Allosteric regulation of the calcium-sensing receptor in obese individuals

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Abstract. We have previously reported that the calcium-sensing receptor (CaSR) plays an important role in modulating lipid metabolism under low calcium conditions. The aim of this study was to identify possible regulators of CaSR and the mechanisms of action of CaSR in obese individuals. Subcutaneous fat samples were obtained from 10 obese and 10 non-obese males undergoing elective abdominal surgery. Visceral fat pads were obtained from 12 obese and 12 non-obese male rats. Serum lipid, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) concentrations, as well as the gene and protein expression of CaSR in the white adipose tissue of obese subjects and rats were determined. Serum total calcium, vitamin D, parathyroid hormone (PTH) and amino acid levels in human subjects were measured. Intracellular calcium and cyclic adenosine monophosphate (cAMP) levels in rat adipocytes were measured by laser scanning confocal microscopy and ELISA, respectively. The results revealed that serum triglyceride (TG), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), TNF-α, IL-6 and PTH levels were significantly higher in the obese individuals versus the controls. By contrast, serum vitamin D and amino acid concentrations were lower in the obese individuals versus the controls. In addition, intracellular calcium levels were higher, while cAMP levels were lower in the obese rat adipocytes compared with the control group. However, the gene and protein expression of CaSR in white adipose tissue did not differ between the obese groups and the controls. Thus, these results suggest that CaSR functions not through its expression, but rather through allosteric regulation in obese individuals.

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Introduction

The prevalence of obesity worldwide has progressively increased over the past few decades in the majority of high- and low-income countries (1). Obesity and the metabolic pathologies associated are the most common and detrimental metabolic diseases, affecting over 50% of the adult population. Obesity is a multi-factorial disease that develops from the interaction between genetic, environmental and psychosocial factors (2). Accordingly, identifying any of these factors is beneficial for the prevention and/or treatment of obesity.

The calcium-sensing receptor (CaSR) is a G-protein-coupled receptor that plays a critical role in modulating calcium homeostasis (3). It was cloned from the bovine parathyroid gland by Brown *et al* in 1993 (4). Subsequently, CaSR has been reported to be expressed in several other organs, where it plays various roles (3). In 2005, Cifuentes *et al* first cloned CaSR in human omental adipose tissue (5). The activation of CaSR has been shown to induce an antilipolytic effect in adipose cells (6). In a previous study, we demonstrated that the activation of CaSR inhibits lipolysis by mediating the intracellular calcium ($[Ca^{2+}]_i$) and cyclic adenosine monophosphate (cAMP) pathways in human SW872 cells (7). In addition, we also reported that CaSR activation promotes adipogenesis by regulating key transcription factors, including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT element-binding protein α (C/EBPα) (8). In another previous study, we demonstrated that CaSR activation affects fat accumulation in rats fed a low calcium diet (9). These findings collectively suggest the important role of CaSR in the development of obesity.

CaSR can be stimulated allosterically, by changes in its expression, or by a combination of both (3). CaSR regulates calcium homeostasis by sensing extracellular calcium concentrations and by mediating alterations in parathyroid hormone (PTH) secretion (10). The activation of CaSR produces rapid, transient increases in cytosolic calcium levels by mobilizing calcium from intracellular stores and by increasing calcium influx through voltage-sensitive calcium channels in the cell membrane (11). $[Ca^{2+}]_i$ plays an important role in the metabolic disorders of obesity and insulin resistance (12-14). Obese patients usually exhibit elevated basal $[Ca^{2+}]_i$ levels in adipocytes (14). Therefore, it is reasonable to assume that CaSR may play a role in the development of obesity by mediating the increase in $[Ca^{2+}]_i$ levels and affecting lipogenesis. In addition, extracellular calcium and L-amino acids have been shown to activate CaSR, as evidenced by $[Ca^{2+}]_i$ mobilization (15).

