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ORIGINAL PAPER

Effect of clenbuterol on apoptosis, adipogenesis, and lipolysis in adipocytes

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Abstract Clenbuterol, a beta$_2$-adrenergic receptor (β_2-AR) selective agonist, has been shown to decrease body fat in animals and can induce apoptosis in adipose tissue in mice. We hypothesized that direct actions of a β-adrenergic receptor agonist on adipocytes could trigger the observed apoptotic effect. The hypothesis was inspected by investigating the direct effect of clenbuterol on apoptosis, adipogenesis, and lipolysis in vitro using the 3T3-L1 cell line and rat primary adipocytes. Cells were treated with 10^{-9} to 10^{-5}M clenbuterol depending on the experiments. There was no apoptotic effect of clenbuterol both in 3T3-L1 cells and rat primary adipocytes. Adipogenesis monitored by Oil Red O staining and AdipoRed™ assay was modestly decreased by clenbuterol treatment ($p<0.05$). In fully differentiated primary adipocytes, clenbuterol increased basal lipolysis compared with the control ($p<0.01$). In summary, direct stimulation of β_2-AR by clenbuterol does not cause apoptosis in adipocytes, despite a direct lipolytic stimulation and attenuation of adipogenesis.

Keywords Adipocyte apoptosis · β-adrenergic receptor agonist · Clenbuterol

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Introduction

Adipose tissue plays a central role in maintaining lipid homeostasis and its mass is determined by a balance of lipolysis and adipogenesis in general; however, adipocyte deletion by apoptosis has been recently suggested as a contributor to body fat loss [18, 28].

Leptin, a cytokine-like peptide, has been shown to induce adipose tissue apoptosis in rats as well as activating lipolysis [10, 25]. Based on the finding that centrally effective doses are much lower than peripherally effective doses [25, 26], it was initially suggested that adipose tissue apoptosis was centrally mediated. However, the central pathways and effectors of adipose tissue apoptosis have not yet been determined, although melanocortin receptors and neuropeptide Y have been identified as downstream mediators of leptin signaling in the brain [6, 16]. On the other hand, leptin has been shown to stimulate

sympathetic outflow to some tissues [13, 26] and adipose tissue has extensive sympathetic innervation with both α and β adrenoceptors [3].

β-adrenergic receptor (β-AR) agonists have been shown to decrease body fat and increase muscle mass in rodents and livestock [15, 31]. The mechanism for decreased fat mass is not fully understood. Clenbuterol, a selective β_2-AR agonist, has been used off-label as a weight loss aid [24]. We also found that oral administration of clenbuterol increased white adipose tissue apoptosis in mice [23]. Based on these findings, it can be hypothesized that adipose tissue apoptosis is mediated through central nervous system (CNS) stimulation of sympathetic nervous system activity. It remains to be discovered how sympathetic stimuli carry the signal from brain to initiate apoptosis in white adipose tissue to link the CNS and adipose tissue. We hypothesized that peripheral sympathetic stimulation by a β-AR agonist could trigger adipocyte apoptosis and tested this hypothesis by investigating the direct effect of clenbuterol on apoptosis, adipogenesis, and lipolysis on 3T3-L1 and rat primary adipocytes.

Materials and methods

Materials and cell culture Clenbuterol (Sigma, St. Louis, MO, USA) was reconstituted as a stock solution prepared in PBS, filter sterilized, and stored at −20°C.

3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as described elsewhere [14]. Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Green Island, NY, USA) containing 10% bovine calf serum until confluent. Two days after confluency (D0), the cells were stimulated to differentiate with 0.5 μM isobutylmethylxanthine, 1 μM dexamethasone, and 167 nM insulin added to DMEM containing 10% fetal bovine serum (FBS) for 2 days (D2). Cells were then maintained in 10% FBS/DMEM medium with 167 nM insulin for another 2 days (D4), followed by culturing with 10% FBS/DMEM medium for an additional 4 days (D8). Approximately 5 days after induction of differentiation, more than 90% of the cells displayed the characteristic lipid-filled adipocyte phenotype. All media contained 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 292 μg of glutamine/ml (Invitrogen, Carlsbad, CA, USA).

