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Involvement of calcium-sensing receptor in inhibition of lipolysis through intracellular cAMP and calcium pathways in human adipocytes

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ABSTRACT

The calcium-sensing receptor (CaSR) was cloned initially from the bovine parathyroid and its primary physiological role is maintaining constant blood Ca^{2+} levels. Subsequently, CaSR was found to be expressed in human adipose tissue, however, its physiological functions remain unclear. In this study, the effect of CaSR on lipolysis and the mechanisms by which it functions were explored in SW872 cells. The results showed an inhibitory effect of CaSR on lipolysis after its being activated by $GdCl_3$, a CaSR agonist. CaSR stimulation decreased both cyclic AMP (cAMP) level and cAMP-dependent protein kinase A (PKA) activity. $GdCl_3$ treatment led to an increase in intracellular calcium ($[Ca^{2+}]_i$) and mRNA level of phosphodiesterase3B (PDE3B). Furthermore, the downstream key enzymes of lipolysis, HSL and ATGL, were downregulated at both the transcription and translation levels by treatment with $GdCl_3$. Compared to the control group, the above effects were prevented by either NPS2390, a CaSR antagonist, or CaSR gene silencing by small interfering RNA (siRNA). These findings suggest that CaSR plays an antilipolytic role by mediating potential $[Ca^{2+}]_i$ and cAMP pathways and resultant downregulation of lipolysis key enzymes in adipocytes.

1. Introduction

The calcium-sensing receptor is a seven transmembrane G-protein-coupled receptor which plays a central role in regulating calcium homeostasis. It was cloned from the bovine parathyroid gland by Brown in 1993 [1]. The human CaSR is a 120 kDa protein, consisting of 1078 amino acids [2]. Activation of the CaSR by extracellular Ca^{2+} inhibits parathyroid hormone (PTH) secretion, stimulates calcitonin secretion, and promotes urinary Ca^{2+} excretion, and thereby maintains serum Ca^{2+} at normal levels [3,4]. In recent years, CaSR has been reported to be expressed in many other tissues, such as the kidneys [5], bone [6], gastrointestinal tract [7], human placenta [8] etc., where it exerts different effects. This suggests that CaSR may be a multifunctional protein. In 2005, Cifuentes et al. first cloned CaSR in human omental adipose tissue, opening the possibility to investigate the physiological implications of CaSR in adipose tissue [9]. However, the mechanisms by which CaSR functions in lipid metabolism *in vitro* has not been elucidated clearly.

CaSR was reported to mediate activation of G_q-PI-PLC pathway leading to $[Ca^{2+}]_i$ increase, and G_i-cAMP signaling causing inhibition of cAMP accumulation in several cell types [3,10]. As a critical pathologic basis of lipid metabolism disorders, that the rise of $[Ca^{2+}]_i$ exhibits antilipolytic effects in adipocytes has been reported [11]. cAMP controls crucial physiological cell functions such as differentiation, cell growth, transcriptional regulation, etc. [12]. The cAMP pathway is one of the best known mechanisms that activate lipolysis in adipocytes. cAMP activates PKA which activates HSL by promoting its phosphorylation [13]. Hormone-sensitive lipase (HSL) has been generally considered as a rate-limiting enzyme for lipolysis and been modulated by a list of biochemical conditions [14]. Adipose triglyceride lipase (ATGL) has been reported to be another key enzyme involved in triglyceride hydrolysis [15]. ATGL catalyzes the initial step in triglyceride lipolysis, while HSL degrades diglyceride to monoacylglycerol (MG) and free fatty acid (FFA). It has been showed that HSL protein and mRNA expressions are major determinants of the maximum lipolytic capacity of human fat cells [14]. Whether CaSR affects lipolysis by mediating $[Ca^{2+}]_i$ and cAMP pathways, or by regulating the expressions of two key lipolysis enzymes were not yet clear. The purpose of the present study is to investigate the possible signaling pathways by which CaSR plays a role in lipid metabolism.

