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Clenbuterol Inhibits SREBP-1c Expression by Activating CREB1

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As a β_2-adrenergic agonist, clenbuterol decreases body fat, but the molecular mechanism underlying this process is unclear. In the present study, we treated 293T and L-02 cells with clenbuterol and found that clenbuterol downregulates SREBP-1c expression and upregulates CREB1 expression. Considering SREBP-1c has the function of regulating the transcription of several lipogenic enzymes, we considered that the downregulation of SREBP-1c is responsible for body fat reduction by clenbuterol. Many previous studies have found that clenbuterol markedly increases intracellular cAMP levels, therefore, we also investigated whether CREB1 is involved in this process. The data from our experiments indicate that CREB1 overexpression inhibits SREBP-1c transcription, and that this action is antagonized by CREB2, a competitive inhibitor of CREB1. Furthermore, since PPARs are able to repress SREBP-1c transcription, we investigated whether clenbuterol and CREB1 function via a pathway involving PPAR activation. However, our results showed that clenbuterol or CREB1 overexpression suppressed PPARs transcription in 293T and L-02 cells, which suggested that they impair SREBP-1c expression in other ways.

Keywords: CREB, Insulin, PPARs, SREBP-1c

Introduction

SREBPs (Sterol regulatory element binding protein) exists in 3 forms, named SREBP-1a, SREBP-1c and SREBP-2 (Gondret *et al.*, 2001). The SREBP-1 forms are crucial for the regulation of lipogenic genes, whereas SREBP-2 chiefly controls gene expression responsible for cholesterol synthesis (Horton *et al*., 2002). SREBP-1a and SREBP-1c are derived from a common gene with different promoters (Brown and Goldstein, 1997). SREBP-1c, a well characterized transcription factor belongs to the basic helix-loop-helix leucine zipper family (Yokoyama *et al*., 1993; Brown and Goldstein, 1997), and is ubiquitously highly expressed in liver and adipose tissue (Shimomura *et al*., 1997), which implies that SREBP-1c is essential in energy homeostasis. It is generally accepted that SREBP-1c plays a part in the transcriptional regulations of lipogenic genes, including acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl CoA desaturase-1 (SCD-1), *et al*. (Shimano *et al*., 1997), by binding to their SRE or related sequences (Kim *et al*., 1995). Recent research indicates that SREBP-1c plays crucial roles in glucose/lipid metabolism (Osborne 2000) as well as in insulin signal transduction (Ide *et al*., 2004).

Cyclic AMP response element-binding protein 1(CREB1) is a member of the CREB/ATF family in addition to at least 10 other genes with a basic leucine zipper, which is required for protein dimerization (Mayr and Montminy, 2001). However, CREB was the first to be isolated, and was initially presumed to be involved in learning and memory (Silva *et al*., 1998). Currently, evidence obtained *in vitro* and *in vivo* demonstrates that CREB1 also functions in glucose homeostasis and adipocyte differentiation and that it regulates the expressions of various genes (Reusch *et al*., 2000). CREB2, also called ATF-4 (Hai *et al*., 1989), was originally described as a CRE-dependent transcription inhibitor (Karpinski *et al*., 1992) that interferes with CREB1 action by forming heterodimers with CREB1, and is thereby also known as repressor of CREB1 (Abel *et al*., 1998).

Clenbuterol is a long-acting β_2-adrenoceptor agonist, and can increase intracellular cAMP levels and reduce body fat levels. In the present study, to investigate the molecular mechanism involved in this process, we treated 293T and L-02 cells with clenbuterol. The mRNA levels of studied genes and glucose concentrations were measured. Data collected indicated that clenbuterol impairs SREBP-1c expression and stimulates CREB1 expression in cells. CREB1 and SREBP-1c are known to performed many functions during gene

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transcription, cell differentiation, and glucose/lipid metabolism, but the interrelationships between them have not been elucidated. To clarify whether CREB1 is involved in the process, CREB1 and CREB2 were co-transfected into cells to probe the molecular mechanism involved. Our results supported a negative-regulation between CREB1 and SREBP-1c, which may provide a base that enables the relations between these two transcriptional factors to be established.

