Calcium Mobilization Inhibits Lipid Accumulation During the Late Adipogenesis via Suppression of PPARγ and LXRα Signalings

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Abstract

Calcium plays a role as a signaling molecule in various cellular events. It has been reported that calcium suppresses adipocyte differentiation only in the early phase of adipogenesis. Herein, we demonstrate that treatment of A23187, a mobilizer of intracellular calcium, on day 4 post adipocyte differentiation could still reduce lipid accumulation in differentiating 3T3-L1 cells for 48 h. In addition, luciferase reporter gene and RT-Q-PCR assays demonstrate that A23187 can selectively inhibit transcriptional activities and expression of PPARγ and LXRα, suggesting that A23187 may reduce lipid accumulation in the late phase of adipogenesis via downregulation of PPARγ and LXRα expression and transactivation. Moreover, inhibition of HDAC activity by trichostatin A (TSA) partially blocked A23187-mediated downregulation of transcriptional activities of PPARγ and LXRα. Together, our data demonstrate that calcium mobilization inhibits expression and transcriptional activities of PPARγ and LXRα, resulting in reduced lipid accumulation in differentiating adipocytes, and thus, mobilization of intracellular calcium in adipocytes may serve as a new preventive and therapeutic approach for obesity.

Key words: Calcium, adipocyte, PPARγ, LXRα

Introduction

Adipocytes are important cells for lipid homeostasis in our body by regulating flux of lipids into circulation in response to nutrition status (Guilherme et al., 2008). In addition, adipocytes also play a role as highly specialized endocrine cells secreting a variety of adipocytokines such as TNFα, leptin, adiponectin and resistin. These adipocytokines have been known to participate in various physiological processes including insulin sensitivity, cell proliferation, immune response and food intake (Kershaw and Flier, 2004; Rosen and Spiegelman, 2006). Increased lipid accumulation by dysregulated lipid metabolism in adipose tissues resulted in obesity and is frequently related with metabolic diseases including insulin resistance and type 2 diabetes (Fu et al., 2005; Kershaw and Flier, 2004; Gregor and Hotamisligil, 2007; Shi and Burn, 2004).

Several transcription factors have been known to be involved in adipocyte differentiation (Gregoire et al., 1998; Lane et al., 1999; Rosen et al., 2000). During adipogenesis, profile of a variety of genes has been changed and PPARγ and C/EBPα have been shown to play key roles in this process (Darlington et al., 1998). PPARγ, the master regulator of adipocyte differentiation, belongs to the superfamily of nuclear receptors (NRs) (Chawla et al., 1994). NR superfamily includes traditional endocrine receptors for steroids, thyroid hormones, vitamin D3, and retinoids, as well as a large number of orphan receptors of which ligands and physiological functions are initially unknown (Evans, 1988; Giguere, 1999). In addition to PPARγ, LXRα, a NR activated by oxysterol, has been known to function as a lipid sensor that responds to dietary lipids and their metabolic derivatives in liver and adipose tissues. Previous studies have demonstrated that LXRα enhances fatty acid synthesis by LXR-SREBP-1c-lipogenic gene pathway or direct upregulation of acetyl-CoA carboxylase and fatty acid synthase (FAS) expression (Chen et al., 2004; Joseph et al., 2002; Liang et al., 2002).

Several studies have shown that high calcium intake from dairy products results in prevention of obesity and insulin resistance, implicating link between calcium and adiposity (Papakonstantinou et al., 2003; Pilvi et al., 2003).
Recently, it was reported that mobilization of intracellular calcium ([Ca$^{2+}$]$\text{i}$) resulted in complete inhibition of adipogenesis in the early phase of adipocyte differentiation (Draznin et al., 1988; Shi et al., 2000). However, several studies reported conflicting results about calcium effect on adipogenesis in the late phase of adipocyte differentiation (Jensen et al., 2004; Shi et al., 2000; Whitehead et al., 2001). Thus, calcium effect on lipid accumulation in differentiating adipocytes is still not established. In this study, we demonstrated that increase of [Ca$^{2+}$]$\text{i}$ in the late phase of adipogenesis suppressed lipid accumulation in differentiating adipocytes and this inhibitory effect was due to decreased expression of lipogenic genes via reduced expression and transcriptional activities of PPAR$\gamma$ and LXR$\alpha$. Suppressive effect of [Ca$^{2+}$]$\text{i}$ on transcriptional activities of these nuclear receptors is partially blocked by a histone deacetylase (HDAC) inhibitor, suggesting that mobilization of [Ca$^{2+}$]$\text{i}$ may increase HDAC activity on transcriptional machineries of lipogenic NRs.

