

Glucose tolerance test. At Day 22 of the treatment, three mice of each group were used for glucose tolerance test. The mice were fasted for 15 hr, and given glucose orally at a dose of 1.5g/kg body weight at 09:00. Blood was taken from the caudal vein at 30, 60, 90, 120, 180, 240, and 300 min after the glucose administration, and subjected to measurement of blood glucose level.

Statistical analysis. Values are indicated as means ± standard deviations. Differences between the groups were assessed by *F*-test, followed by Student's *t*-test or Welch's *t*-test (SAS Institute, Tokyo, Japan). The results were considered significant at $p<0.05$.

RESULT

Effects of β_3 adrenergic receptor agonist on glucose and lipid metabolisms in KK-A^y mice. KK-A^y mice were given CL316,243 or saline once a day for 21 days, and blood glucose, TG, FFA, lactate, and insulin levels were determined in the fed state at Days 0, 3, 7, 14 and 21. As shown in Table 1, the drastic drops of TG and FFA concentrations were observed at Day 3 in the CL316,243-treated mice, and these effects lasted during the examination period (TG, $p<0.001$; FFA, $p<0.001$ at Day 21). The treatment of CL316,243 also significantly lowered non-fasted blood glucose level. The decrease in blood glucose level was apparent at Day 3 and continued up to Day 21, reaching a maximum decrease of about 70% finally ($p<0.001$). Plasma insulin level was also notably reduced in the CL316,243-treated mice, compared with that of the saline-treated control mice ($p<0.001$ at Day 21). Throughout this experiment, no significant differences in body weight, lactate or food intake were observed between the groups.

TABLE 1

Effects of CL316,243 on Body Weight, Food Intake, Blood Glucose, Triglyceride, Free Fatty Acid, Plasma Insulin, and Lactate in KK-A^y Mice

	Saline-treated KK-A^y mice				
	Day 0	Day 3	Day 7	Day 14	Day 21
Body weight (g)	47.94 ± 1.92	47.36 ± 1.45	46.52 ± 1.77	46.40 ± 1.21	46.82 ± 1.57
Food intake (g/day)	6.72 ± 0.40	6.38 ± 0.49	6.74 ± 0.53	7.14 ± 0.42	6.70 ± 0.35
Blood glucose (mg/dL)	488.86 ± 7.04	498.88 ± 25.45	486.80 ± 18.46	483.52 ± 36.28	483.44 ± 27.92
Triglyceride (mg/dL)	362.70 ± 42.11	357.68 ± 23.66	344.46 ± 26.24	342.42 ± 34.85	334.92 ± 22.17
Free fatty acid (mEq/L)	0.816 ± 0.20	0.706 ± 0.10	0.832 ± 0.15	0.913 ± 0.07	0.946 ± 0.09
Insulin (ng/ml)	27.19 ± 6.85	23.70 ± 4.02	24.25 ± 7.10	24.55 ± 6.38	25.64 ± 7.39
Lactate (mg/dL)	20.52 ± 2.84	24.99 ± 3.32	34.66 ± 1.83	29.64 ± 4.61	28.32 ± 3.22
	CL316,243-treated KK-A^y mice				
	Day 0	Day 3	Day 7	Day 14	Day 21
Body weight (g)	47.66 ± 1.88	47.16 ± 1.47	47.74 ± 1.21	46.48 ± 1.78	46.28 ± 1.72
Food intake (g/day)	6.76 ± 0.36	6.82 ± 0.46	6.74 ± 0.84	7.16 ± 0.72	6.72 ± 0.37
Blood glucose (mg/dL)	491.62 ± 4.85	411.72 ± 29.50**	268.46 ± 34.51***	171.70 ± 30.23***	159.68 ± 17.39***
Triglyceride (mg/dL)	336.62 ± 13.83	130.26 ± 33.99***	131.86 ± 15.23***	132.32 ± 16.53***	124.38 ± 18.57***
Free fatty acid (mEq/L)	0.792 ± 0.17	0.420 ± 0.13**	0.455 ± 0.12**	0.485 ± 0.05***	0.482 ± 0.10***
Insulin (ng/ml)	23.41 ± 5.19	17.43 ± 4.41*	12.67 ± 2.98**	9.95 ± 3.54**	6.92 ± 2.08***
Lactate (mg/dL)	23.84 ± 3.77	22.63 ± 3.89	27.53 ± 1.79	22.99 ± 3.98	24.36 ± 2.68

Note. Values are indicated as means ± standard deviations. Differences between the groups were assessed by *F*-test, followed by Student's *t*-test or Welch's *t*-test (**$p < 0.01$, ***$p < 0.001$).

