The Calcium-Sensing Receptor Affects Fat Accumulation via Effects on Antilipolytic Pathways in Adipose Tissue of Rats Fed Low-Calcium Diets1–3

Yong-Han He,4 Yue Song,4 Xi-Lu Liao,4 Liang Wang,4 Gang Li,5 Alima,4 Ying Li,4* and Chang-Hao Sun4*

4Department of Nutrition and Food Hygiene, Public Health College, and 5Department of General Surgery, The Second Affiliated Hospital, Harbin Medical University, Harbin, China

Abstract
Low-calcium intake is associated with increased risk of obesity, but the mechanism underlying this is not clear. We previously reported that the calcium-sensing receptor (CaSR) plays an important role in modulating the expression of rate-limiting lipolysis enzymes in human adipocytes. In the present study, rats were fed diets containing normal [0.50% (NC)], low [0.30% (LC)], or very low [0.15% (VLC)] calcium for 15 wk. Ten rats of each group were killed at wk 5, 10, and 15 of the intervention. The LC-fed rats had greater visceral fat mass, lower serum FFA and glycerol concentrations, and greater CaSR expression in white adipose tissue than did those fed the NC diet at wk 10 and 15. Hormone-sensitive lipase (HSL) and adipose TG lipase (ATGL) protein levels were lower, whereas fatty acid synthase mRNA in white adipose tissue was greater in the LC-fed rats compared with the NC-fed rats. These differences from the NC group were greater in the VLC group than in the LC group at wk 15. In vitro experiments showed that 1,25-dihydroxycholecalciferol stimulated the expression of CaSR through the nuclear vitamin D receptor (nVDR). This resulted in an antilipolytic effect by increasing intracellular calcium, decreasing the intracellular cAMP level, and downregulating HSL and ATGL protein expression in adipocytes. These effects were suppressed by either nVDR or CaSR small-interfering RNA. These results suggest that CaSR affects fat accumulation by mediating antilipolytic pathways in adipose tissue of rats fed low-calcium diets. J. Nutr. 141: 1938–1946, 2011.

Introduction
The CaSR6 plays a central role in the regulation of extracellular calcium homeostasis. It was cloned by Brown et al. (1) in 1993 from the bovine parathyroid gland. In humans, the CaSR is composed of 1078 amino acids (2). The CaSR regulates calcium homeostasis within a narrow physiological range by sensing extracellular calcium concentrations and by mediating alterations in PTH secretion and renal calcium reabsorption (3,4). The human CaSR mutations cause familial hypocalciuric hypercalcemia and severe neonatal hyperparathyroidism, 2 inherited disorders characterized by altered calcium homeostasis (5).

Recently, the CaSR has been cloned from many other tissues, including the bone, kidneys, gastrointestinal tract, and human placenta, with different effects being observed in each (3). In 2005, Cifuentes et al. (6) first described CaSR expression in human adipocytes. Using antagonists or siRNA of CaSR, it was demonstrated that CaSR inhibited lipolysis by mediating [Ca2+]i and cAMP signaling pathways in the human SW872 cell line (7). However, the role of CaSR in lipid metabolism in vivo has not yet been elucidated.

Many studies have shown that calcium intake in many countries is much lower than the international recommendations (8,9). Low-calcium intake is considered to be a risk factor for some disorders, including osteoporosis (10), hypertension (11), cancer (12), and insulin resistance (13). Low-calcium intake was also reported as a potential contributor to obesity (14,15). However, until now there have been no reasonable explanations for the relationship between low-calcium intake and fat accumulation. The present study was carried out to test the hypothesis that CaSR is a possible link between low dietary calcium intake and the regulation of fat accumulation or lipid metabolism, and the potential relationship between CaSR...
expression and its effects on fat storage under low-calcium conditions has been investigation.