Changes in the expression of CaSR may also induce various effects in different tissues (3). The mRNA and/or protein expression of CaSR is modulated by a variety of substances and biochemical conditions, such as extracellular calcium (16), vitamin D (17) and phosphorus (18). Obesity is associated with a state of low-grade chronic inflammation and this may represent a link between the obese state and health complications associated with obesity, such as cardiovascular disease and insulin resistance (19,20). In 2010, Cifuentes *et al* reported that obesity-associated pro-inflammatory cytokines increase CaSR protein expression in adipocytes (21) and that CaSR activation elevates pro-inflammatory cytokine expression in human adipose cells and adipose tissue (22), suggesting that inflammatory factors are possible regulators of CaSR in obese individuals. In a previous study, we also found that CaSR expression was induced in the white adipose tissue of rats fed a low calcium diet (9). However, to our knowledge, no studies to date have identified the specific mechanisms of action of CaSR in the white adipose tissue of obese subjects.

In this study, we investigated the gene and protein expression of CaSR in the white adipose tissue of obese human individuals and rats. We also determined the possible regulators of CaSR, such as serum calcium, vitamin D, inflammatory cytokines and amino acids. Our results revealed that obesity produces a state of lower vitamin D and amino acids and a state of higher inflammation, with the adipose tissue CaSR expression remaining unaltered. These data, in conjunction with those from our previous studies, suggest that CaSR functions in the white adipose tissue of human subjects and rats through an allosteric mechanism.

Materials and methods

Human subjects. Ten obese [aged 18-50 years; body mass index (BMI) ≥28 kg/m^2] and 10 non-obese males (aged 18-50 years; BMI ≥18 to ≤24 kg/m^2) undergoing elective abdominal surgery in the Second Affiliated Clinical Hospital of Harbin Medical University, Harbin, China were recruited in this study. To avoid the influence of sex hormones on CaSR gene and protein expression, we recruited only male subjects in this study. Height was measured without wearing shoes by using a steel tape with a maximum of 2 m and an accuracy of 0.1 m. Body weight was measured using an electronic scale with a dial showing a maximum of 136 kg and an accuracy of 0.5 kg. Fat mass was measured using the bioelectric impedance method with a body FM analyzer (Tanita TBF-300; Tanita Corp., Tokyo, Japan) according to the manufacturer's instructions. This method has been validated for Asian children (23) and adults (24) and has been used by us in previous studies (25,26). The subjects were instructed to stand barefoot on the metal sole plates of the machine. Gender and height details were entered manually into the system. The measurement of impedance uses a standard 50 kHz-0.8 mA sine wave constant current. BMI was calculated as weight (kg) divided by the square of height (m). Subjects with a BMI ≥28 kg/m^2 were categorized as obese according to the criteria for the Chinese population (27). Blood samples were collected after 12 h of overnight fasting. The samples were left to coagulate at 4˚C and then centrifuged at 3,000 rpm for 15 min to extract serum. None of the patients were acutely ill, or showed any clinical evidence of endocrine diseases. The clinical characteristics of the subjects are presented in Table I. Information on age, height, alcohol consumption, cigarette smoking and physical activity at work and at leisure was obtained from questionnaires. Following anesthesia, samples of subcutaneous adipose tissue (average, 1.0-1.5 g) were obtained from the incision site 10-20 min after surgery commenced and frozen below -80˚C prior to the detection of CaSR mRNA and protein expression. Informed consent was obtained from the donors and the study was approved by the Ethics Committee of Harbin Medical University.

Table I. Clinical characteristics and serum lipids of obese and normal weight subjects.

Characteristics	Group	
	Control (n=10)	Obese (n=10)
Age (years)	42.81±6.96	41.22±6.84
Body weight (kg)	63.51±5.25	81.56±6.22[a]
BMI (kg/m^2)	22.79±1.43	30.36±0.94[a]
Fat mass (kg)	13.33±3.82	29.85±5.21[a]
Triglyceride levels (mmol/l)	1.45±0.22	1.85±0.31[a]
Total cholesterol levels (mmol/l)	4.01±0.83	5.32±0.62[a]
HDL-C levels (mmol/l)	1.13±0.41	0.82±0.21[a]
LDL-C levels (mmol/l)	2.53±0.42	3.36±0.63[a]
TNF-α levels (pg/ml)	2.52±0.36	3.91±0.52[a]
IL-6 levels (pg/ml)	4.26±0.95	7.53±1.26[a]

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. [a]P<0.05 compared with the control group.