We next studied the effects of CL316,243 on glucose tolerance in KK-A^y mice after the administration for 21 days. The blood glucose level in KK-A^y mice treated with CL316,243 reached a maximum level at 30 min after the glucose administration (243.2 ± 9.3 mg/dL), and then decreased. On the other hand, the blood glucose level of KK-A^y mice treated with saline peaked at 60 to 90 min after the administration (428.4 ± 14.9 mg/dL), and diminished gradually. The overall blood glucose level in the control mice was much higher than that of the drug-treated mice, leading to larger area under the curve (AUC) value. These results showed that the postprandial hyperglycemia and insulin resistance were markedly improved by the CL316,243 treatment (Fig. 1).

Effects of β_3 adrenergic receptor agonist on UCPs mRNA, TNFα mRNA, and leptin mRNA expressions. We investigated the effects of the chronic treatment of the β_3 adrenergic receptor agonist on the mouse UCPs mRNA expressions in KK-A^y mice. In the BAT, CL316,243 markedly increased the UCP1, UCP2 and UCP3 mRNA expressions by 14-fold, 6-fold and 16-fold, respectively. We also found the increase of the UCP1 mRNA expression by 12-fold and that of the UCP3 mRNA by 9-fold in the WAT, but the UCP2 mRNA was not changed in this tissue. Interestingly, the UCP2 and UCP3 expressions were strikingly decreased in the skeletal muscle and heart. Especially, the UCP3 mRNA expression level in the skeletal muscle was dropped to 10% of that of the saline-treated mice. In the liver, none of UCPs expressions were detected (Fig. 2a, b). To reconfirm that the UCP2 and UCP3 mRNA expression were decreased in the skeletal muscle and heart by CL316,243 treatment, we analyzed their expression level by RT-PCR method. As shown in

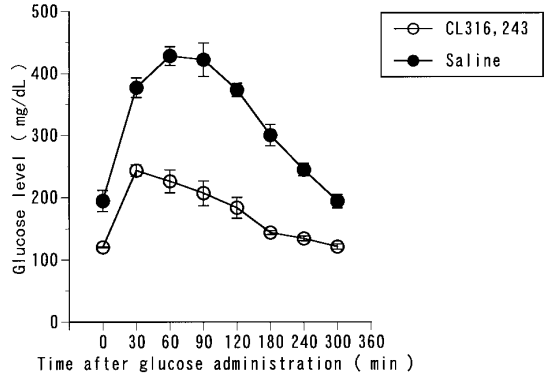

FIG. 1. Effects of β_3 adrenergic receptor agonist on glucose tolerance in KK-A^y mice. The mice were given CL316,243 at a dose of 0.2mg/kg body weight s.c. or the same volume of saline daily for 21 days, and glucose tolerance was assessed as described in *Materials and Methods.* Values represent mean glucose levels (mg/dL) ± standard deviations. (n=5 per treatment in each group). Saline = >KK-A^y mice treated with saline; CL316,243 = KK-A^y mice treated with CL316,243.