2. Materials and methods

2.1. Chemicals and reagents

$GdCl_3$, NPS2390, caffeine and DMEM/F-12 were purchased from Sigma (St. Louis, MO, USA). The fluo-3 AM was from Molecular

Abbreviations: CaSR, calcium-sensing receptor; PVDF, polyvinylidene difluoride; $GdCl_3$, gadolinium chloride; BSA, bovine serum albumin; PMSF, phenylmethyl sulfonylfluoride; HEPES, hydroxyethyl piperazine ethanesulfonic acid; PBS, phosphate buffered solution; Tris, tris (hydroxymethyl) aminomethane.

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Probes Inc. (Eugene, Oregon, USA). The glycerol assay kit was from Applygen Technologies Inc. (Beijing, China). The BCA Protein Assay Kit, RIPA lysis buffer, and PMSF were from Beyotime Institute of Biotechnology (Haimen, China). The cAMP Assay Kit was from Assay Designs, Inc. (Ann Arbor, Michigan, USA). Polyclonal antibodies against CaSR, HSL, ATGL, and β-actin were obtained from Abcam (Cambridge, UK). The cDNA Reverse Transcription Kit and SYBR Green Mix were from Applied Biosystems (Foster City, CA). The PKA assay kit was from Promega (Madison, WI, USA). siRNAs were from Ribobio (Guangzhou, China). All the primer sets were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Lipofectamine 2000 reagent, Trizol reagent and OPTI-MEMI media were bought from Invitrogen life technologies (Carlsbad, CA, USA).

2.2. SW872 cells

The SW872 cell line was previously characterized and has been shown to be a good cell model for adipocyte gene expression [16]. It was obtained from American Tissue Culture Collection (Rockville, MD) and cultured in DMEM/F-12 (3:1) containing 10% fetal bovine serum and 50 mg/mL penicillin/streptomycin in 5% CO_2/95% air at 37 °C [17]. Confluent monolayers of preadipocytes were induced to differentiate according to the method of Hui-ling [18].

2.3. CaSR gene silencing by siRNA

siRNA targeted to human CaSR (CaSR-siRNA) (sense 5′-GCA ACU GAG UAA GGA GCAAdTdT-3′ and antisense 5′-UUG CUC CUU ACU CAG UUGCdTdT-3′) and non-specific control siRNA (NS-siRNA) were purchased from Ribobio (Guangzhou, China). The transfection was carried out according to the method of Cao et al. [19]. Both reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR) were used to determine the effectiveness of the CaSR siRNA knockdown according to the manufacturer's recommendations.

2.4. Lipolysis assay

Cells were cultured in each well of a 24-well plate. The medium was collected and incubated at 70 °C for 10 min to inactivate residual lipases. Glycerol released into the medium was determined by the glycerol assay kit (GPO Trinder reaction) at 490 nm. The medium was centrifuged at 10,000*g* for 5 min at 4 °C before determination. The total protein concentration was estimated by the BCA method according to the manufacturer's instructions. Experiments were replicated at least three times. Lipolysis data were expressed as micromoles of glycerol released per microgram of total protein.

2.5. Measurement of the intracellular cAMP concentration

Cells were cultured in each well of a 6-well plate. Cells were treated with $GdCl_3$ for 30 min, or pretreated with NPS2390 for 5 min and were then incubated with $GdCl_3$ and NPS2390 together for 30 min. After medium removal, cells were washed with PBS at 4 °C, 0.1 M HCl was added to the cells and incubated for 10 min at room temperature using a plate shaker to facilitate cell lysis. The intracellular cAMP concentration was measured using cAMP Assay Kit according to the manufacturer's instructions. The data were expressed as picomoles of cAMP per microgram of total protein.

2.6. PKA activity assay

SW872 adipocytes were grown in 6-well plates and stimulated with $GdCl_3$ in the presence or absence of NPS2390, and were then lysed in 100 μl RIPA lysis buffer (Beyotime). The lysate was centrifuged for 5 min at 4 °C at 14,000*g* in a microcentrifuge and the supernatant was saved. PKA activity was determined by measuring the transfer of [^{32}P]-labeled phosphates to a phosphocellulose filter-bound peptide substrate using the SignaTECT PKA assay kit (Promega) according to the manufacturer's recommendations. Each plate was assayed in triplicate. PKA activity was reported as a percentage of the maximal activity in the presence of 1 mM cAMP.