Materials and Methods

Materials. TRIzol was purchased from Sangon Co., Ltd.; Mammalian Cell Protein Extraction kit from Shenergy Biocolor BioScience & Technology Co., Ltd.; Glucose Assay Kit from Shanghai Shenergy-diasys Diagnostic Technology Co. Ltd.; and Dulbecco's modified eagle medium (DMEM) and Lipofectamine™ 2000 from Invitrogen/GIBCO. All restriction endonucleases were purchased from TaKaRa Co.

Plasmid construction. All vectors were constructed as described previously (Zhou *et al*., 2006). Briefly, full-length CREB1 and CREB2 were obtained by RT-PCR. PCR products that contained *Bam*HI-*Not*I restriction sites were subcloned into pEGFP-N1 vector instead of enhanced green fluorescent protein (EGFP) gene. These plasmids were named p-CREB1 and p-CREB2, respectively.

Plasmid extraction. Plasmids were reproduced in *Escherichia coli* DH5a and extracted as described previously (Sambrook and Russell, 2001). Briefly, the whole process included two steps: first step, Plasmid DNA was isolated by lysis from 100 ml bacterial cultures by treating with alkali and SDS; second step, plasmid DNA was purified by precipitation with polyethylene glycol 8000. The concentration of plasmid DNA was diluted to 1 mg/ml.

Cell culture and treatment. 293T cells and L-02 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 5% CO_2 and 37°C. The L-02 cell line was derived from adult human liver (Yeh *et al*., 1980). For the relevant experiments, cell density was about 5×10^5 cells/well in 24-well culture plates for RNA extraction or 5×10^6 cells/dish in 60-mm Petri dishes for glucose concentration assays. 293T cells were divided into four groups, TC (293T cells Control), TCT (293T cells with Clenbuterol Treatment), TT1 (293T cells Transfected by p-CREB1) and TT12 (293T cells co-Transfected with p-CREB1 and p-CREB2). L-02 cells were divided into five groups, LC (L-02 cells Control), LCT (L-02 cells with Clenbuterol Treatment), LT1 (L-02 cells Transfected by p-CREB1), LT12 (L-02 cells co-Transfected by p-CREB1 and p-CREB2) and IT (Insulin Treatment). The control group was cultured in basal medium- DMEM supplemented with 10% fetal bovine serum. Reagent concentrations were as follows: insulin 10 nM and clenbuterol 10 nM. All treatments are listed in Table 1.

Transient transfection of 293T cells and L-02 cells. Expression vectors were transiently transfected into 293T cells and L-02 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, vectors were first transfected into cells in serum-free medium which was changed for DMEM containing 10% FBS six hours later. Cells were collected 48 h after transfection for RNA isolation.

RNA isolation. Total RNAs were isolated from 293T cells and L-02 cells using TRIzol. All RNA samples were treated with Dnase I to digest genomic DNA and stored at −80°C for further processing.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control was performed to determine the mRNA levels of G6Pase, GLUT2, GLUT4, IRS-2, SREBP-1c, PPARα and PPARγ in 293T and L-02 cells. All primers are listed in Table 2. Preliminary experiments were carried out using various PCR cycles to determine the linear ranges of amplification of all genes. The PCR conditions used were 94°C for 5 min, with amplification for 30 cycles for PPARα, IRS-2, GLUT2, SREBP1c and G6Pase {40 s at 94°C, 40 s at 58°C, and 30 s at 72°C} or for 30 cycles for PPARγ and GLUT4 {40 s at 94°C, 40 s at 53°C and 30 s at 72°C; 94°C for 5 min}, or for 25 cycles for GAPDH {40 s at 94°C, 40 s at 58°C and 40 s at 72°C}.

Protein isolation and concentrations. Proteins in L-02 cells were isolated using Mammalian Cell Protein Extraction kits, and concentrations were determined using the Bradford assay (Bradford, 1976).

Glucose concentrations. Glucose concentrations in L-02 cell media were assayed using the Glucose Assay Kit. Absorbance was assayed at 340 nm using a BECKMAN COULTER DU 800 UV/Visible spectrophotometer. All sample concentrations were normalized by each protein amount. The consumption of glucose was calculated by subtracting final glucose concentrations from initial glucose concentrations.