To obtain rat primary adipocytes, inguinal fat pads were excised aseptically from 8-week-old male Sprague–Dawley rats weighing 80–100 g. The isolation and culture procedure was performed as described previously [12]. Briefly, adipocytes were isolated by collagenase digestion and filtration. Cells were washed and plated in 10% FBS/DMEM/F12 medium (Sigma). After 24 h, the plating medium was replaced with a differentiation medium comprised of serum-free DMEM/F12 medium supplemented with ITTS (Sigma, containing 850 nM insulin, 64 nM transferrin, 29 nM sodium selenite, and 2 nM T3) for 8 days. Cells were maintained at 37°C in a humidified 5% CO_2 atm.

Concentrations of clenbuterol tested were selected based on previous reports in the literature [22] and preliminary dose-range experiments. Time points selected were based on preliminary time course experiments (data not shown).

Cell viability assay The MTS assay (Celltiter 96 Aqueous One Solution, Promega, Madison, WI, USA) was performed to determine the number of viable cells in culture. Cells were treated with 0 (vehicle only), 10^{-7}, or 10^{-6}M clenbuterol for 6, 12, 24, or 48 h. The absorbance was measured at 490 nm in a plate reader (μ Quant™ Bio-Tek Instruments, Winooski, VT, USA) to determine the formazan concentration, which is proportional to the number of living cells in culture.

Apoptosis assay Annexin V-FITC (AV)/propidium iodide (PI) staining combined with laser scanning cytometry (LSC; CompuCyte, Cambridge, MA, USA) detected cell membrane phosphatidylserine externalization and was used as an apoptotic assay in 3T3-L1 cells. Preadipocytes and mature adipocytes were treated with 0, 10^{-7}, or 10^{-6}M clenbuterol and incubated for 24 or 48 h. The detailed procedure and validation as a tool of apoptosis assay have been described elsewhere [19]. Single-stranded DNA (ssDNA) ELISA kit was used for detection of apoptosis in rat primary preadipocyte cultures (ApoStrand™, BIOMOL, Madison, WI, USA). This assay is based on the selective denaturation of DNA in apoptotic cells by formamide, which reflects changes

in chromatin associated with apoptosis. Preadipocytes and mature cells were treated with 0, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6}M clenbuterol for 6, 12, or 24 h.

Adipogenesis assay Clenbuterol (0, 10^{-7}, 10^{-6}M) was added to the cells with the differentiation medium for 6 days (D0 to D6). To visualize lipid content, treated cells were stained with Oil Red O and hematoxylin as described by Suryawan and Hu [29]. After mounting with glycerol gelatin, three images for each dish were captured and analyzed for lipid droplet size and total droplet number using ImagePro software (Mediacybernetics, Silver Spring, MD, USA). Intracellular lipid content was also measured using a commercially available kit (Adipored^{TM} Assay Reagent, Cambrex Bio Science Walkersville, Inc).

Lipolysis assay Mature rat primary adipocytes were treated for 4 h with vehicle (control), isoproterenol (β1 and β2 adrenoceptor agonist; 10^{-7}M) or clenbuterol (10^{-7} and 10^{-5}M). The conditioned medium was removed from each well and assayed for glycerol content with Free glycerol determination kit (Sigma). The time point was chosen from a preliminary test, in which it was within the linear range of glycerol release.

Statistical analysis All values were expressed as means±SEM and one-way ANOVA was used to determine significance of treatment effects at each time point. Differences among treatment means were determined by Tukey's test, and a p value of <0.05 was considered significant.

Results

Effect of clenbuterol on adipocyte viability and apoptosis Clenbuterol did not significantly affect viability of either preadipocytes or mature adipocytes, although there was a tendency towards a decrease in viability in the clenbuterol treated cells after 48 h incubation. To determine the direct effect of clenbuterol on cellular apoptosis, we used AV/PI staining combined with LSC assay in 3T3-L1 cells. The percentages of early and late apoptotic cells were not different from those of control cultures in preadipocytes or mature adipocytes (Table 1). We next investigated whether clenbuterol had an apoptotic effect in primary rat adipocytes, using ssDNA ELISA assay. Clenbuterol (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) did not show any effect on apoptosis after 6, 12, or 24 h incubation (Fig. 1), although a preliminary experiment with 10 nM tumor necrosis factor α showed that the cells could be induced to undergo apoptosis (data not shown).