Animals and diet. In this study, 48 male Wistar rats (weight, 180-220 g) from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (SLACCAS; Shanghai, China) were housed individually in stainless steel cages in an animal room at a constant temperature (22±3˚C) and a 12-h light/dark cycle. For a 10-week period, 12 rats were fed a standard diet (STD) and 36 rats were fed a high-fat diet (HFD) based on

Table II. Ingredients of the diets used for feeding the rats.

Ingredients	Amount (g/100 g diet)	
	STD	HFD
Casein	20	20
L-cysteine	0.3	0.3
L-methionine	0.16	0.16
Carbohydrates	66.84[a]	55.84[b]
Fat	7.00[c]	18.00[d]
Cellulose	1	1
Vitamin mix, AIN-93G	1	1
Mineral mix, AIN-93G	3.5	3.5
Choline bitartrate (50% choline)	0.2	0.2
Sources of energy (%)		
Protein	20	17
Carbohydrates	65	48
Fat	15	35

[a]Cornstarch (42.75 g), dextrinized cornstarch (14.2 g) and sucrose (11 g); [b]cornstarch (22.75 g), dextrinized cornstarch (14.2 g) and sucrose (15 g); [c]soybean oil; [d]lard (14.0 g) plus soybean oil (4.0 g). STD, standard diet group; HFD, high-fat diet group. n=12 per group.

a purified AIN-93G diet (28). Distilled water was provided *ad libitum*. The ingredients of the diets are presented in Table II. The rats were weighed weekly during the 10-week experimental period. Food consumption was measured daily. After the 10-week period, 36 rats were divided into 3 groups according to weight gain, with the lower 33% of the rats having gained no additional weight, the upper 33% of the rats having become obese, and the remaining 33% of the rats having gained moderate weight. The obese and control rats were fasted for 12 h, then anesthetized using pentobarbital (15-20 mg/kg, intraperitoneal) and sacrificed by exsanguination from the abdominal aorta. The blood samples were centrifuged at 3,000 rpm for 15 min to extract serum. Peri-renal, omental and epididymal fat pads were dissected from each animal. Tissues were weighed immediately after dissection to avoid evaporative weight loss, and then frozen at -80˚C for subsequent analysis. Visceral fat content was calculated as follows: 100 (perirenal + epididymal + omental fat pads)/body weight. The animal care and experimental procedures were approved by the Animal Experimental Committee of Harbin Medical University.

Measurement of serum lipid concentrations. The human and rat serum triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels were assayed by standard enzymatic colorimetric methods using commercial kits (BioSino Biotechnology, Beijing, China) and with an auto-analyzer (Autolab PM 4000; AMS Corp., Rome, Italy). Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the equation presented in the study by Friedewald *et al* (29).

Measurement of serum inflammatory cytokine levels. Human and rat serum levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Measurement of serum amino acid levels. Human serum amino acid concentrations were determined using high performance liquid chromatography (HPLC) as previously described (30). Briefly, serum samples were spiked with acetonitrile, vortex-mixed, centrifuged and the supernatant was recovered. An Alliance 2695 Separations Module and a 2487 UV detector from Waters Corp., (Milford, MA, USA) were used for the HPLC analysis. Derivatization and gradient of serum amino acids were performed according to the manufacturer's instructions.