Table 1. The description of cell treatment

Reagent \ Group	293T				L-02				
	TC	TCT	TT1	TT12	LC	LCT	LT1	LT12	IT
Basal medium	+	+	+	+	+	+	+	+	+
Insulin (10 nM)									+
Clenbuterol (10 nM)		+				+			
p-CREB1			+	+			+	+	
p-CREB2				+				+	

Table 2. The primers use for semi-quantitative RT-PCR

Gene name	Size (bp)	Forward and Reverse primer (5'-3')	Accession number
PPARα	234	F: CCTAAAAAGCCTAAGGAAACCGTTC R: AAGATATCGTCCGGGTGGTTGC	**AY206718**
PPARγ	195	F: TCTCCAGTGATATCGACCAGC R: TTTTATCTTCTCCCATCATTAAGG	**BT007281**
IRS-2	383	F: CACCTCCCCACGACAGTTGC R: GGTGGGACAAGAAGTCAATGCTG	**NM_003749**
GLUT2	398	F: TTTTCAGACGGCTGGTATCAGC R: CACAGAAGTCCGCAATGTACTGG	**J03810**
GLUT4	187	F: AGGGAACAGGAAAGTATGTGC R: ATCCCCTCTTTGCCTTAGTTG	**M91463**
SREBP-1c	248	F: CACCGTTTCTTCGTGGATGG R: CCCGCAGCATCAGAACAGC	**BC057388**
G6Pase	244	F: CGACCTACAGATTTCGGTGCTTG R: AGATAAAATCCGATGGCGAAGC	**NM_000151**
GAPDH	452	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	**BC083511**

Statistical analysis. Results are expressed as means ± SE of 3 independent experiments performed in triplicate. Comparisons were made using the Student's *t* test, and significance was accepted at the $P < 0.05$ level.

Results

Clenbuterol inhibited SREBP-1c expression and stimulated CREB1 expression in 293T cells and L-02 cells. Clenbuterol increased intracellular cAMP levels. 10 nM clenbuterol was added to media and total RNAs were isolated 48 h later. mRNA levels of SREBP-1c and CREB1 were measured. The results indicated that clenbuterol markedly repressed SREBP-1c expression and stimulated CREB1 expression (Fig. 1), which suggested that cAMP signaling suppressed SREBP-1c transcription. Since CERB1 and SREBP-1c are highly related to glucose metabolism and insulin signaling, we measured the expressions of IRS-2, GLUT2, GLUT4 and G6Pase in L-02 cells. The results (Fig. 2A) obtained demonstrated that clenbuterol induced GLUT2 and GLUT4 expression and markedly stimulated the expressions of IRS-2 and G6Pase (Fig. 2B).

CREB1 inhibited SREBP-1c expression and changed the expression of studied genes in L-02 cells. It is generally acknowledged that the action of CREB1 is dominated by a cAMP signaling pathway. To determine whether CREB1 is involved in the transcriptional regulation of SREBP-1c, p-CREB1 was transfected into both 293T cells and L-02 cells (Fig. 3). After 48 h, RNAs were extracted, and SREBP-1c mRNA levels were measured. The data obtained showed that CREB1 overexpression in both cells inhibited SREBP-1c transcription (Fig. 3), which indicated that CREB1 is involved in the regulation of SREBP-1c transcription. Furthermore, we determined the relative expressions of genes in L-02 cells and