Effect of clenbuterol on 3T3-L1 cell adipogenesis Oil Red O staining was used to visualize lipid accumulation in the differentiating adipocytes. The images showed that clenbuterol did not affect mean lipid droplet size or lipid droplet numbers (data not shown), but did cause a modest decrease in lipid content in treated cells. This was supported by quantitative data on D6 using the AdipoRed assay. The lipid content had a tendency to decrease with clenbuterol treatment; however, this effect was statistically significant only at 10^{-6}M ($p<0.05$; Fig. 2).

Effect of clenbuterol on adipocyte lipolysis Clenbuterol increased lipolysis in rat primary adipocytes compared with control (Fig. 3). Free glycerol release into the culture medium was 158% and 190% of control values in cultures containing 10^{-7} and 10^{-6}M clenbuterol, respectively ($p<0.01$). The increase in lipolysis was comparable to the effect of isoproterenol, a synthetic agonist for β1 and β2 adrenergic receptors.

Discussion

The primary objective of the present study was to determine whether direct actions of clenbuterol on adipocytes might explain the previously observed apoptotic effect in clenbuterol-fed mice [23]. Based on evidence that leptin and adrenergic stimulation increased adipose tissue apoptosis and centrally administered leptin caused increase in peripheral sympathetic activity [8, 11], we predicted that peripheral sympathetic stimulation by a β-AR agonist could initiate adipocyte apoptosis. Contrary to our prediction, however, clenbuterol did not induce apoptosis in either 3T3-L1 preadipocytes or mature adipocytes. Clenbuterol also did not induce apoptosis in rat primary adipocytes, which had

Table 1 Effect of clenbuterol on apoptosis of 3T3-L1 preadipocytes and mature adipocytes

Treatment	Early apoptotic cells (%)		Late apoptotic cells (%)	
	24 h	48 h	24 h	48 h
Preadipocytes				
Control	5.58±1.39	12.05±1.82	2.50±0.41	2.15±0.41
CB 10^{-7}M	5.08±1.71	9.78±1.46	3.95±0.67	3.08±0.63
CB 10^{-6}M	7.33±2.04	7.12±1.03	3.05±1.14	2.33±0.53
Mature adipocytes				
Control	1.20±0.19	1.18±0.08	0.30±0.20	0.30±0.17
CB 10^{-7}M	0.58±0.29	1.63±0.66	0.28±0.24	0.48±0.21
CB 10^{-6}M	1.50±0.46	0.85±0.10	0.33±0.07	0.25±0.12

This experiment was performed on four replicates and data expressed as mean±SEM. Cellular apoptosis was determined by AV/PI combination assay using LSC

previously a more normal exposure to sympathetic innervation.

It was shown that leptin can deplete adipocyte fat even after complete separation from nerve supply to white adipose tissue [30]. Although it suggests the significance of the direct effect of leptin, we have shown previously that leptin does not act directly on adipocytes with regard to apoptotic deletion of adipocytes [1]. Thus, we cannot rule out the possibility that some humoral factor secreted by sympathetic nerve system stimulation acts to induce apoptosis. The lack of in vitro apoptotic effect in our study suggests that clenbuterol, by acting on tissues other than adipose tissue, increases the susceptibility of adipose tissue to other apoptosis-inducing factors or conditions rather than acting directly on adipocytes in vivo.

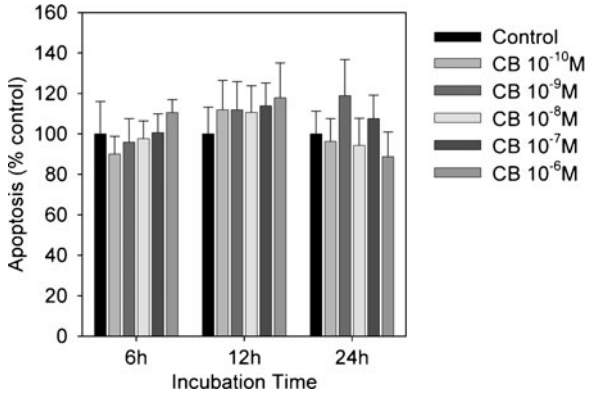

Fig. 1 Effect of clenbuterol on apoptosis of rat primary adipocytes. Cells were incubated with control or clenbuterol (*CB*) at various concentrations for 6, 12, or 24 h. Cell apoptosis was determined by ssDNA ELISA. Assays were performed twice with eight replicates