**Materials and Methods**

**Animals and diets.** Four-week-old male Sprague-Dawley rats (n = 30/diet group; total 90 rats; 60–80 g) from SLACCAS were housed individually in stainless wire-bottom metallic cages in an animal room at constant temperature and a 12-h-light/12-dark cycle. The rats were fed a purified diet (TROPHIC Animal Feed High-tech) for an adaptation period of 1 wk and were then divided randomly into 3 groups that consumed ad libitum diets based on the purified AIN-93G diet (16) containing 0.5% [wt:wt; normal (NC)], 0.30% [wt:wt; low (LC)], or 0.15% [wt:wt; very low (VLC)] calcium. Distilled water was consumed ad libitum. Rats were weighed weekly during the 15-wk experimental period. Food consumption was measured daily. At wk 5, 10, and 15 of feeding, 10 rats from each group were feed deprived for 12 h, anesthetized with pentobarbital (15–20 mg/kg, i.p.), and killed by exsanguination from the abdominal aorta. Blood samples were left to coagulate at 4°C and then centrifuged at 3000 × g for 15 min to extract serum. Liver, kidney, perirenal, epididymal, and omental fat pads were dissected from each rat. Tissues were weighed immediately after dissection to avoid evaporative weight loss and then frozen at −80°C for subsequent analysis. Body fat content was calculated as follows:

\[
\text{Body fat content} = \frac{\text{Weight of fat tissue}}{\text{Body weight}} \times 100
\]

The left femur from each rat was removed to measure BMD. Animal care and experimental procedures were approved by the Animal Experimental Committee of Harbin Medical University.

**Blood analysis.** Serum TG, total cholesterol, HDL-cholesterol, and LDL-cholesterol were determined using commercial assay kits (Biosino Bio-Technology and Science) and with an auto-analyzer (Autolab). Serum FFA and LPL were measured using commercial assay kits (Applygen Technologies; Nanjing Jiancheng Bioengineering Institute) following the manufacturers’ protocols. LPL activity is expressed as mmol FFA produced/h/L serum.

**BMD.** Femur BMD was measured by DXA using a GE Lunar Prodigy densitometer (GE LUNAR Radiation) at the Second Affiliated Clinical Hospital of Harbin Medical University.

**Adipose tissue morphometry.** The epidymal fat pad was fixed with 10% formaldehyde and paraffin embedded. The mean adipocyte size was measured using hematoxylin-stained sections. The number of adipocytes per microscopic field (density) was determined at a magnification of ×100. The mean surface area of the adipocytes (μm²) was calculated using image analyzer software (Motic China Group).

**Western blotting.** The protein expression of CaSR, HSL, and ATGL in liver and muscle, vitamin D hydroxylase (CYP27B1) in kidney, and FAS in adipose tissue were determined using the ABI Prism 7500 Fast Real-Time PCR system with SYBR Green PCR Master mix (Applied Biosystems). The quantitative real-time PCR amplification procedure was as follows: samples were preincubation at 95°C for 10 min followed by 40 cycles of amplification consisting of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C; β-actin was used as the internal control. The reaction was followed by melting curve analysis to verify specificity. The expression of each gene was evaluated using the 2^(-∆∆CT) method as described by Livak and Schmittgen (19). Dilution curves were used to test the PCR efficiency according to the guidelines for qRT-PCR proposed by Bustin et al. (20). The sequences and annealing temperatures of the primers (Sangon Biotech) are given in **Supplemental Table 1**.

**Serum [Ca²⁺] and calcium-related hormones.** Serum total calcium was measured by the arsenazo III dye method with a commercial calcium kit (Kehua Bio-Engineering). PTH was measured using an Intact PTH ELISA kit (Alpco Diagnostics) and 1,25-di(OH)₂D₃ was determined using a rat 1,25-di(OH)₂D₃ ELISA kit (Uscn Life Science) according to the manufacturer’s instructions. The concentrations were determined by calculations based on a standard curve. All standards and samples were run in duplicate.