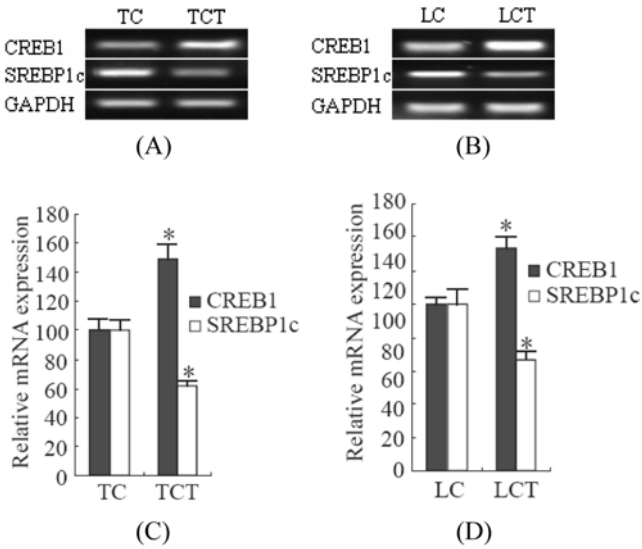

Fig. 1. Effects of clenbuterol on the expressions of CREB1 and SREBP-1c in 293T and L-02 cells. After plating, cells were cultured as described in Materials and Methods, and after culturing for 48h, total RNA was extracted and analyzed for the expressions of CREB1 and SREBP-1c. (A) 293T cells were cultured in basal medium (TC) or treated with clenbuterol (TCT). (B) L-02 cells were cultured in basal medium (LC) or treated with clenbuterol (LCT). (C) and (D) quantifications of blots corresponding to these genes in 293T and L-02 cells, respectively. GAPDH was used as an internal control. Data are means ± SE of 3 independent experiments performed in triplicate. *($p < 0.05$) indicates that the expression of the gene concerned in the treatment group was significant different from that than in the control group (TC or LC).

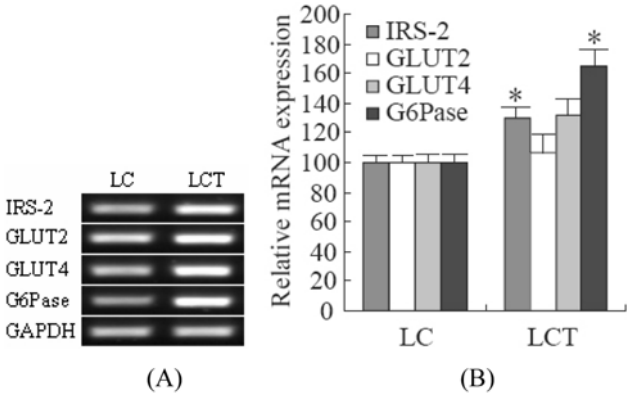

Fig. 2. Effects of clenbuterol on the expressions of genes in L-02 cells. After plating, cells were cultured as described in Materials and Methods. After 48 h, total RNA was extracted and analyzed for the expressions of IRS-2, GLUT2, GLUT4 and G6Pase. (A) L-02 cells were cultured in basal medium (LC) or treated with clenbuterol (LCT). (B) The quantification of blots corresponding to the expressions of these in L-02 cells. GAPDH was used as an internal control. Data were presented as means ± SE of 3 independent experiments performed in triplicate. *($p < 0.05$) indicates that expression level of the gene concerned in the treatment group was significant different from that in the control group (LC).

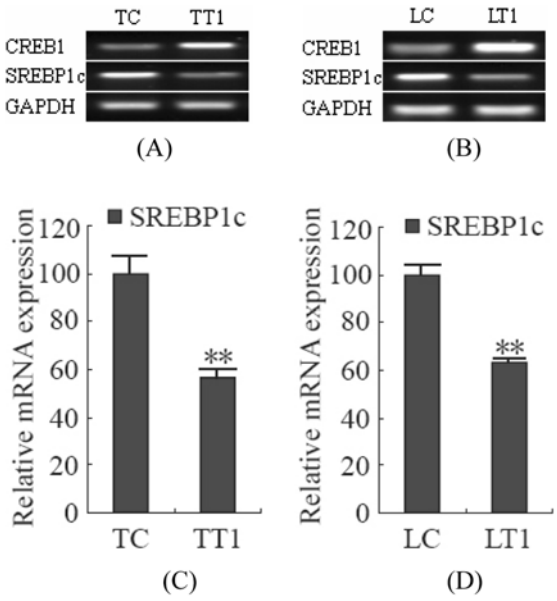

Fig. 3. Effects of CREB1 overexpression on SREBP-1c transcription in 293T and L-02 cells. After plating, cells were cultured as described in Materials and Methods. After 48 h, total RNA was extracted and analyzed for the expressions of CREB1 and SREBP-1c. (A) 293T cells were cultured in basal medium (TC) or transfected with p-CREB1 (TT1). (B) L-02 cells were cultured in basal medium (LC) or transfected with p-CREB1 (LT1). (C) and (D) Quantification of blots corresponding to SREBP-1c expression in 293T or L-02 cells. GAPDH was used as an internal control. Data are provided as means ± SE of 3 independent experiments performed in triplicate. **($p < 0.01$) indicates that expression level of the gene in the treatment group was significant different from that in the control group (TC or LC).

the results obtained showed that CREB1 stimulated the expressions of IRS-2, GLUT4 and G6Pase, but had no effect on GLUT2 (Fig. 4).