2.7. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement

$[Ca^{2+}]_i$ in SW872 adipocytes was measured using laser scanning confocal microscope according to Zemel's methods [20] with minor modifications. Cells were loaded with more sensitive fluo-3 AM instead of fura-2. The florescence intensity was recorded by Nikon inverted microscope ECLIPSE TE2000-E equipped with a CCD camera. The florescence intensity value is expressed as the ADC-units. Images were analyzed with Nikon EZ-C1 FreeViewer 3.00.502 imaging software.

2.8. Extraction of total RNA and PCR analyses

Total RNA was isolated using Trizol reagent and cDNA was synthesized with random primer according to the manufacturer's instructions. The qRT-PCR amplification procedure was as follows. Samples were pre-denatured at 95 °C for 10 min and then subjected to 40 cycles of amplification consisted of 15 s at 95 °C, 30 s at 55 °C for CaSR (58 °C for HSL; 55 °C for ATGL; 50 °C for PDE3B; 60 °C for β-actin, which was used as the internal control), and 30 s at 72 °C. The expression levels of each mRNA were determined with the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) using a SYBR Green PCR Master Mix (Applied Biosystems). For each sample, a ΔC_T value was obtained by subtracting β-actin C_T values from those of the gene of interest. The average ΔC_T value of the control group was then subtracted from the sample to derive a $\Delta - \Delta C_T$ value. The expression of each gene was then evaluated by $2^{-(\Delta-\Delta C_T)}$. The detailed methods of RT-PCR in our laboratory have previously been described [21]. The sequences of the primers are given in Table 1.

2.9. Western blot analysis

Following incubation with the various treatments, SW872 adipocytes were washed three times with PBS at 4 °C and harvested. The detailed protocol of Western blot in our laboratory was described in Lu's published article [22]. The immune complexes were detected with an alkaline phosphatase-coupled secondary antibody. Experiments were replicated at least three times and a representative blot is shown.

2.10. Statistical analysis

Differences were analyzed for significance using the one-way ANOVA test. Data were expressed as mean ± SD, and *P* values less than 0.05 were considered statistically significant. Each value is the mean of at least three repetitive experiments in each group. Statistical analyses were performed using SPSS 13.0 statistical program (version 13.01S; Beijing Stats Data Mining Co. Ltd).

3. Results

3.1. Expression of CaSR and effectiveness of CaSR knockdown in the SW872 cell line

The SW872 cell line was initiated from a surgical specimen of a fibrosarcoma. The CaSR mRNA and protein are present in both SW872 preadipocytes and mature adipocytes by RT-PCR and Western blot analyses (Fig. 1A, B). The result of qRT-PCR demonstrated the level of CaSR mRNA in mature adipocytes was more than three times higher than in preadipocytes (Fig. 1C). The levels of CaSR

Table 1
The sequence of primers, annealing temperature and number of cycle.

Primer		Sequence	Length (bp)	Annealing temperature (°C)	Number of cycle
CaSR	Sense	5′-ATGACTTCTGGTCCAATGAG-3′	156	55	40
	Antisense	5′-TGCGGAACTTGATAAACAC-3′			
HSL	Sense	5′-GTGCAAAGACGGAGGACCACTCCA-3′	298	58	40
	Antisense	5′-GACGTCTCGGAGTTTCCCCTCAG-3′			
ATGL	Sense	5′-GTGTCAGACGGCGAGAATG-3′	119	55	40
	Antisense	5′-TGGAGGGAGGGAGGGATG-3′			
PDE3B	Sense	5′-CTTTGGGATTGGGACTTA-3′	105	50	40
	Antisense	5′-CACCATATTGCGAGCCT-3′			
β-actin	Sense	5′-ACTATCGGCAATGAGCG-3′	220	60	40
	Antisense	5′-GAGCCAGGGCAGTAATCT-3′			

mRNA were inhibited after knockdown by siRNA, with a 80% reduction (Fig. 1D, F).