**Materials and Methods**

**Cell culture, Adipocyte differentiation and Oil Red O staining**

3T3-L1 and HEK293T cells obtained American Type Culture Collection (ATCC). 3T3-L1 was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% bovine serum and 100 µg/mL penicillin-streptomycin and maintained on the petri-dishes. HEK293T was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and same condition of antibiotics as described above. 3T3-L1 cells cultured to 90% confluence. Two days later, the medium was replaced with the differentiation induction medium (MDI) containing 5 µg/mL insulin, 0.25 mM 3-Isobutyl-1-methylxanthine (IBMX) and 0.25 µM dexamethasone in DMEM containing 10% FBS. The cells were treated with MDI during 72 h. The medium was replaced with MDI except 0.25 mM IBMX. Cells cultured mature adipocyte until 8 d. Cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) and stained with Oil Red O (Sigma, USA).

**Reporter gene assay**

Reporter genes pGL3-TK-Cyp7a1-LXRE$_{-3}$-Luc, pGL3-TK-Acol-PPRE$_{-3}$-Luc, pGL3-TK-HCR1-TR4RE$_{-3}$-Luc and pGL3-TK-HCR1 were previously described (Peet et al., 1998; Forman et al., 1995; Kim et al., 2003). Transfections were performed using SuperFect reagent (Qiagen) according to the manufacturer’s instructions in HEK293T cells. Luciferase activities were measured in a luciferase reporter assay system (Berthold). Relative luciferase activity (fold) was expressed based on the induction fold relative to transfection of an empty vector (set as 1-fold) and the results were expressed as means ± SD of three separate experiments.

**RNA isolation, cDNA synthesis and RT-Q-PCR**

Table 1. Primer sequence of genes for RT-Q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR4</td>
<td>CAGCAGTTCTCATCTAACCAGCCC</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>CTGCTCCGACAGCTGTAGTC</td>
<td></td>
</tr>
<tr>
<td>PPAR$\gamma$</td>
<td>TGCTGTATGGGTAAGAATCTGGG</td>
<td>(Cariou et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>CGCTTGATGCAAAGGAATGCG</td>
<td></td>
</tr>
<tr>
<td>FXR$\alpha$</td>
<td>CCAACCTGGGCTTACC</td>
<td>(Cariou et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>CACACAGCTCATCCCTT</td>
<td></td>
</tr>
<tr>
<td>PEPCK</td>
<td>TCAACACCACCTCCTTAC</td>
<td>(Kitamura et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>CTTAGCCTGTTCCTGTGC</td>
<td></td>
</tr>
<tr>
<td>aP2</td>
<td>GATTCCGATGAAAATCACCAGCA</td>
<td>(Berger et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>CTTATTGTGGTGCCTTACC</td>
<td></td>
</tr>
<tr>
<td>LXR$\alpha$</td>
<td>TGCCATACGATCTCTCTCT</td>
<td>(Volle et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>GAGACCAGCAGCTCATAGC</td>
<td></td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>GATCAAAAGAGGAGCAGTGC</td>
<td>(Cariou et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>TAGATGGTGCGCTGTAGTG</td>
<td></td>
</tr>
<tr>
<td>FAS</td>
<td>AGATGCTGAGAAGCAGCAT</td>
<td>(Schmid et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>GAGACGCTGCACCTCGAGCTG</td>
<td></td>
</tr>
<tr>
<td>36B4</td>
<td>AGATGCAGCAGTCGCCAT</td>
<td>(Moniotte et al., 2001)</td>
</tr>
</tbody>
</table>
Inhibition of Lipid Accumulation in Adipocytes by Ca\(^{2+}\) Suppression of PPAR\(\gamma\) and LXR\(\alpha\) Signalings

determined by RT-Q-PCR. Total RNA was isolated from 3T3-L1 cells using RiboEX (Geneall, Korea), and cDNA was synthesized using MMLV-RTase (Promega) and Oligo DT primers (Promega) according to the manufacturer’s instructions. Primer sequences of mouse genes for RT-Q-PCR are shown in Table 1. Relative expression levels of Adipogenic genes were determined by relative to the internal control 36B4 transcripts using a Gel doc XR system (Bio-Rad).