It has been shown that adrenergic stimulation induces different effects on apoptosis depending on tissues. For instance, norepinephrine, a β-AR agonist, was found to induce apoptosis of myocytes, while it protects against apoptosis in brown adipose tissue [20, 27]. In addition, it is interesting that the apoptosis induced by clenbuterol has different mechanisms in different tissues. In a recent study, clenbuterol treatment induced apoptosis in the heart and soleus muscle in the rat, and it was found that myocardial apoptosis was mediated through neuromodulation of the sympathetic system, while in the soleus muscle, direct stimulation of β-AR was involved [4]. However, the study on the mechanism and effects of β-AR agonists on adipose tissue apoptosis has been very limited, and further research is needed.

There is also evidence that β-adrenergic stimulation triggers apoptosis of endothelial cells [9]. Because adipocyte survival in adipose tissue is dependent on an adequate blood supply, regression of blood vessels as a result of endothelial cell death could secondarily result in apoptosis of adipocytes. Thus, adipose tissue apoptosis that occurred in mice following treatment with clenbuterol may have been a result of a primary effect on blood vessel endothelial cells followed by secondary apoptosis of adipocytes. Furthermore, adipose tissue apoptosis that occurs following leptin treatment may also be secondary to increased adrenergic stimulation leading to endothelial cell apoptosis and blood vessel regression. Indeed, Cohen et al. [7] have shown that leptin treatment of rats caused apoptosis of endothelial cells in adipose tissue.

We did find that clenbuterol could have a direct effect on lipolysis and lipogenesis. In this study, β-adrenergic stimulation by clenbuterol led to in-