Measurement of serum calcium, vitamin D and PTH concentrations. Human serum total calcium levels were measured by the arsenazo III dye method using a commercial calcium kit (Kehua Bio-Engineering Co., Ltd., Shanghai, China). 25-Hydroxy vitamin D_3 ($25(OH)D_3$) was measured by ultra performance liquid chromatography (UPLC). Proteins in 100 μl serum were precipitated by the addition of 200 μl acetonitrile. Samples were vortexed and centrifuged at 10,000 rpm. Supernatants were separated into new 2-ml tubes and diluted by the addition of 100 μl distilled water. $25(OH)D_3$ was extracted using an Oasis HLB 96-well μElution Plate (Waters Corp.) and detected by Acquity UPLC (Waters Corp.). PTH levels were measured using an Intact PTH ELISA kit (Alpco Diagnostics, Salem, NH, USA) according to the manufacturer's instructions.

Measurement of $[Ca^{2+}]_i$ and cAMP concentrations. Rat adipocytes were isolated from epididymal fat pads from the obese and control rats by washing, mincing, collagenase (Invitrogen Life Technologies, Grand Island, NY, USA) digestion and filtration of the cells, as previously described (9) according to the method presented in the study by Rodbell (31). Adipocytes were loaded with fluo-3 AM and then $[Ca^{2+}]_i$ levels were measured by laser scanning confocal microscopy (ECLIPSE TE2000-E; Nikon, Tokyo, Japan) as previously described (9). The florescence intensity value of the obesity group is expressed as a percentatage of the control group. The intracellular cAMP concentration was measured using a cAMP assay kit (Assay Designs, Ann Arbor MI, USA) according to the manufacturer's instructions. The data are expressed as pmol cAMP/mg total protein. Protein concentrations were measured using a BCA kit (Beyotime Institute of Biotechnology, Haimen, China).

Extraction of total RNA and quantitative reverse transcription PCR (qRT-PCR) analyses. Total RNA was isolated from 0.2 g human subcutaneous fat, or rat perirenal, epididymal or omental fat pads using TRIzol reagent (Invitrogen Life Technologies) and 1 μg of total RNA was used to synthesize cDNA using random primers according to the manufacturer's instructions (Invitrogen Life Technologies). The qRT-PCR amplification procedure was carried out as follows: samples were pre-denatured at 95˚C for 10 min and then subjected to 40 cycles of amplification consisting of 15 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C. β-actin was used as the

Table III. Primer sequences, annealing temperature and number of cycles.

Primer		Sequence	Length (bp)	Annealing temperature (˚C)	No. of cycles
CaSR	Sense	5'ATGACTTCTGGTCCAATGAG3'	156	60	40
	Antisense	5'TGCGGAACTTGATAAACAC3'			
β-actin	Sense	5'ACTATCGGCAATGAGCG3'	220	60	40
	Antisense	5'GAGCCAGGGCAGTAATCT3'			

CaSR, calcium-sensing receptor.

internal control. The expression levels of each mRNA were determined with the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using a SYBR-Green PCR Master mix (Applied Biosystems). Samples were analyzed using the $2^{-\Delta\Delta Ct}$ method (32). The sequences of the primers (Sangon Biotech Co., Ltd., Shanghai, China) are presented in Table III.

Western blot analysis. CaSR protein expression in white adipose tissue was determined by western blot anlysis. Human subcutaneous fat, or rat perirenal, epididymal or omental adipose tissue (0.5 g) was washed twice with ice-cold PBS, crushed in a mortar with liquid nitrogen, and then lysed in cold lysis butter (Beyotime) for 30 min. The lysates were centrifuged at 12,000 rpm for 20 min at 4˚C. Protein concentrations were measured using a BCA protien assay kit (Beyotime). A CaSR band of 121 kDa was identified under reducing conditions (samples were heat denatured in SDS-PAGE loading buffer supplemented with 5% β-mercaptoethanol). Equal amounts of protein were separated by SDS-PAGE and electro-transferred onto polyvinylidene difluoride membranes (Invitrogen Life Technologies). The membranes were blocked with 1% BSA and probed overnight with primary antibodies against CaSR and β-actin (Abcam, Cambridge, UK). The membranes were then washed 3 times with TBS-T buffer (150 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 7.4, 0.05% Tween-20) for 10 min, incubated with rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37˚C, and then washed again 3 times with TBS-T buffer. The blots were detected using alkaline phosphatase (Promega Corporation, Madison, WI, USA). The protein bands were subjected to autoradiography (Champ-Gel-3200, Sage Creation, China), and densitometry was quantified by Alpha EaseFc software (FluorChem, Alpha Innotech, San Leandro, CA, USA). Data are presented as the ratios of target protein to β-actin. Experiments were replicated at least 3 times and representative blots are shown.