3.2. Antilipolytic effect in SW872 adipocytes by CaSR activation

The level of glycerol released in the medium of adipocytes is generally used to assess the lipolytic effect. In this study, cells were treated with different concentrations of $GdCl_3$ for 12 h. The lipolysis was significantly decreased in a dose-dependent manner. Pretreatment with NPS2390 (20 μM), an antagonist for CaSR, for 30 min, or knockdown of CaSR with siRNA, reversed the antilipolytic effect (Fig. 2A, B).

3.3. Cyclic AMP levels and PKA activity after stimulation of CaSR

In the present study, cAMP levels and PKA activity were significantly decreased in a dose-dependent manner incubating cells with different concentrations of $GdCl_3$ for 30 min, compared to the control group. Pretreatment of cells with NPS2390 overturned the reduction of cAMP level and PKA activity induced by $GdCl_3$ (Fig. 2C, D). These findings indicate the CaSR activation affects cAMP/PKA pathway in adipocytes.

3.4. Oscillation of $[Ca^{2+}]_i$ and change of PDE3B mRNA level elicited by activation of CaSR

The fluorescence intensity was found increased significantly when incubating cells with 125 μM $GdCl_3$. Pretreatment of cells with NPS2390 inhibited the $[Ca^{2+}]_i$ increase compared to the baseline level (Fig. 3A, B). Caffeine has been reported to be a chemical that causes calcium release from internal stores [23]. Here 5 mM caffeine induced an apparent increase in $[Ca^{2+}]_i$, which was used to be the positive control group. That $[Ca^{2+}]_i$ inhibits lipolysis by activation of PDE3B has been illuminated in Zemel's studies [11]. In the present study, PDE3B mRNA was increased significantly by treatment of 125 μM $GdCl_3$ for 12 h. This effect was blocked by either inactivation or knockdown of CaSR (Fig. 3C).

3.5. HSL and ATGL gene and protein expressions after activation of CaSR

Adipocytes were treated with 31.25, 61.5, and 125 μM $GdCl_3$ for 24 h. The HSL and ATGL expressions were significantly down-regulated in a dose-dependent manner compared with the control group (Fig. 4A, B). Pretreatment of cells with NPS2390 for 30 min, then incubated with 125 μM $GdCl_3$ in the continued presence of NPS2390 for a further 24 h, inhibited the reductions of HSL and ATGL expression significantly (Fig. 4C, D). 125 μM $GdCl_3$ led to a 50% decrease in HSL mRNA level, nearly 80% in ATGL level. The reductions of both mRNA abundance were blocked by either inactivation or knockdown of CaSR by siRNA (Fig. 4E, F).

4. Discussion

The energy supply is produced by triglycerides stored in a large vacuole representing approximately 95% of adipocyte volume. The