CREB2 resumed SREBP-1c expression. It has been previously shown that CREB2 can disturb CREB1 function by forming heterodimers with CREB1. Provided that CREB1 acted in SREBP-1c regulation, its effect would be neutralized by CREB2 overexpression. To further confirm that CREB1 is involved in the transcriptional repression of SREBP-1c, p-CREB2 was co-transfected with p-CREB1 into both 293T cells and L-02 cells (Fig. 5). After 48 h, RNAs were isolated and SREBP-1c mRNA levels were analyzed. The data obtained confirmed this hypothesis, i.e., CREB2 overexpression antagonized the action of CREB1 and resumed SREBP-1c transcription in both cells (Fig. 5).

CREB1 inhibited SREBP-1c expression other than via PPARs. Recent studies have found that PPARs interfere with SREBP-1c transcription by suppressing LXR signaling, and that PPAR overexpression attenuates SREBP-1c transcription (Yoshikawa *et al*., 2003). In this study, we investigated whether clenbuterol and CREB1 function through PPARs. Clenbuterol or p-CREB1 were added to L-02 cells. After 48 h, RNAs were isolated and PPAR mRNA levels were determined. The results revealed that both clenbuterol and CREB1 suppressed the expressions of PPARs and SREBP-1c (Fig. 6), thus demonstrating that they impair SREBP-1c transcription other than via PPARs.

Clenbuterol and CREB1 expedited glucose consumption, but were inferior to insulin in this aspect. Since CREB1 plays a crucial role in SREBP-1c expression, we examined the effect of clenbuterol and CREB1 on glucose consumption by L-02 cells. Glucose concentrations in media were assayed at 0, 24 and 48 h, respectively. The data obtained indicated that both increased glucose consumption, but with efficacies lower than that of insulin (Fig. 7), which suggests that they could partially compensate for insulin deficiency.

Discussion

CREB1 and SREBP-1c are well characterized transcription factors that are required for adipocyte differentiation. Some members of the C/EBP family also share the CRE sequence in their promoters. Unlike CREB1, SREBP-1c controls the expression of lipogenic genes and is involved in insulin regulation. In addition, accumulated evidence demonstrates that these two factors contribute to glucose and lipid metabolism.

In this study, it was found that clenbuterol represses SREBP-1c expression (Fig. 1), and thus would be expected to

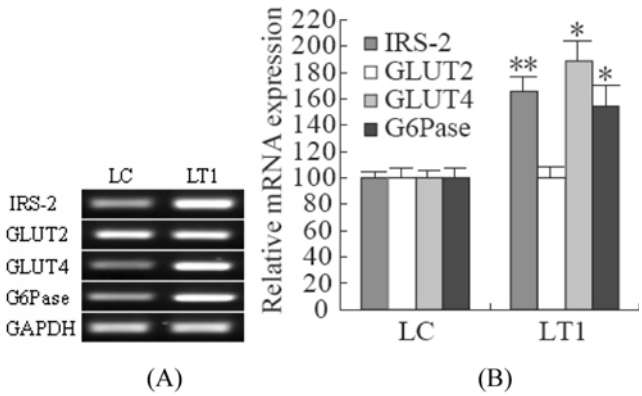

Fig. 4. Effects of CREB1 overexpression on gene expression in L-02 cells. After plating, cells were cultured as described in Materials and Methods. After 48 h, total RNA was extracted and analyzed for the expressions of IRS-2, GLUT2, GLUT4 and G6Pase. (A) L-02 cells were cultured in basal medium (LC) or transfected with p-CREB1 (LT1). (B) Quantification of blots corresponding to the expressions of these genes in L-02 cells. GAPDH was used as an internal control. Data are provided as means ± SE of 3 independent experiments performed in triplicate. *($p < 0.05$), **($p < 0.01$) indicates that the expression level of the gene concerned in the treatment group was different from that in the control group (LC).