Statistical analysis. Differences were analyzed for significance using the Student's t-test. Data were expressed as the means ± SD and a P-value <0.05 was considered to indicate a statistically significant difference. ANOVA was used to compare serum amino acid levels between the obese and normal weight controls, adjusting for age, smoking history, alcohol consumption and physical activity at work and at leisure. Each value is the mean of at least 3 repetitive experiments in each group. Statistical analyses were performed using the SPSS 13.0 statistical program (version 13.01S; Beijing Stats Data Mining Co., Ltd., Beijing, China).

Table IV. Body weight, fat content and serum lipid concentrations in the obese and control rats.

Characteristics	Control (n=12)	Obese (n=12)
Body weight (g)	471.22±28.9	520.8±39.4[a]
Fat content (%)	3.92±1.24	5.42±1.05[a]
Triglyceride levels (mmol/l)	0.73±0.24	1.26±0.34[a]
Total cholesterol levels (mmol/l)	1.72±0.31	2.12±0.23[a]
HDL-C levels (mmol/l)	0.84±0.23	0.64±0.12[a]
LDL-C levels (mmol/l)	0.54±0.22	0.90±0.16[a]
TNF-α levels (pg/ml)	42.38±4.61	56.22±6.21[a]
IL-6 levels (pg/ml)	10.53±2.68	18.62±2.65[a]

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. [a]P<0.05 compared with the control group.

Results

Lipids and inflammatory factors in obese human and rat serum. The concentrations of TG, TC and LDL-C, but not HDL-C, were significantly higher in the obese versus the non-obese individuals. The serum levels of TNF-α and IL-6 were also significantly higher in the obese versus non-obese individuals (Tables I and IV), suggesting a hyperlipidemic and inflammatory status in obese individuals. There were no significant differences between the groups as regards age, smoking history, alcohol consumption and physical activity (data not shown).

Calcium, vitamin D and PTH concentrations in human serum. Calcium is the primary agonist of CaSR. In this study, serum calcium concentrations in the obese group did not differ from those in the control group (Fig. 1A). Vitamin D has been reported to regulate the expression of CaSR in several types of tissue. $25(OH)D_3$ is the main metabolite of vitamin D in serum, and is regarded as the best indicator of the overall vitamin D status (33). Our results revealed that serum $25(OH)D_3$ levels were significantly lower in the obese versus non-obese individuals (Fig. 1B). The main role of CaSR is to maintain

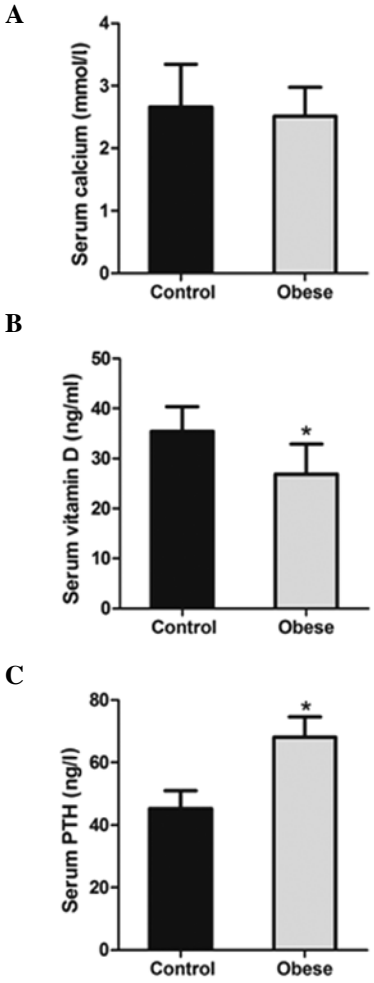

Figure 1. Serum concentrations of total calcium, vitamin D and parathyroid hormone (PTH). (A) Serum total calcium, (B) vitamin D and (C) PTH levels in 10 obese males and 10 non-obese controls were measured using the arsenazo III dye method, UPLC and ELISA, respectively. Values are the means ± SD. *P<0.05 versus the control group.