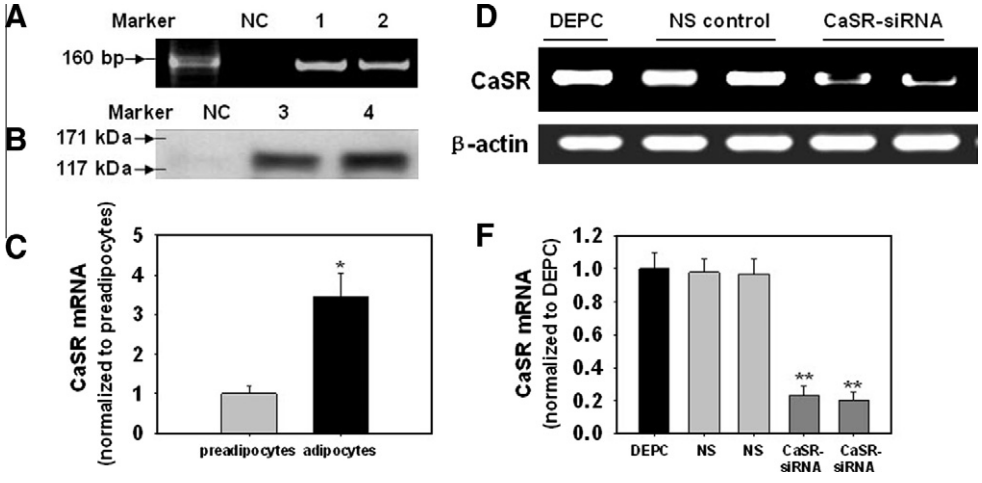

Fig. 1. Expression and silencing of CaSR in SW872 adipocyte. (A) RT-PCR analysis for CaSR transcripts in total RNA from preadipocytes (lane 1), mature adipocytes (lane 2). An 156-bp fragment of the CaSR cDNA was amplified. A negative control (NC) without cDNA in the PCR is included. (B) Western blot analysis of CaSR in preadipocytes (lane 3), mature adipocytes (lane 4). An approximately 130-kDa band of CaSR was identified. A negative control (NC) without first antibody in the Western blot is included. (C) qRT-PCR analysis for CaSR mRNA level in preadipocytes and adipocytes. (D) RT-PCR and qRT-PCR analyses for the effectiveness of CaSR knowdown. DEPC indicates the control group using DEPC water in substitution for siRNA. NS means the control group in which cells were treated with non-specific control siRNA. The experiment was performed in triplicate. Values are means ± SD. $^{*}p < 0.05$ vs. the control group, $^{**}p < 0.01$ vs. the control group.

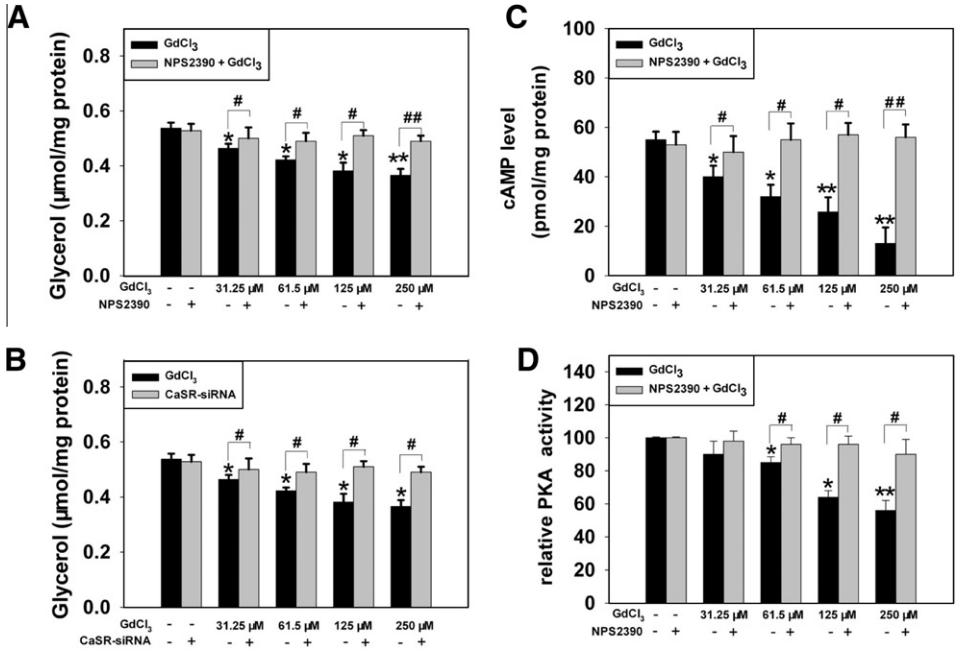

Fig. 2. Effect of CaSR agonist on lipolysis, intracellular cAMP levels and PKA activity. (A,B) Adipocytes were treated for 12 h with different concentrations of $GdCl_3$ in the presence or absence of NPS2390, or after the silencing of CaSR. (C) Adipocytes were treated for 30 min with different concentrations of $GdCl_3$, or pretreated for 30 min with NPS2390, then incubated with $GdCl_3$ in the continued presence of NPS2390 for a further 30 min. Levels of glycerol, cAMP and PKA activity were detected as described in the materials and methods section. The experiment was performed in triplicate. Values are means ± SD. $^{*}p < 0.05$ vs. the control group, $^{**}p < 0.01$ vs. the control group, $^{\#}p < 0.05$ vs. the NPS2390 free group or CaSR-siRNA group, $^{\#\#}p < 0.01$ vs. the NPS2390 free group or CaSR-siRNA group.