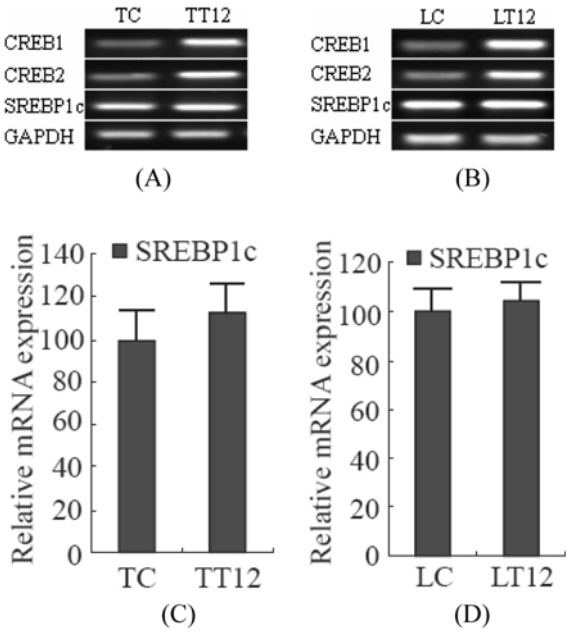

Fig. 5. CREB2 antagonized the action of CREB1 during the transcriptional regulation of SREBP-1c in 293T and L-02 cells. After plating, cells were cultured as described in Materials and Methods. After 48 h, total RNA was extracted and analyzed for the expressions of CREB1, CREB2 and SREBP-1c. (A) 293T cells were cultured in basal medium (TC) or co-transfected with p-CREB1 and p-CREB2 (TT12). (B) L-02 cells were cultured in basal medium (LC) or co-transfected with p-CREB1 and p-CREB2 (LT12). (C) and (D) Quantification of blots corresponding to SREBP-1c expression in 293T and L-02 cells, respectively. GAPDH was used as an internal control. Data are presented as means ± SE of 3 independent experiments performed in triplicate.

attenuate fat deposits by reducing the expressional levels of some lipogenic genes. We considered this is a rationale that could explain the mechanism of meat/fat ratio increases in obese animals by clenbuterol. Moreover, because clenbuterol can stimulate intracellular cAMP levels, and CREB1 is central to second messenger regulated transcription, we investigated whether CREB1 is involved in the transcriptional regulation of SREBP-1c. To ensure the generality of our results, we use two cell lines. The results obtained from 293T and L-02 cells indicated that CREB1 overexpression inhibits SREBP-1c transcription (Fig. 3). To further confirm this hypothesis, CREB2 (a competitive inhibitor of CREB1) was co-transfected with CREB1 into both cells. The data obtained showed that CREB2 antagonized the action of CREB1 and prevents its inhibiting SREBP-1c expression (Fig. 5). Thus, all of the data obtained during the present study support the notion that CREB1 regulates SREBP-1c transcription.

Some groups have reported that SREBP-1c transcription requires LXR ligands (DeBose-Boyd *et al*., 2001; Repa *et al*., 2000), which were identified originally as orphan nuclear hormone receptors that are activated by a variety of sterols. Moreover, SREBP-1c expression was found to be impaired as PPAR levels increased (Yoshikawa *et al*., 2003). However, in the present study, CREB1 overexpression not only repressed SREBP-1c expression, but also decreased PPAR expression (Fig. 6). Our results concur with those of Herzig's, who found that CREB1 suppresses PPARg expression (Herzig *et al*., 2003), but contradict those of Yoshikawa, who concluded that PPARs and SREBP-1c have the opposite effect (Yoshikawa *et al*., 2003). Our data suggest that CREB1 inhibits SREBP-1c expression through ways not involving PPARs.

Considering the significant effects of CREB1 and SREBP-1c on glucose metabolism and insulin signaling, we examined the expressions of related genes and glucose concentrations in L-02 cells. It was found when SREBP-1c was suppressed that the mRNAs of glucogenic genes and IRS-2 increased, and when this suppression was abolished these gene expressions decreased (Fig. 2 and Fig. 4). Moreover, we found that both CREB1 and clenbuterol are able to expedite glucose consumption, although their efficacies were lower than that of insulin (Fig. 7).