Results

Calcium mobilization inhibits lipid accumulation in the late phase of adipogenesis

To study the role of [Ca\(^{2+}\)]\(_i\) in the adipocyte differentiation, a calcium ionophore A23187 were used to mimic physiological [Ca\(^{2+}\)] mobilization in 3T3-L1 cells (Berridge, 1993; Campbell, 1983; Duffus and Patterson, 1974). We first treated A23187 to differentiating 3T3-L1 cells for 48 h at different time points and then determined lipid accumulation in 3T3-L1 adipocytes on day 8 post adipocyte differentiation by Oil-Red O staining. While treatment of A23187 on day 2 post adipocyte differentiation completely blocked adipogenesis, addition of A23187 on day 4 only partially suppressed lipid accumulation in differentiated 3T3-L1 adipocytes. In contrast, we were not able to see any suppressive effect of A23187 on lipid accumulation when A23187 was treated to differentiating 3T3-L1 cells on day 6 (Fig. 1A). Since Fig. 1A showed that addition of A23187 was still able to partially suppress adipogenesis even on day 4 post adipocyte differentiation on which PPAR\(\gamma\) and C/EBP\(\alpha\) are already expressed, we evaluated calcium mobilization effect on expression of lipogenic genes using RT-Q-PCR. As shown in Fig. 1B, 48 h treatment of 2 \(\mu\)M A23187 to differentiating 3T3-L1 cells on day 4 resulted in significant reduction of mRNA levels of aP2, FAS and PEPCK genes in day 8 adipocytes as compared with vehicle-treated adipocytes. Data from Fig. 1 suggests that suppressive effect of calcium mobilization on lipid accumulation in the late phase of adipocyte differentiation may be at least partially due to reduced expression of lipogenic genes.

Calcium mobilization selectively downregulates PPAR\(\gamma\) and LXR\(\alpha\) signaling

Our studies demonstrated that [Ca\(^{2+}\)] mobilization

Fig. 1. [Ca\(^{2+}\)] inhibits lipid accumulation during the late phase of adipogenesis. A. A23187 reduced lipid accumulation in 3T3-L1 adipocytes during the late phase of adipogenesis. Two \(\mu\)M A23187 was treated for 48 h to 3T3-L1 cells at different time points after MDI stimulation and mature adipocytes were stained with the triglyceride specific dye Oil Red O and photographed on day 8. Lower panel, Quantitative analysis of lipid accumulation in adipocytes by measuring OD\(_{500}\) of Oil Red O eluted from A23187- or vehicle-treated adipocytes as indicated. B. A23187 suppressed expression of adipogenic marker genes. After treatment of vehicle or 2 \(\mu\)M A23187 for 48 h on day 4 after MDI stimulation, mRNA levels of adipogenic genes in vehicle- or A23187-treated adipocytes were determined by RT-Q-PCR. Representative RT-Q-PCR data shown here is from three independent experiments with similar results.
inhibits lipid accumulation even in the late phase of adipogenesis via downregulation of lipogenic genes. Various NRs have been known to be involved in lipid metabolism via regulation of lipogenic gene expression. To test whether calcium inhibits lipid accumulation in differentiating 3T3-L1 cells via reduction of lipogenic NR expression, we first evaluated effect of A23187 on mRNA levels of several NRs including PPARγ and LXRα in differentiating adipocytes using RT-Q-PCR. As shown in Fig. 2A, 48 hour treatment of 2 μM A23187 to day 4 3T3-L1 cells resulted in reduction of mRNA levels of PPARγ and LXRα but not of FXRα and TR4 (Fig. 2A). Next, we tested whether addition of rosiglitazone, a PPARγ agonist, could overcome A23187-mediated suppression of lipid accumulation in differentiating adipocytes during the late phase of adipogenesis by Oil Red O staining. As expected, 48 h treatment of A23187 on day 4 3T3-L1 cells reduced lipid accumulation in day 8 adipocytes (Fig. 2B). When rosiglitazone was co-treated with A23187, inhibitory effect of A23187 on lipid accumulation in differentiating adipocytes was partially derepressed, suggesting the possibility that during the late phase of adipogenesis, A23187 may suppress lipid accumulation in differentiating adipocytes by downregulation not only of expression but also of transcriptional activity of lipogenic NRs. In addition, previous report proposed that calreticulin, a major Ca^{2+}-buffering protein in the lumen of the ER which is induced by PPARγ, might inhibit PPARγ activity by negative feedback mechanism (Szabo et al., 2008). Thus, by using reporter gene assay, we investigated if [Ca^{2+}], mobilization could regulate transactivation of NRs implicated in lipid metabolism. Addition of A23187 resulted in suppression of ligand (1 μM GW3965 or 1 μM rosiglitazone) induced LXRα and PPARγ transcriptional activities in HEK293T cells (Fig. 3). However, we were not able to see any significant effect of A23187 on TR4- and FXRα-mediated transcriptional activities, suggesting that A23187 may inhibit lipid accumulation in differentiating 3T3-L1 cells via specific modulation of PPARγ and LXRα activities.