**Cell culture and treatment with low calcium, PTH, and 1,25-di(OH)₂D₃.** Primary-cultured rat adipocytes (hereafter rat adipocytes) and a human adipocyte cell line (SW872) were used as cell models in this experiment. Rat adipocytes were isolated from epidymal fat pads from rats fed the AIN93G diet by washing, mincing, collagenase (Invitrogen) digestion, and filtration of the cells, as previously described (21) according to the method of Rodbell (22). Isolated adipocytes were cultured in phenol red-free DMEM/F12 (Sigma) supplemented with 1% FBS and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology) in a humidified incubator with an atmosphere of 5% CO₂ at 37°C. The SW872 cell line was obtained from American Tissue Culture Collection and cultured according to Richardson et al. (23). The medium was changed every other day. Confluent monolayers of adipocytes were induced to differentiate into mature cells using 0.5 mmol/L oleic acid (Sigma) for 3 d according to the method of Lu et al. (24). A calcium-free Eagle’s Minimum Essential Medium (Sigma) was used supplemented with various concentrations of CaCl₂ (0.25, 0.50, and 1.0 mmol/L) relative to the expression of CaSR in in vitro experiments. The [Ca²⁺⁺] was the control medium was 1 mmol/L. When the cells were incubated with PTH (Sigma) or 1,25-di(OH)₂D₃ (Sigma), DMEM/F12 (3:1) was used as the control medium with 1.0 mmol/L calcium. After incubations with low calcium, PTH, or 1,25-di(OH)₂D₃ for the indicated times, the protein expression of CaSR, HSL, ATGL, and β-actin were detected by Western blotting as described by Ueki and Stipanuk (18).

**Lipolysis assay.** For rat adipocytes, a total of 50 μL of packed adipocytes were suspended in 500 μL of phenol red-free DMEM/F-12, as previously described (21). Lipolysis data are expressed as mmol glycerol released/L packed cell volume. For SW872 adipocytes, the cells were treated with 1,25-di(OH)₂D₃ for 12 h and glycerol release in the culture medium was measured. The cells were washed gently 3 times with cold PBS (4°C) and then lysed with lysis buffer, as used in Western blotting. The total protein concentration was estimated using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Lipolysis data are expressed as mmol glycerol released/mg total protein.

**Gene silencing of CaSR and nVDR by siRNA.** Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEMI (Invitrogen) according to the method of Cao et al. (25). siRNA targeting CaSR (sense 5′-GCA AUC GAG UUA GGA GCAAdTdTT-3′ and antisense 5′-UUG CUC CUU ACU ACU CAG CAGUdTdTT-3′), nVDR (sense 5′-GGA UCU GAG UGA AGA AGAAdTdTT-3′ and antisense 5′-AUC UUC UUC ACU CAG CAGUdTdTT-3′), and nonspecific control siRNA were purchased from Ribobio. Both quantitative real-time PCR and RT-PCR were used to determine the effectiveness of siRNA knockdown. RT-PCR was conducted as described by Jean et al. (26). The sequences and annealing temperatures of the primers are given in Supplemental Table 1.

**Measurement of the intracellular cAMP concentration.** The intracellular cAMP concentration was measured using a cAMP Assay kit...
(Assay Designs) as previously described (7). The data are expressed as pmol cAMP/mg total protein.

$[Ca^{2+}]_{i}$, $[Ca^{2+}]_{o}$, in SW872 adipocytes was measured by laser scanning confocal microscopy (Nikon) as described by Shi et al. (27) with minor modifications. The cells were cultured on 24-well plates with round coverslips at a density of 30,000 cells/cm$^2$. Cultured primary rat adipocytes and SW872 adipocytes were loaded with fluo-3 AM (Invitrogen) in HBSS containing 138 mmol/L NaCl, 1.8 mmol/L CaCl$_2$, 0.8 mmol/L MgSO$_4$, 0.9 mmol/L NaH$_2$PO$_4$, 4 mmol/L NaHCO$_3$, 20 mmol/L HEPES, 5 mmol/L glucose, 1% BSA (Amersco), and 50 mg/L Pluronic F-127 (Invitrogen). Each analysis evaluated the responses of 6–8 representative whole cells. The fluorescence intensity value is expressed in analogue to digital converter units. Images were analyzed using EZ-C1 FreeViewer 3.00.502 imaging software (Nikon).

HSL and ATGL protein expression in adipocytes treated with 1,25-diol(D)$_2$D$_3$. SW872 adipocytes were treated with 1,25-diol(D)$_2$D$_3$ after nVDR or CaSR knockdown by gene silencing. After incubation with 1,25-diol(D)$_2$D$_3$ for the indicated time, cells were homogenized with ice-cold lysis buffer and the homogenates were subjected to Western blotting analysis as described by Ueki and Stipanuk (18).