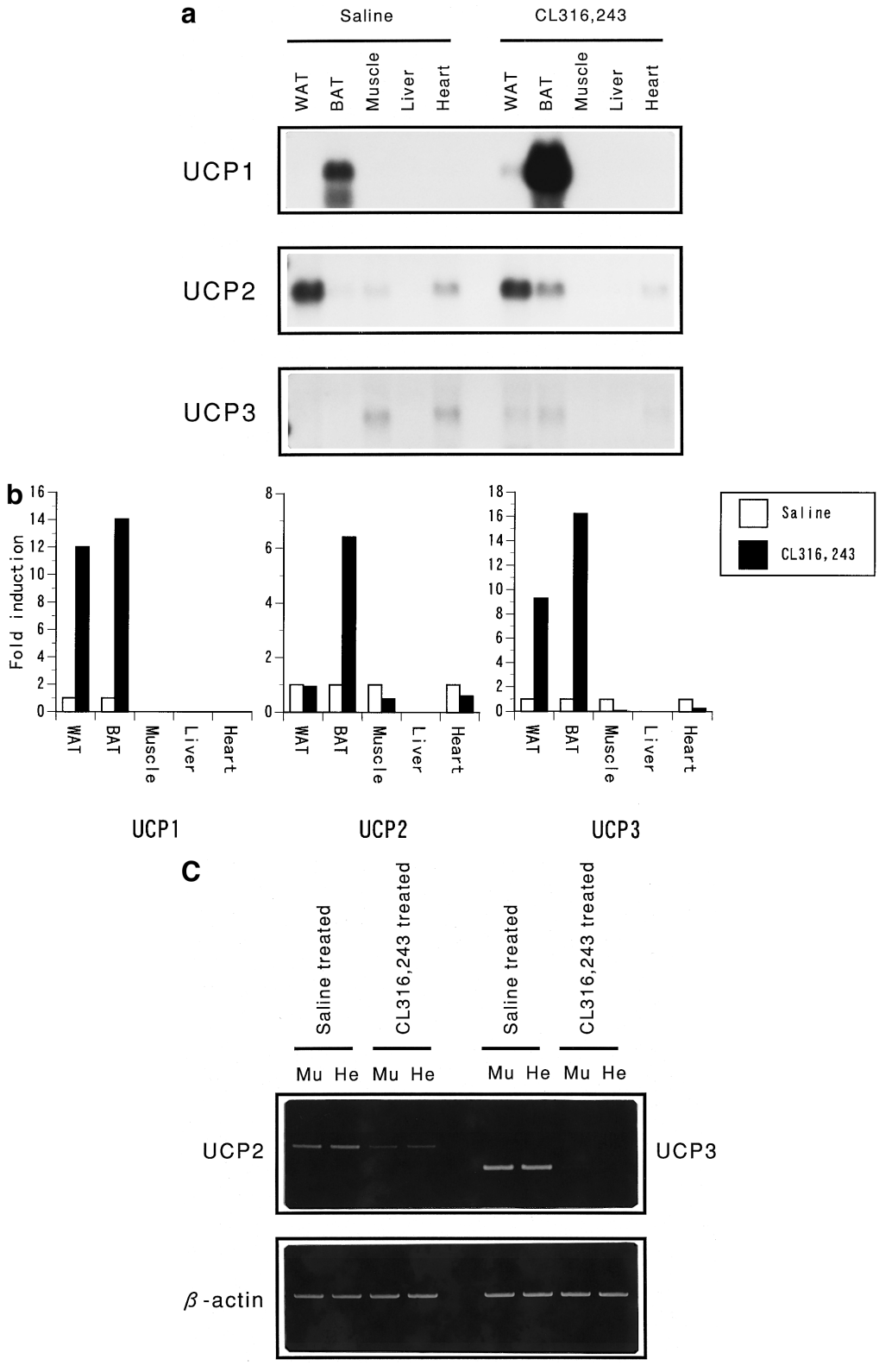

FIG. 2. Effects of β_3 adrenergic receptor agonist on UCP1, 2 and 3 mRNA expressions in various organs of KK-A^y mice. The mice were given CL316,243 at a dose of 0.2mg/kg body weight s.c. or the same volume of saline daily for 21 days, and Northern blot analysis and RT-PCR analysis were performed as described in Materials and Methods. (a) Northern blots containing total RNA from the indicated mice tissues were probed successively for mouse UCP1, UCP2 and UCP3 probes. Each lane contains total RNA from a single mice. (b) Comparison of mouse UCP1, 2 and 3 mRNA expressions in various tissues between the saline-treated and CL316,243-treated KK-A^y mice based upon the quantitation of Northern blot gels. Data represent the mean (n=2 per the treatment in each group). WAT, white adipose tissue; BAT, brown adipose tissue. (c) RT-PCR analysis of the UCP2 and UCP3 expression in the skeletal muscle and heart of the mice treated with CL316,243 or saline. Mouse β-actin was used for an internal control. Mu, skeletal muscle; He, heart.