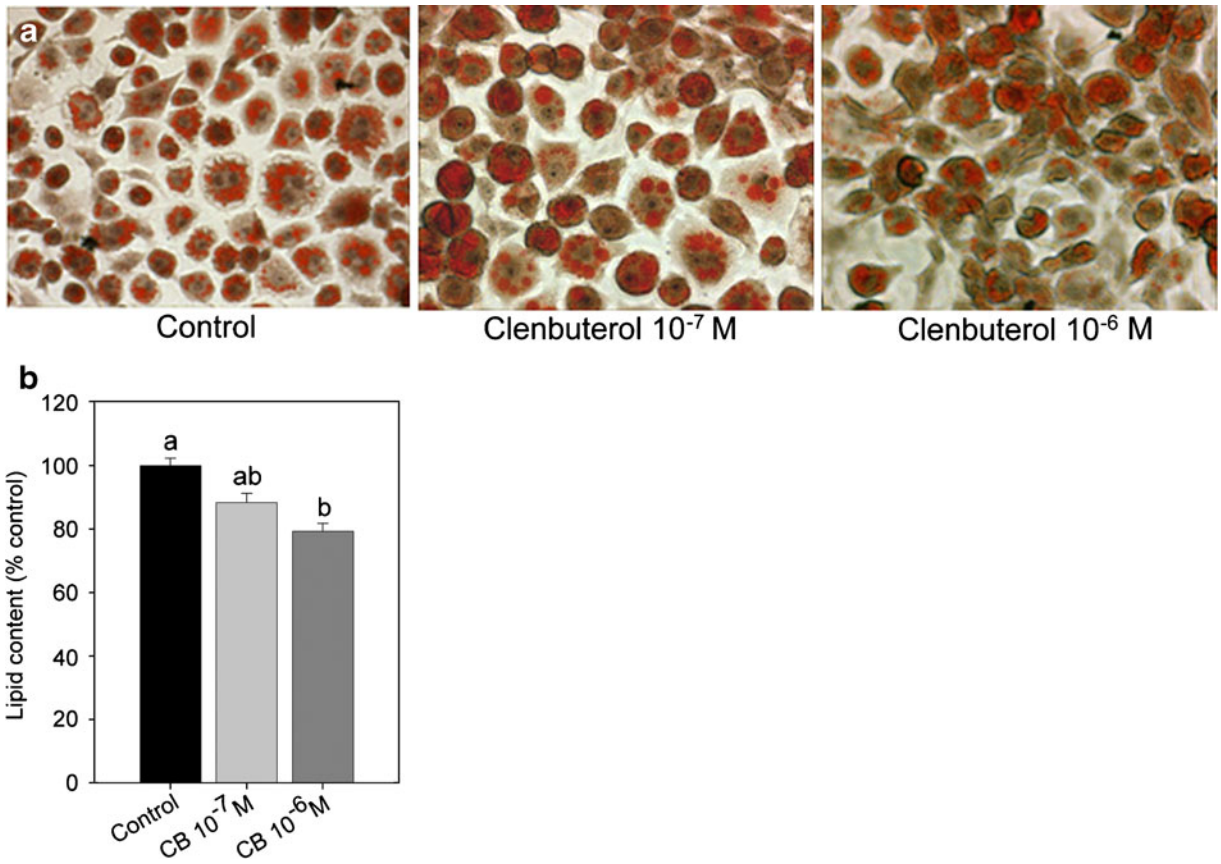

Fig. 2 Effect of clenbuterol on lipid accumulation of maturing adipocytes. 3T3-L1 cells were treated with clenbuterol (10^{-7}, 10^{-6}M) in differentiation medium for 6 days (D0–D6). **A** Representative Images of Oil Red O staining. The experiments were performed on four replicates and three images were captured for each dish. **B** Lipid content was measured by AdipoRed assay on D6. Assays were performed twice with eight replicates. One-way ANOVA was used to determine the significance of treatment effects. Means with different letters (*a* and *b*) are different by Tukey's test, $p<0.05$

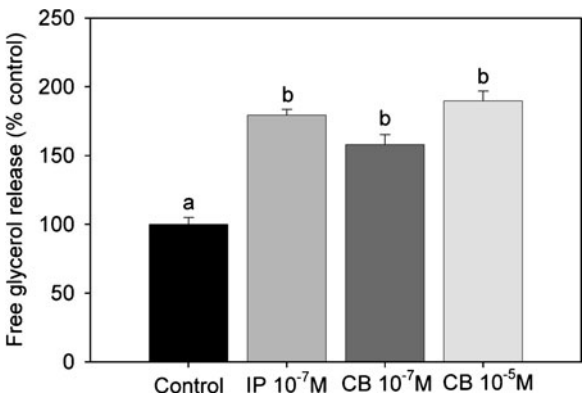

Fig. 3 Effect of clenbuterol on adipocyte lipolysis. Mature rat primary adipocytes were treated for 4 h with *control*, clenbuterol (*CB*), or isoproterenol (*IP*). Values are presented as percentage of control. The experiments were performed on four replicates. One-way ANOVA was used to determine the significance of treatment effects. Means with different letters (*a* and *b*) are different by Tukey's test, $p<0.05$

creased basal lipolysis and a modest decrease of adipogenesis in adipocytes. This result is consistent with the report showing lipolysis is more sensitive to beta-adrenergic stimulation than is lipogenesis [21]. Beta receptors are linked to Gs proteins, which are in turn linked to adenyl cyclase. The intracellular cAMP rise by agonist binding to β receptors and downstream effectors of cAMP are directly involved in lipolysis. The clenbuterol treatment increased lipolysis in mature adipocytes as much as isoproterenol. However, there were less prominent effects on lipogenesis. Considering that the main role of insulin in adipose tissue is promoting glucose uptake, and β-adrenergic agonists can counteract the physiologic response of adipocytes to insulin [2, 17], we predicted that clenbuterol would attenuate adipogenesis, but the effect was weaker than expected. It seems partly due to the fact that β_1,β_2-AR agonists initiate weak

inhibition of glucose transport and β_3-AR appear to have an important role in insulin-stimulated glucose transport at least in rat adipocytes [5]. Thus, clenbuterol, a β_2-AR agonist, seems to cause a modest suppression of lipogenesis. In summary, these results show that β_2-AR are involved in lipolysis stimulation and modest attenuation of adipogenesis in adipocytes; however, direct stimulation of β_2-AR does not induce adipocyte apoptosis, which was observed in clenbuterol-fed mice.

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