Statistical analysis. Statistical analyses were performed using SPSS 13.0 software (Beijing Stats Data Mining). Data are expressed as means ± SEM and differences of $P < 0.05$ were considered significant. Data from rat experiments and glycerol release in vitro were analyzed by 2-way factorial ANOVA. One-way ANOVA was used to analyze other data from in vitro experiments. Repeated-measures ANOVA was used to test for differences in $[Ca^{2+}]_{i}$ elicited by 1,25-di(OH)$_2$D$_3$. When interaction and/or the main effects were significant, means were compared using the Bonferroni post hoc test.

Results

Food intake, weight gain, and visceral fat. Food intake did not differ among the 3 groups at any time (Fig. 1A). Body weight was greater in the VLC group than in the NC and LC groups at wk 15 ($P < 0.05$) (Fig. 1B). Visceral fat mass increased in all 3 groups ($P < 0.05$); however, the interaction of diet and time was not significant. Fat mass was greater in the LC and VLC groups than in the NC group at wk 10 and 15 ($P < 0.05$). The LC and VLC groups did not differ in fat mass at wk 10. At wk 15, fat mass differed among the 3 groups ($P < 0.05$) (Fig. 1C). Adipocyte size was greater in the LC and VLC rats compared with the NC rats at wk 10 ($P < 0.05$) (data not shown) and 15 ($P < 0.05$), but the LC and VLC groups did not differ (Supplemental Fig. 1A,B).

BMD. Rats fed the VLC diets had lower femur BMD than did the NC-fed rats at wk 5 ($P < 0.05$). BMD differed among the 3 groups at wk 10 and 15 ($P < 0.05$) and there was no interaction between time and diet (Supplemental Fig. 1C).

Serum lipids and LPL activity. The interaction between diet and time affected the FFA and glycerol concentrations, with the 3 groups differing from one another at wk 10 and 15 ($P < 0.05$) but not at wk 5 (Table 1). There were no differences among the 3 groups in serum LPL activity or total cholesterol, HDL-cholesterol, LDL-cholesterol, or TG concentrations at any time (Table 1).

HSL and ATGL protein expression and FAS and Cpt-1 mRNA expression. The expression of HSL was lower in the LC and VLC groups than in the NC group at wk 10 and decreased in a dose-dependent manner in the 3 groups at wk 15 ($P < 0.05$) (Fig. 2A). The expression of ATGL decreased (Fig. 2B), whereas FAS mRNA expression increased (Fig. 2D) in a dose-dependent manner in the 3 groups at wk 10 and 15 ($P < 0.05$). Determination of the levels of Cpt-1 mRNA in rat liver and skeletal muscle showed no differences among the 3 groups at any time (Supplemental Fig. 2A).

CaSR expression in white adipose tissue. The interaction between diet and time affected the expression of CaSR in white adipose tissue ($P < 0.05$). There was no difference among the 3 groups in CaSR expression at wk 5. CaSR expression was greater in the LC and VLC groups than in the NC group at wk 10 ($P < 0.05$); the LC and VLC groups did not differ. At wk 15, CaSR expression dose dependently increased in the 3 groups ($P < 0.05$) (Fig. 2C).

Serum calcium, PTH, 1,25-di(OH)$_2$D$_3$, and vitamin D hydroxylase concentrations. At wk 5, 10, and 15, serum total calcium levels in the VLC group, but not the LC group were lower than in the NC group ($P < 0.05$); the LC and VLC groups did not differ. The PTH and 1,25-di(OH)$_2$D$_3$ concentrations and
FIGURE 2

HSL (A), ATGL (B), and CaSR (C) protein levels and the FAS (D) mRNA level in white adipose tissue of rats fed a NC, LC, or VLC diet for 5, 10, or 15 wk. Values are means ± SEM, n = 10. Means at a time without a common letter differ, P < 0.05. *Different from wk 5, P < 0.05; #different from wk 10, P < 0.05. ATGL, adipose TG lipase; CaSR, calcium-sensing receptor; FAS, fatty acid synthase; HSL, hormone sensitive lipase; LC, low calcium; NC, normal calcium; VLC, very low calcium.