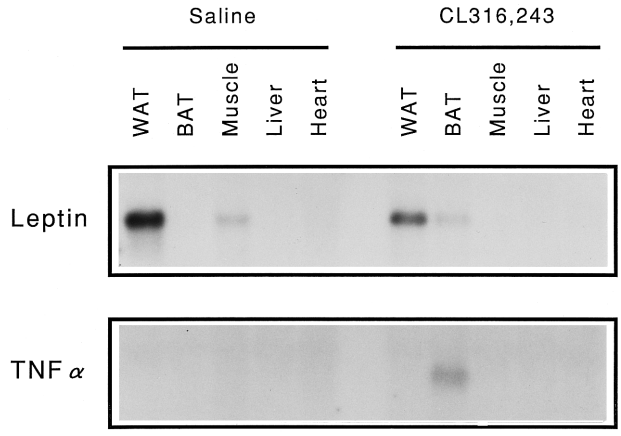

FIG. 3. Different TNFα and leptin mRNA expressions in various tissues of KK-A^y mice induced by CL316,243 treatment. The mice were treated with CL316,243 (0.2 mg/kg body weight s.c.) or the same volume of saline daily for 21 days. Total RNA from the white adipose tissue (WAT), brown adipose tissue (BAT), skeletal muscle, liver and heart was used for a Northern blot, which was probed successively for mouse UCP1, UCP2, and UCP3 probes. Each lane contains total RNA from a single mice.

Fig. 2c, both UCP2 and UCP3 mRNA expressions were clearly down-regulated in the skeletal muscle and heart.

We also examined the expressions of TNFα and leptin mRNA in each tissue. As shown in Fig. 3, no tissue showed the TNFα mRNA expression in the saline-treated mice, but we found the small amount of its expression in the BAT after the CL316,243 treatment. The expression of the leptin mRNA was decreased by the drug treatment in the WAT and skeletal muscle, but was increased in the BAT.

DISCUSSION

To study the regulations of all UCPs members when diabetic and obese KK-A^y mice were treated with β_3 adrenergic receptor agonist, we examined the effects of CL316,243 on the expression patterns of the UCP1, UCP2 and UCP3 in various organs, as well as determination of blood glucose, lactate, plasma insulin level, TG, FFA and glucose tolerance.

It has been well-known that high level of circulating lipid plays important roles in whole-body insulin action. Randle *et al.* proposed that inappropriate high lipid inhibits glucose utilization in the skeletal muscle and increases hepatic glucose output (24). We observed that β_3 adrenergic receptor agonist treatment sharply decreased plasma TG and FFA at Day 3, followed by gradual but significant decrease in the blood glucose until Day 21. It indicated that the glucose utilization was restored by the drastic drops of the plasma TG and FFA. Glucose tolerance test also showed the significant improvement of insulin sensitivity. The mechanisms by which β_3 adrenergic receptor agonist reduces plasma lipid concentrations may be via activation of the hormone sensitive lipase and the UCPs.

By Northern blot analysis, we found that the expressions of all UCPs were markedly increased in the BAT of the drug-treated mice. The expressions of the UCP1 and UCP3 mRNA were also increased in the WAT, suggesting that both adipose tissues are main targets in the chronic treatment of β_3 adrenergic receptor agonist in KK-A^y mice. Our results showed that the UCP3 mRNA expression is regulated in similar way of the UCP1, and it is supported by the fact that the chronic cold acclimation increases the UCP1 and UCP3 mRNA expressions in the BAT. However, the cold acclimation does not increase the UCP3 mRNA expression in the skeletal muscle (17, 25–27). Further, the UCP3 mRNA expression was increased in the BAT, but was decreased in the skeletal muscle and heart in our study. These results show that different mechanisms are responsible for the control of the UCP3 mRNA expression among the BAT, skeletal muscle and heart. There are two possible explanations: one is that the skeletal muscle does not contain β_3 adrenergic receptor in appreciable amount relative to the BAT and WAT, and the other is that the UCP3 mRNA expression in the skeletal muscle and heart is mainly controlled by insulin and/or FFA. The expression of the UCP3 mRNA is modulated by food intake and its composition (17, 25, 27): high-fat diet increases the UCP3 mRNA expression in the BAT; Fasting decreases the UCP3 mRNA expression in the BAT, while it increases its expression in the skeletal muscle. Fasting induces metabolic changes including the increase of circulating FFA without changes of catecholamines. We performed Northen blot analysis using the tissues removed from the mice when plasma TG and FFA had completely decreased as shown in Table 1, indicating that circulating FFA could regulate the UCP3 mRNA expression in the skeletal muscle.