Recent studies have shown that SREBP-1c overexpression promotes the accumulation of fat, inhibits insulin secretion (Wang *et al*., 2003), damages b-cell function, and impairs glucose tolerance (Takahashi *et al*., 2005). More pointed evidence proving that SREBP-1c regulates insulin signaling was provided by the finding that SREBP-1c directly suppresses IRS-2 transcription (Ide *et al*., 2004). In the present study, we obtained the same result as Ide *et al*. (2004), concerning the opposite trends shown by the expressions of SREBP-1c and IRS-2. It should be noted that Jhala found deficient CREB

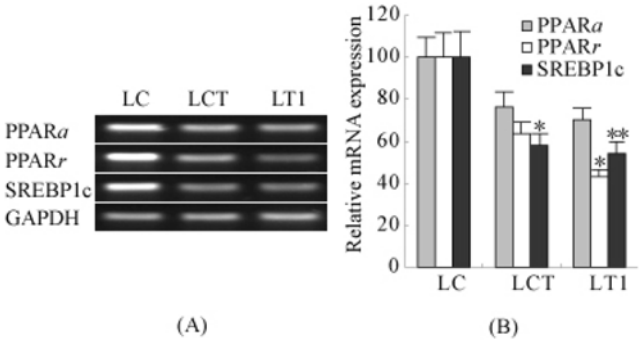

Fig. 6. Effects of clenbuterol and CREB1 overexpression on the expressions of PPAR and SREBP-1c in L-02 cells. After plating, cells were cultured as described in Materials and Methods. After 48h, total RNA was extracted and analyzed for the expressions of PPARα, PPARγ and SREBP-1c. (A) L-02 cells were cultured in basal medium (LC), treated with clenbuterol (LCT) or transfected with p-CREB1 (LT1). (B) Quantification of blots corresponding to the expressions of these in L-02 cells. GAPDH was used as an internal control. Data are presented as means ± SE of 3 independent experiments performed in triplicate. *($p < 0.05$), and **($p < 0.01$) indicate that the expression level of the gene concerned in the treatment group differed significant from than in the control group (LC).

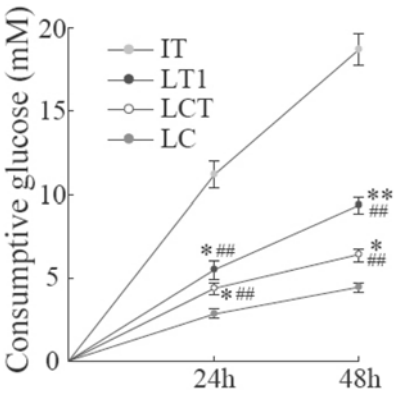

Fig. 7. Clenbuterol or CREB1 overexpression expedited glucose consumption in L-02 cells. After 0, 24, and 48 h, glucose concentrations were determined. Data were normalized versus total protein and are presented as means ± SE of 3 independent experiments performed in triplicate. *($p < 0.05$) and **($p < 0.01$) indicate that glucose consumption in the treatment group differed significantly from that observed in the control group (TC). $^{\#}$($p < 0.05$) and $^{\#\#}$($p < 0.01$) indicate that glucose consumption in the treatment group differed significantly from that observed in the insulin-treatment group (IT).

activity induced diabetes development and severely disrupted the expression of IRS-2 in mice pancreatic β-cells (Jhala *et al*., 2003). We infer that the potential reason for this is that cells failed to administrate SREBP-1c expression by CREB. Summarizing, we consider that CREB1 is involved in the transcriptional regulation of SREBP-1c and suggest that SREBP-1c is a negative regulator of insulin signaling.

Previous researches have indicated that cAMP inhibits SREBP-1c expression at the transcriptional (Deng *et al*., 2002) and post-translational levels (Yellaturu *et al*., 2005). Unlike these studies, we affirm the negative-regulation link between CREB1 and SREBP-1c, which does much to enrich interactions between transcription factors.

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