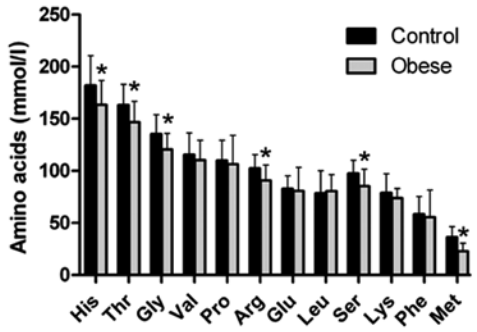

Figure 2. Serum concentrations of amino acids. Serum levels of amino acids from 10 obese and 10 non-obese controls were determined by HPLC. Values are the means ± SD. *P<0.05 versus the control group, adjusted for age, smoking history, alcohol consumption and physical activity at work and at leisure. His, histidine; Thr, threonine; Gly, glycine; Val, valine; Pro, proline; Arg, arginine; Glu, glutamate; Leu, leucine; Ser, serine; Lys, lysine; Phe, phenylalanine; Met, methionine.

calcium concentrations by regulating the secretion of PTH. In this study, PTH levels were found to be higher in the obese subjects compared with the control group (Fig. 1C).

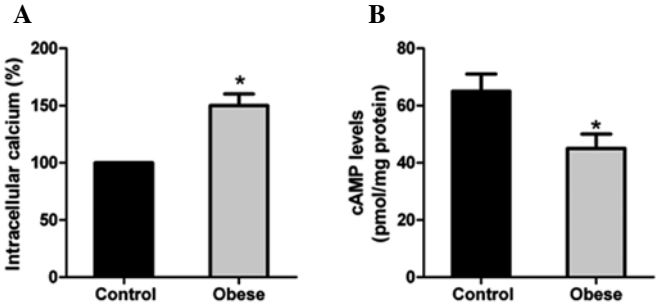

Figure 3. Levels of (A) intracellular calcium and (B) cyclic adenosine monophosphate (cAMP) in rat adipocytes. Intracellular calcium and cAMP were measured by laser scanning confocal microscopy and ELISA, respectively. Values for intracellular calcium in the obese group are expressed as a percentatage of the control group. Values for cAMP are the means ± SD. *P<0.05 versus the control group.

Amino acid levels in human serum. Amino acids can regulate a number of cellular responses, such as controlling rates of transcription and translation through the activation or inhibition of specific signaling pathways. It has been shown that CaSR is a molecular target for L-amino acids (15). Accordingly, in this study, 10 amino acids in human serum were measured using HPLC. Among these, serum histidine, threonine, glycine, arginine, serine and methionine were found to be significantly lower in the obese versus non-obese individuals, after adjusting for age, smoking history, alcohol consumption and physical activity at work and at leisure (Fig. 2). Other amino acids measured showed no differences between these 2 groups.

$[Ca^{2+}]_i$ and cAMP levels in rat adipocytes. It has been reported that $[Ca^{2+}]_i$ and cAMP are 2 critical secondary messengers modulated by the activation of CaSR (3). In this study, we observed that $[Ca^{2+}]_i$ concentrations increased, while cAMP levels decreased in obese rat adipocytes compared with the control rat adipocytes (Fig. 3).

CaSR gene and protein levels in human and rat white adipose tissue. As calcium, vitamin D, amino acids and the inflammatory cytokines, TNF-α and IL-6, have been reported to affect the expression of CaSR *in vitro* (3,21), we further investigated the expression of CaSR in the obese state. The results revealed that the gene and protein expression of CaSR in both the human and rat white adipose tissue did not differ between the obese and non-obese groups (Fig. 4). These results suggest that CaSR expression in fat tissue is not altered in obese individuals.