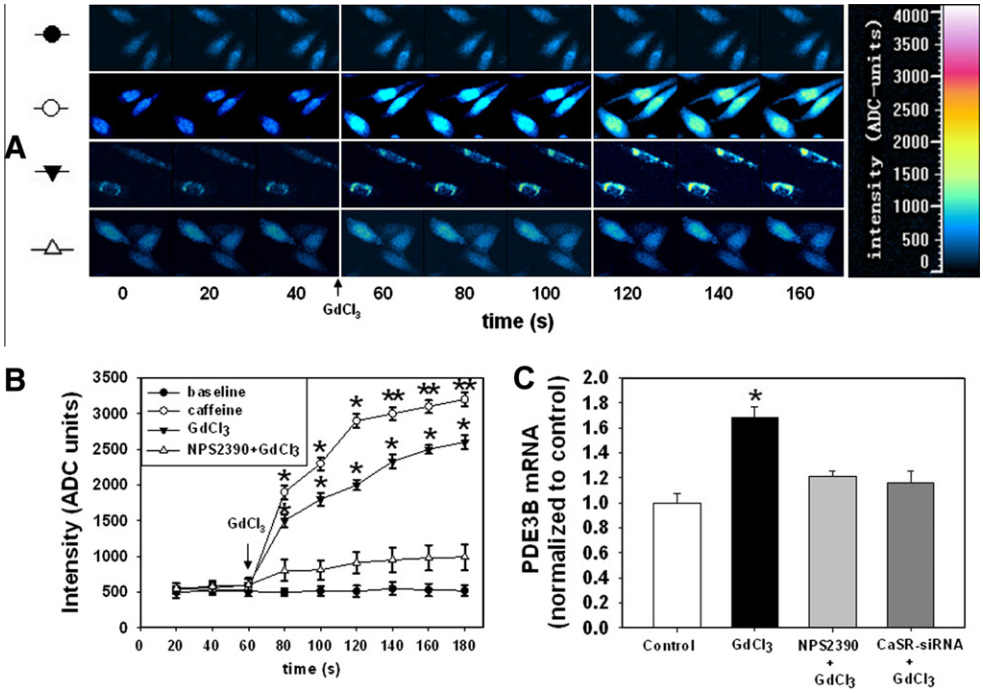

Fig. 3. Changes of concentration of $[Ca^{2+}]_i$ and mRNA levels of PDE3B in adipocytes. Adipocytes were incubated with $GdCl_3$ in the presence or absence of NPS2390. 5 mM caffeine was used as a positive control. (A) After establishing a baseline image, $GdCl_3$ was added immediately to a final concentration of 125 μM (arrow head indicates the addition of $GdCl_3$). Cells were pre-treated with NPS2390 (20 μM) for 30 min, and then scanned for 5 min on addition of 125 μM $GdCl_3$. (B) Fluorescent intensities in $[Ca^{2+}]_i$ were recorded by laser scanning confocal microscope in different treatment. (C) The mRNA levels of PDE3B when cell were given 125 μM $GdCl_3$ for 12 h in the presence or absence of NPS2390, or after the silencing of CaSR. The experiment was performed in triplicate. Values are means ± SD. $^{*}p < 0.05$ vs. baseline level (control group in Fig. 3C); $^{**}p < 0.01$ vs. baseline level.

lipolytic reaction in adipocytes is one of the most important reactions in the management of bodily energy reserves. A reduced lipolytic activity may lead to the accumulation of adipose tissue stores and may contribute to the symptoms of obesity. The best known mechanism mediating lipolysis is the cAMP pathway [13].