**Inhibition of HDAC activity reduces suppressive effect of A23187 on PPARγ and LXRα transcriptional activities**

Previous studies have demonstrated that increased HDAC activity enforces corepressor complex and thus, blocks recruitment of coactivators to liganded NRs within their target gene promoter, resulting in repression of NR function in the regulation of their target gene expression. To test whether HDACs mediate suppressive effect of
Inhibition of Lipid Accumulation in Adipocytes by Ca\(^{2+}\) Suppression of PPAR\(\gamma\) and LXR\(\alpha\) Signalings

A23187 on PPAR\(\gamma\) and LXR\(\alpha\) transactivation, we evaluated effect of TSA, a HDAC inhibitor, on A23187-mediated inhibition of PPAR\(\gamma\) and LXR\(\alpha\) activities. As shown in Fig. 4, ligand treatment (1 \(\mu\)M GW3965 or 1 \(\mu\)M rosiglitazone) to HEK293T cells transfected with LXR\(\alpha\) or PPAR\(\gamma\) induced LXR\(\alpha\) or PPAR\(\gamma\) transcriptional activities, respectively. These ligand effects on LXR\(\alpha\) and PPAR\(\gamma\) activities disappeared when A23187 was co-treated with their ligands (1 \(\mu\)M GW3965 or 1 \(\mu\)M rosiglitazone).

However, TSA treatment together with A23187 reduced A23187-mediated suppression of LXR\(\alpha\) and PPAR\(\gamma\) activities. This result suggests that A23187 inhibits lipid accumulation at least partially via inhibition of LXR\(\alpha\) and PPAR\(\gamma\) transactivation by increase of HDAC activity. Together, these data indicate that A23187 plays a role as a functional modulator of PPAR\(\gamma\) and LXR\(\alpha\) transcriptional activities in differentiating adipocytes.

Fig. 3. Increased [Ca\(^{2+}\)]\(_i\) selectively suppressed transcriptional activities of PPAR\(\gamma\) and LXR\(\alpha\). HEK293T cells were transfected with expression vectors for NRs (50 ng pCMX-LXR\(\alpha\), 50 ng pCMX-RXR\(\alpha\), 50 ng pSG5-PPAR\(\gamma\), 50 ng pSG5-FXR\(\alpha\) or 100 ng pCMX-TR4) and the reporter genes containing appropriate response element for corresponding NR (pGL3-TK-Cyp7a-LXRE\(_{3}\)-Luc, pGL3-TK-AcoI-PPRE\(_{3}\)-Luc, pGL3-TK-HCR1-TR4RE\(_{3}\)-Luc or pGL3-TK-HCR1 for FXR, 300 ng of each). Cells were then treated with 1 \(\mu\)M GW3965, 1 \(\mu\)M rosiglitazone or 100 \(\mu\)M CDCA for 24 h in the presence or absence of 2 \(\mu\)M A23187 as indicated and assayed for luciferase activity.