Discussion

CaSR plays a vareity of roles in different types of tissue in the body. In adipose tissue, ours, as well as other studies have demonstrted that after being activated, CaSR plays an antilipolytic role in adipose cells (6,7). CaSR has been shown to affect adipocyte differentiation through the regulation of transcriptional factors (8). *In vivo*, we have reported that the expression of CaSR in white adipose tissue is upregulated by a low calcium diet (9), but we did not elucidate its mechanisms of action in obesity.

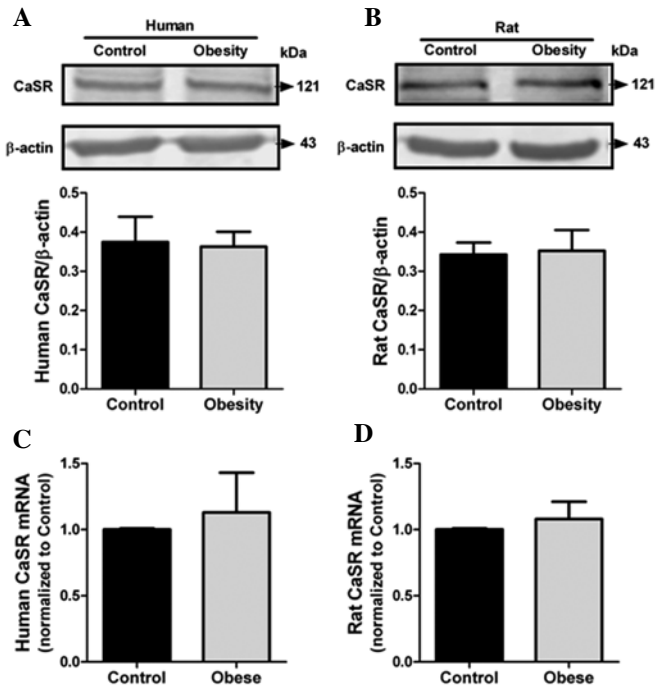

Figure 4. Protein and gene expression of the calcium-sensing receptor (CaSR) in human adipose tissue. The expression of (A and B) CaSR protein and (C and D) gene expression in human and rat adipose tissue were determined by western blot analysis and qRT-PCR, respectively. The experiments were performed in triplicate. Values are the means ± SD.

CaSR, discovered in 1993, is best known for its role in regulating the synthesis and secretion of PTH in the parathyroid glands. Since then, it has been detected in many cell types and has been shown to have various cell-dependent functions (3). One of these functions is its involvement in inflammatory events which are characterized by high levels of inflammatory cytokines (34,35). The expression of CaSR has been reported to be upregulated in the presence of IL-6 and TNF-α in different cell types (36-38). This has led to a speculation that the obesity-induced inflammatory status may play a crucial role in the upregulation of CaSR expression. Recently, Cifuentes *et al* demonstrated that the obesity-associated inflammatory cytokines, TNF-α and IL-6, increase CaSR expression in adipocytes (21). However, there has been a lack of *in vivo* evidence to support this conclusion. Therefore, in this study, we determined the status of CaSR in adipose tissue from obese humans and rats. Our results revealed that there were higher levels of TNF-α and IL-6 in obese individuals; these data are in agreement with those from previous studies showing elevated serum cytokine levels in humans and animals with excess adiposity (39). However, CaSR expression was not significantly altered in the obese versus non-obese individuals in the present study. These data are inconsistent with those presented in the study by Cifuentes *et al,* who demonstrated that CaSR expression was increased by obesity-associated pro-inflammatory cytokines (21). The mRNA and/or protein expression of CaSR can change under various circumstances. For example, calf parathyroid cells show rapid and marked reductions in CaSR mRNA and protein levels after being cultured (40). Furthermore, the renal expression of CaSR has been shown to increase following treatment with 1,25-dihydroxyvitamin D (17). Phosphorus intake has also been reported to be associated with a reduced CaSR mRNA and protein expression in the parathyroid glands (18). As described above, although obesity is associated with a state of inflammation, the level is chronic and low. Moreover, it is difficult to evaluate the local physiological concentrations of cytokines within obese adipose tissue, which depends on the secretion and clearance capacity of the individual. The cytokine concentrations in presented in the study by Cifuentes *et al* were much higher than other reported circulating levels (21). In addition, there are many other active components that may also play roles in modulating CaSR expression. As such, we believe our results to be more consistent with the actual physiological state of obesity.