The CaSR, a G protein coupled receptor that signals through G_i, G_q, and sometimes $G_{12/13}$ pathways, is best known and understood for its role in regulating the secretion and synthesis of PTH in the parathyroid glands [1]. CaSR activates different signal transduction pathways, depending on the cell types. CaSR was reported to be expressed in human adipose tissue in 2005 [9]. Recently, Cifuentes et al. reported that obesity-associated proinflammatory cytokines increased CaSR protein expression in primary human adipocytes and LS14 human adipose cell line [24]. These studies indicate CaSR is closely related with the altered state of adipose tissue in obesity. In the present study, we showed that activation of CaSR contributed to a reduction in cAMP level and PKA activity in the process of antilipolysis. Chang et al. have shown that CaSR agonists inhibit cAMP accumulation in HEK293 cells stably transfected with the human CaSR [25]. We speculate the reduced cAMP levels and PKA activity account for the antilipolytic effect in adipocytes to a large extent.

In our experiment, we also found that activation of CaSR led to a significant increase in $[Ca^{2+}]_i$. As early as in 1988, Draznin et al. found basal adipocyte $[Ca^{2+}]_i$ increased in obese patients compared with normal subjects [26]. Later, Zemel et al. reported increased

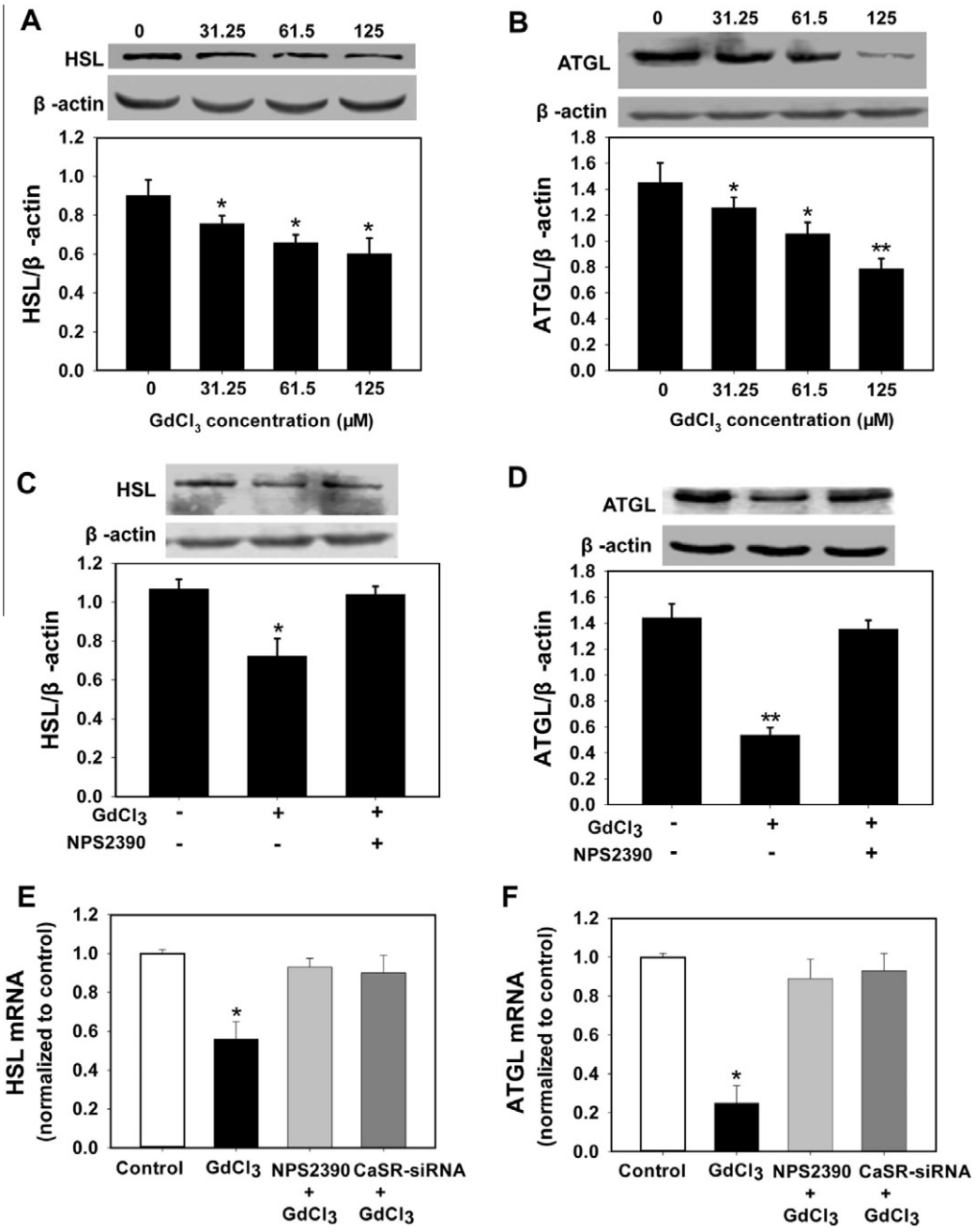

Fig. 4. Effect of CaSR agonist on HSL and ATGL gene and protein expressions. (A)–(D) HSL and ATGL expressions were detected after adipocytes were treated for 24 h with different concentrations of $GdCl_3$ in the presence or absence of NPS2390. (E,F) The mRNA levels of HSL and ATGL were determined after cells were treated with 125 μM $GdCl_3$ for 12 h in the presence or absence of NPS2390, or after the silencing of CaSR. The experiment was performed in triplicate. Values are means ± SD. $^{*}p < 0.05$ vs. the control group, $^{**}p < 0.01$ vs. the control group.

adipocyte $[Ca^{2+}]_i$ inhibited lipolysis, stimulated lipogenesis, and increased adiposity [11,27]. As a newly discovered protein, CaSR has been found to mediating calcium release from internal store in series of cell types [28,29]. Our data showed that activation of CaSR caused an increase in $[Ca^{2+}]_i$ level, inhibiting basal lipolysis in adipocytes. These effects could be reversed by using CaSR antagonist. Increased $[Ca^{2+}]_i$ in adipocytes has been reported to activate PDE3B, leading to a decrease in cAMP and, consequently, inhibition of lipolysis [11]. Down-regulation of PDE3B mRNA was closely correlated with the decrease in PDE3 activity [30,31]. Therefore, we investigated the levels of PDE3B mRNA in mature adipocytes after the CaSR activation. As we expected, CaSR agonist increased the level of PDE3B mRNA, which was reversed by either inactivation or silencing of CaSR. The upregulation of PDE3B catalyzed cAMP hydrolysis, leading to the reduction of cAMP level and a resultant antilipolysis. The findings suggest that increased $[Ca^{2+}]_i$ induced by activation of CaSR plays an essential role in the process of lipolysis in adipocytes.

HSL is the enzyme responsible for the hydrolysis of triacylglycerol from the lipid droplet of adipocytes into glycerol and FFA [32]. The activity of HSL is thought to be primarily regulated by phosphorylation–dephosphorylation. However, it has been reported that HSL protein expression is a major determinant of the maximum lipolytic capacity of human fat cells [14]. Besides HSL, ATGL is a recently described adipose-enriched protein with triglyceride-specific lipase activity. It has been demonstrated to be critical for both basal and catecholamine-stimulated triglyceride hydrolysis. Over-expression of ATGL in 3T3-L1 adipocytes increased basal lipolysis [33]. In the present study, stimulation of CaSR was observed to inhibit HSL and ATGL expressions in SW872 adipocytes. The mechanisms behind the change of both protein expressions could involve many factors. cAMP was originally shown to induce gene transcription through activation of PKA, and subsequent phosphorylation of the transcription factor cAMP response element-binding protein (CREB) [34–36]. In our experiment, the decreased cAMP level induced by activation of CaSR may account for, to some extent, the reduction of HSL and ATGL protein expressions.

In conclusion, our findings suggest that CaSR plays an antilipolytic role by mediating potential $[Ca^{2+}]_i$ and cAMP pathways and resultant downregulation of lipolysis key enzymes in adipocytes. The CaSR mediated signaling pathway may represent an important target for development of therapeutic interventions in obesity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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