Insulin is the second possible factor to control the UCP3 mRNA expression. The strong relationship was observed between the UCP3 mRNA expression in the skeletal muscle and the plasma insulin level (Table 1). The roles of insulin on the UCP1 mRNA expression in the BAT have been reported previously (28, 29) and the UCP1 mRNA expression in diabetes induced by streptozotocin was decreased (28). Moreover, it was shown that insulin has direct effects on the UCP1 mRNA expression in brown preadipocytes of *Phodopus sungorus* (30). From these and our results, it is possible that insulin also play roles in the regulation of the UCP3 mRNA expression in the skeletal muscle. Thus, FFA and insulin are possible factors to regulate the UCP3 mRNA expression in the skeletal muscle and heart. Recently, we succeeded in cloning of 5′-flanking region of the mouse UCP3 gene, and found putative cAMP response elements (31). This finding suggests

that the UCP3 mRNA expression in the BAT and WAT is regulated by β_3 adrenergic stimulation via the sympathetic nervous system. But the regulation of the UCP3 mRNA expression in the skeletal muscle and heart may rather be controlled by FFA, insulin or both.

The UCP2 mRNA expression was up-regulated in the BAT, but unchanged in the WAT. The absence of the effects of β_3 adrenergic receptor agonist on the UCP2 mRNA expression in the WAT was unexpected, since this tissue contains large amount of β_3 adrenergic receptor. Previous experiments also showed that CL316,243 has no effects on the UCP2 mRNA expression in the mouse WAT (13). The reason why the UCP2 responses to β_3 adrenergic receptor agonist are different between the BAT and WAT are still unclear. Interestingly, leptin and TNFα mRNA expressions were up-regulated in the BAT, but were not in WAT (Fig. 3). Therefore, we hypothesize that FFA is a key factor in the differential UCP2 expression. The following findings indicate relationships among the leptin, TNFα, FFA and UCP2 expressions: 1) leptin inhibits lipogenesis and accelerates the lipolysis in intracellular pancreatic islets in the direct extraneural way (32), 2) high blood level of leptin, produced by adenovirus mediated-leptin gene therapy, results in a dramatic increase of the UCP2 mRNA expression in the WAT (33, 34), 3) TNFα is produced from adipocytes and also causes lypolysis (35), 4) FFA acts as a positive transcriptional regulator of lipid-related genes by means of peroxisome proliferator-activated receptors (PPAR) (36), 5) TNFα can play important roles in obesity and diatetes in addition to well-known roles in cachexia (37, 38) and its production is strongly correlated with fat mass. The reduction of fat mass by leptin leads to decrease of the TNFα expression (39), 6) Thiazolidinediones stimulate the UCP2 mRNA expression in cell lines representing the WAT and skeletal muscle (40). Therefore, the expression of the UCP2 mRNA in the BAT could be caused via PPAR activation by FFA increment, which is activated by the leptin and TNFα expressions in the BAT. Taken together with the unchanged UCP2 mRNA expression in WAT, in which the leptin mRNA was decreased and TNFα was undetected, the UCP2 mRNA expression could be regulated by leptin and TNFα. However, the reason why β_3 adrenergic receptor agonist stimulates the leptin and TNFα mRNA expressions remains uncertain, and further studies will be required.

In conclusion, the chronic treatment of β_3 adrenergic receptor agonist increased all UCPs mRNA expressions in the BAT of KK-A^y mice along with insulin sensitivity improvement. This treatment had no effects on the UCP2 mRNA expression in the WAT, but decreased the UCP2 and UCP3 mRNA expressions in the skeletal muscle and heart. The decrease of the UCP2 and UCP3 mRNA in the skeletal muscle and heart correlated with the plasma insulin and FFA levels. Therefore, it is suggested that the UCP1 and UCP3 mRNA expressions are mainly controlled by the hypothalamus via the sympathetic nervous system in the BAT and WAT. On the other hand, plasma insulin, FFA or both can play roles to regulate the UCP2 and UCP3 mRNA expressions in the skeletal muscle and heart.

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