TABLE 1
Serum lipid, calcium, and calcium-related hormone concentrations in rats fed a NC, LC, or VLC diet for 5, 10, or 15 wk

<table>
<thead>
<tr>
<th></th>
<th>wk 5</th>
<th>wk 10</th>
<th>wk 15</th>
<th>P value</th>
<th>Calcium</th>
<th>Time</th>
<th>Calcium × time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC</td>
<td>LC</td>
<td>VLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>0.64 ± 0.09</td>
<td>0.62 ± 0.07</td>
<td>0.59 ± 0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.35 ± 0.09</td>
<td>1.32 ± 0.18</td>
<td>1.37 ± 0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>0.72 ± 0.16</td>
<td>0.65 ± 0.18</td>
<td>0.73 ± 0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>0.36 ± 0.08</td>
<td>0.39 ± 0.07</td>
<td>0.38 ± 0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPL, mmol/L/t/l</td>
<td>17.1 ± 3.22</td>
<td>15.9 ± 2.54</td>
<td>16.4 ± 3.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFA, mmol/L</td>
<td>5.24 ± 0.82</td>
<td>5.28 ± 0.58</td>
<td>5.30 ± 0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycolipids, µmol/L</td>
<td>215 ± 17.3</td>
<td>208 ± 22.2</td>
<td>222 ± 16.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum calcium, mmol/L</td>
<td>2.60 ± 0.31b</td>
<td>2.54 ± 0.23b</td>
<td>2.31 ± 0.09a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH, ng/L</td>
<td>42.6 ± 6.08a</td>
<td>55.4 ± 3.28b</td>
<td>62.1 ± 6.97c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25-(OH)2D3, µmol/L</td>
<td>0.15 ± 0.04a</td>
<td>0.25 ± 0.03b</td>
<td>0.31 ± 0.03c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 10. Means at a time with superscript letters without a common letter differ, P < 0.05. *Different from wk 5, P < 0.05; #different from wk 10, P < 0.05. HDL-C, HDL cholesterol; LC, low calcium; LDL-C, LDL cholesterol; NC, normal calcium; PTH, parathyroid hormone; VLC, very low calcium.
the CYP27B1 mRNA levels in the kidney dose dependently increased in the 3 groups at wk 10 and 15 (P < 0.05) (Table 1; Supplemental Fig. 2B).

**Effects of low calcium, PTH, and 1,25-dihydroxyvitamin D3 (1,25-dihydroxycholecalciferol; 1,25-di(OH)2D3) on CaSR expression in adipocytes.** SW872 and rat adipocytes were treated with different concentrations of CaCl2, PTH, or 1,25-di(OH)2D3 for 24 h. CaSR expression was not affected by low calcium or PTH (Supplemental Fig. 3) but was dose and time dependently increased by 1,25-di(OH)2D3 (P < 0.05) (Fig. 3). To gain further insight into the role of 1,25-di(OH)2D3 in modulating CaSR expression, nVDR was knocked down with siRNA (Supplemental Fig. 4C,D), through which vitamin D regulates its target genes in many tissues (28). The expression of CaSR was greater in the 1,25-di(OH)2D3-treated cells than in the control cells (P < 0.05). This effect was reversed by nVDR siRNA, with lower CaSR expression in the nVDR siRNA cells than in the 1,25-di(OH)2D3-treated cells (P < 0.05) (Fig. 5B).