Fig. 4. Inhibition of HDAC activity reduced suppressive effect of A23187 on PPAR\(\gamma\) and LXR\(\alpha\) transcriptional activities. HEK293T cells were transfected with NRs (LXR\(\alpha\) or PPAR\(\gamma\), 50 ng each) and appropriate reporter genes (pGL3-TK-Cyp7a-LXRE\(_{3}\)-Luc or pGL3-TK-AcoI-PPRE\(_{3}\)-Luc, 300 ng each). Cells were then treated with 1 \(\mu\)M GW3965 or 1 \(\mu\)M rosiglitazone for 24 h in the presence or absence of 100nM TSA or/and 2 \(\mu\)M A23187 as indicated and assayed for luciferase activity.
**Discussion**

Calcium participates in cell signaling pathway by regulation of signaling molecules such as PKC, MAPK and calmodulin-dependent enzymes and calcium mobilization has been known to affect differentiation program of various cell types including adipocytes and osteoclast (Zayzafoon, 2006). Previous studies have shown that calcium mobilization inhibits adipogenic program in the early phase of adipocyte differentiation via suppression of adipogenic transcription factor expression (Shi et al., 2000). However, role of [Ca$$^{2+}$$], in the late phase of adipocyte differentiation remains controversial (Jensen et al., 2004; Whitehead et al., 2001). Several studies reported that [Ca$$^{2+}$$], has its negative effect only in the early phase but not in the late phase of adipogenesis (Shi et al., 2000). In contrast to these studies, Zemel et al. have reported that increased [Ca$$^{2+}$$], suppressed lipolysis in the late phase of adipogenesis, resulting in increased lipid accumulation in adipocytes. In this study, by using 3T3-L1 preadipocytes, we demonstrated that calcium mobilization by A23187 on day 4 post adipocyte differentiation resulted in partial reduction of lipid accumulation and this calcium effect may at least partially go through PPAR$$\gamma$$ and LXR$$\alpha$$ to reduce lipid accumulation during adipogenesis. Adipocyte differentiation is facilitated by serial induction of various transcription factors. Several lines of evidence have established that adipogenic program triggers C/EBP$$\alpha$$ and $$\gamma$$ expression followed by induction of C/EBP$$\alpha$$ and PPAR$$\gamma$$, which in turn, orchestrate adipocyte differentiation and lipid accumulation by induction of adipogenic gene expression (Darlington et al., 1998). Our results showed that A23187 clearly inhibited transcriptional activities of PPAR$$\gamma$$ and LXR$$\alpha$$. PPAR$$\gamma$$ has been well known to be the master regulator of adipocyte differentiation. While LXR$$\alpha$$ is highly expressed in adipocytes and its expression is regulated by PPAR$$\gamma$$, role of LXR$$\alpha$$ in adipogenesis is not well defined. Several reports have shown that activation of LXR$$\alpha$$ facilitates lipid accumulation in adipocytes via induction of lipogenic genes (Juvet et al., 2003; Laffitte et al., 2001). In support of suggested function of LXR$$\alpha$$ in lipid accumulation, LXR$$\alpha$$/$$\gamma$$ deficiency resulted in reduced adipose tissues in older mice. In contrast, in other studies, LXR$$\alpha$$ did not significantly contribute to adipocyte differentiation and lipid accumulation although it could induce SREBP-1c, a well-known lipogenic transcription factor (Sekiya et al., 2007). Additional studies will be needed to clearly determine the role of LXR$$\alpha$$ in adipogenesis. Recent study has proposed that calreticulin is induced by PPAR$$\gamma$$ and inhibits PPAR$$\gamma$$ activity by negative feedback mechanism, leading to suppression of adipocyte differentiation (Szabo et al., 2008). Our findings showed that calcium selectively suppress activities of PPAR$$\gamma$$ and LXR$$\alpha$$ but not of TR4 and FXR$$\alpha$$. Liganded NRs induces target gene expression by direct binding to their response elements within target gene promoters and recruitment of various coactivators are involved in these activities of liganded NRs. In contrast, corepressors inhibit NR function and corepressor effect on NR activity is associated with HDAC activity. A23187 has been reported to increase HDAC activity with reduction of histone H3 acetylation in adiponectin promoter (Kim et al., 2006). In agreement with previous report, reduced transcriptional activities of PPAR$$\gamma$$ and LXR$$\alpha$$ by A23187 were partially recovered when TSA was co-treated with A23187. Based on our findings, mobilization of [Ca$$^{2+}$$], in the late phase of adipogenesis resulted in reduced lipid accumulation by suppression of PPAR$$\gamma$$ and LXR$$\alpha$$ expression and transcription activities and this suppressive effect of calcium on these NR activities may be at least partially through increase of HDAC activity.

Obesity is leading cause of various metabolic diseases including diabetes via insulin resistance. Thus, our study may provide the new strategy for the development of calcium milk products which can prevent obesity via modulation of lipid accumulation in adipocytes.

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**References**


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