Although we did not observe any significant changes in CaSR expression in adipose tissue in obese individuals, the role of CaSR in adipocytes may also be attributed to its allosteric regulation under conditions of obesity. According to its structure and known function, the most direct evidence to judge the allosteric regulation of CaSR is whether it can lead to a reduction in cAMP levels and an increase in $[Ca^{2+}]_i$ release in a short period when its expression remains constant. Our results revealed that cAMP levels were significantly reduced, while $[Ca^{2+}]_i$ levels were increased in obese rat adipocytes compared with the control group. These data are consistent with the data from our previous studies, demonstrating a rapid increase in $[Ca^{2+}]_i$ levels and a decreased cAMP accumulation in response to a CaSR agonist in adipocytes (7,8).

There are a variety of stimuli that can be sensed by CaSR in the obese state. Extracellular calcium has been the primary agonist since the discovery of CaSR. Vitamin D is a factor that has been identified to regulate CaSR expression in the kidneys (41). L-amino acids have also been shown to be CaSR ligands (15). Any changes in the above stimuli may activate or inactivate CaSR allosterically, or modulate its expression, and finally achieve their biological functions. In this study, serum calcium levels were the same between the obese and control groups due to the strong ability of the body to maintain calcium homeostasis. Nevertheless, vitamin D and amino acid concentrations were significantly lower in the obese individuals, as has previously been reported by us, as well as others (30,42). Under such circumstances, the expression of CaSR in adipose tissue remains unaltered, further supporting the possibility of allosteric regulation. In this study, although $25(OH)D_3$ levels were lower in the obese compared with the normal weight subjects, there are some lines of evidence demonstrating that $1,25\text{-di}(OH)_2D_3$, the biologically active form of vitamin D_3, is elevated in obese humans (43-45). We have previously reported that the elevation of $1,25\text{-di}(OH)_2D_3$ stimulates the expression of CaSR in adipocytes under low calcium conditions and that this is associated with an increase in $[Ca^{2+}]_i$ levels (9). However, in this study, no difference in CaSR expression in adipose tissue was observed between the obesity group and the normal weight group.

Free L-amino acids are essential molecules in biological systems. Cellular sensing of L-amino acids modulates diverse cellular responses. Fluctuating blood levels of amino acids have an important impact on body protein, carbohydrate and calcium metabolism and perhaps tissue growth and development. Thus, the low levels of amino acids observed in obese individuals

may be a regulator of body weight. Low amino acid levels have been shown to be associated with high levels of PTH, which potentially involve amino acid sensing by CaSR (46). PTH has been shown to stimulate an increase in $[Ca^{2+}]_i$ levels in adipocytes (47), which may finally lead to fat accumulation mainly by the activation of adipocyte phosphodiesterase (PDE) and a reduction in cAMP levels, leading to a decrease in hormone-sensitive lipase (HSL) phosphorylation, by stimulating the expression and activity of fatty acid synthase (FAS) (48-50). In this study, serum PTH levels were significantly higher in the obese subjects compared with the control group. Therefore, we hypothesized that the increased $[Ca^{2+}]_i$ and the decreased cAMP levels can be mediated by CaSR through sensing the changes in serum amino acids under conditions of obesity. Of course, there are numerous other unknown CaSR regulators in the serum that need to be further identified.

In conclusion, the results from the present study demonstrate that the expression of CaSR in adipose tissue is unaltered in obese individuals with inflammation and low vitamin D and acid amino acid concentrations, providing preliminary evidence that CaSR may play a role through sensing the changes in amino acids in obese individuals rather than through its expression.

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