**Effects of 1,25-dihydroxyvitamin D3 (1,25-dihydroxycholecalciferol; 1,25-di(OH)2D3) on lipolysis, intracellular cAMP, [Ca2+]i, and lipolysis enzymes in adipocytes.** Lipolysis was dose dependently decreased in SW872 and rat adipocytes after incubation with 1,25-di(OH)2D3 (P < 0.05). Pretreating rat adipocytes with 20 μmol/L of the antagonist, NPS2390 (Sigma), or knockdown of CaSR with siRNA in SW872 adipocytes reversed the antilipolytic effects of 1,25-di(OH)2D3 (P < 0.05) (Fig. 4A,B). The intracellular cAMP level was decreased by treatment with 50 nmol/L 1,25-di(OH)2D3 for 12 h (P < 0.05); this effect was reversed by CaSR siRNA (Fig. 4C). Expression of HSL and ATGL was reduced by treatment with 1,25-di(OH)2D3 (P < 0.05) (Fig. 5A,B). The effects of 1,25-di(OH)2D3 on HSL and ATGL expression were reversed by silencing the CaSR or nVDR genes in SW872 adipocytes (Fig. 5A,B). 1,25-dihydroxycholecalciferol increased [Ca2+]i relative to the control cells (P < 0.05); this effect was reversed by CaSR inhibition or gene silencing in SW872 adipocytes (Fig. 6A) or by CaSR inhibition in rat adipocytes (Fig. 6B).

**Discussion**

These results show that diets containing low levels of calcium caused significant visceral fat accumulation and body weight gain compared with the NC diet. Previous studies have shown that calcium supplementation may increase fecal fatty acid excretion (29) or decrease [Ca2+]i (30), resulting in reduced weight gain. However, the mechanism by which calcium plays a pathogenic role in weight control and energy balance was...
acknowledged to be poorly understood in earlier reports (31,32). Moreover, the mechanisms described to date were mainly based on studies of calcium supplementation (33) and the effects of calcium deficiency on fat accumulation remained unclear.

In this study, we observed that the LC diets caused fat accumulation, which was accompanied by decreased serum FFA and glycerol concentrations as well as decreased HSL and ATGL expression in rat adipose tissue. This led us to consider that low-calcium intake might have a role in reducing lipolysis in adipose tissue. To further test this hypothesis, other sources of FFA and glycerol, such as serum TG hydrolysis by LPL, must be excluded. Therefore, serum LPL activity was also measured in the different calcium diet groups but did not differ among them. This suggests that the decreased FFA and glycerol levels were mainly due to decreased lipolysis in adipose tissue and also accounts for the stable serum TG concentrations in the LC- and VLC-fed rats in this study. FFA oxidation also plays an essential role in fat catabolism. Serum FFA are used to produce energy through β-oxidation in the liver or skeletal muscle, and Cpt-1 is the rate-limiting enzyme in this process (34). However, there were no differences in Cpt-1 mRNA levels in skeletal muscle or in the liver among the 3 groups. These findings verified that the LC diets exerted antilipolytic effects by downregulating the expression of HSL and ATGL.

In addition to decreased expression of HSL and ATGL, CaSR expression was significantly increased in adipose tissue in time- and dose-dependent manners. CaSR was reported to be expressed in human adipose tissue and to inhibit lipolysis in human adipocytes (6,7,35). Ho et al. (36) reported significant reductions in body weight in their mouse model with CaSR knockdown. These findings indicate that CaSR may play an important role in modulating lipid metabolism in adipose tissue. In previous in vitro studies, it was shown that the antilipolytic effects of CaSR activation included inhibition of HSL and ATGL expression (7). Therefore, it is reasonable to speculate that the LC diet-induced downregulation of HSL and ATGL was mediated by CaSR in our experiment.

To validate this hypothesis, a further series of experiments were conducted. It was necessary to know how CaSR was upregulated in adipose tissue under low-calcium conditions. Calcium deficiency could trigger many molecular events and hormonal changes, including changes in serum calcium, PTH, and 1,25-di(OH)2D3 levels. There were no changes in CaSR expression following treatment with extracellular calcium or PTH in this experiment. However, CaSR expression was time- and dose-dependently upregulated after incubating cells with 1,25-di(OH)2D3. These findings confirmed that the expression of CaSR in white adipose tissue in calcium-deficient rats is regulated by 1,25-di(OH)2D3 rather than extracellular [Ca2+] or PTH. As a biologically active form of vitamin D3, 1,25-di(OH)2D3 functions through nVDR, a member of the nuclear hormone receptor superfamily, to regulate the transcription of its target genes in many tissues, including the intestine, bone, parathyroid gland, and skin (28). Canaff and Hendy (37) identified the functional vitamin D response elements in the CaSR gene and revealed the mechanism by which 1,25-di(OH)2D3 upregulated CaSR expression in the parathyroid and kidney. To confirm that 1,25-di(OH)2D3 regulates CaSR expression in adipocytes, the nVDR was knocked down using siRNA and the increased CaSR expression was prevented, demonstrating that 1,25-di(OH)2D3 is responsible for the upregulation of CaSR in adipocytes.

Next, experiments were carried out to determine whether upregulated CaSR was involved in the modulation of HSL and ATGL expression, as described above. We found that exposure to 1,25-di(OH)2D3 reduced glycerol release into the medium along with decreased expression of HSL and ATGL. These effects were diminished by either nVDR or CaSR gene knockdown.

In addition, the intracellular cAMP levels were decreased by treating cells with 1,25-di(OH)2D3, an effect that was reversed by CaSR siRNA. cAMP is a second messenger targeting lipolytic pathways (38) and can regulate gene transcription through the phosphorylation of cAMP response element-binding protein (39). The decreased cAMP level caused by upregulated CaSR expression may account for the antilipolytic effects and reduced protein expression of HSL and ATGL.

It has been reported that elevated [Ca2+] can increase the rate of lipogenesis and inhibit lipolysis, consequently leading to
In the current study, 1,25-di(OH)2D3 increased \([\text{Ca}^{2+}]\), levels, decreased cAMP levels, and downregulated adipocyte HSL and ATGL expression.

Although lipolysis plays a major role in fat accumulation, lipogenesis should not be overlooked. FAS is a key enzyme in de novo lipogenesis (41) and is primarily regulated by hormonal and nutritional factors at the transcriptional level. These results showed that the LC diets upregulated FAS mRNA expression in white adipose tissue. \([\text{Ca}^{2+}]\) has been reported to regulate FAS expression in human adipocytes (42) and the intracellular cAMP level also plays an inhibitory role in modulating FAS expression (43,44). Based on these earlier findings, it seems likely that CaSR is involved in the upregulation of FAS, as observed in animal fat tissue. To validate this hypothesis, further studies are needed to explore the role of CaSR in lipogenesis and its association with lipogenic proteins, such as FAS, in the pathogenesis of obesity.

Although substantial fat accumulation occurred in the low calcium–fed groups, food intake did not differ among the 3 groups of rats during the entire feeding period. It was previously reported that UCP2 expression in adipose tissue and UCP3 expression in liver and muscle were significantly downregulated by a LC diet (45–47). UCP2 is abundantly expressed in white adipose tissue and has a unique role in energy balance, body weight regulation, and thermoregulation (48). Hence, downregulation of UCP2 may decrease thermogenesis in calcitonin-deficient rats, explaining the dissipation of dietary energy in the present study.

In summary, in rats fed a calcium-deficient diet, the plasma calcium concentration is tightly controlled by PTH and 1,25-di(OH)2D3, which increase bone resorption and calcium absorption to replenish the blood calcium concentrations when it is low (51). Simultaneously, low calcium-induced secretion of 1,25-di(OH)2D3 upregulates CaSR expression in adipose tissue, which is coupled with changes in serum calcium. This is followed by increases in \([\text{Ca}^{2+}]\), decreases in cAMP levels, and reduced lipolysis or possibly enhanced lipogenesis, ultimately leading to fat accumulation in adipocytes. Accordingly, these antilipolytic and possible lipogenic effects that are mediated by CaSR under low-calcium conditions are involved in the increased fat accumulation.
relationship between calcium intake and fat stores or body weight. The CaSR may represent a new, important target for the development of therapeutic interventions for obesity.

Acknowledgments
Y.H.H., Y.L., and C.H.S. conceived and designed the study; Y.H.H., Y.S., X.L.L., and L.W. conducted the experiments and data collection; G.L., A., and L.W. conducted statistical analysis; Y.H.H., Y.S., L.W., G.L., A., Y.L., and C.H.S. interpreted the data; Y.H.H. wrote the manuscript; and Y.H.H., Y.L., and C.H.S. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited


