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Calcium Sensing Receptor (CaSR) activation elevates proinflammatory factor expression in human adipose cells and adipose tissue

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Abstract

We have previously established that human adipose cells and the human adipose cell line LS14 express the calcium sensing receptor (CaSR) and that its expression is elevated upon exposure to inflammatory cytokines that are typically elevated in obese humans. Research in recent years has established that an important part of the adverse metabolic and cardiovascular consequences of obesity derive from a dysfunction of the tissue, one of the mechanisms being a disordered secretion pattern leading to an excess of proinflammatory cytokines and chemokines. Given the reported association of the CaSR to inflammatory processes in other tissues, we sought to evaluate its role elevating the adipose expression of inflammatory factors. We exposed adipose tissue and in-vitro cultured LS14 preadipocytes and differentiated adipocytes to the calcimimetic cinacalcet and evaluated the expression or production of the proinflammatory cytokines IL6, IL1β and TNFα as well as the chemoattractant factor CCL2. CaSR activation elicited an elevation in the expression of the inflammatory factors, which was in part reverted by SN50, an inhibitor of the inflammatory mediator NFκB. Our observations suggest that CaSR activation elevates cytokine and chemokine production through a signaling pathway involving activation of NFκB nuclear translocation. These findings confirm the relevance of the CaSR in the pathophysiology of obesity-induced adipose tissue dysfunction, with an interesting potential for pharmacological manipulation in the fight against obesity- associated diseases.

Keywords

calcium sensing receptor; obesity; cytokines; inflammation

Introduction

Obesity has reached pandemic proportions globally, and its association with a large number of serious health problems such as type 2 diabetes, cardiovascular disease and certain type of cancers is a great concern. Current investigative effort to understand the link between the disease and its comorbidities, aims to limit the negative consequences of the latter. The expansion of adipose mass is usually accompanied by an inflammatory status that renders a dysfunctional adipose tissue, whose altered physiology brings about the whole body metabolic alterations, increasing cardiovascular and other risks. However, different recent

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studies have shed light on a consistent proportion (about 30%) of obese subjects that seem to be metabolically healthy (1). This phenomenon is of great interest, for it supports the concept that it is not the amount of fat present, but the quality and functionality of the tissue that determines whether excess body fat leads to obesity-associated metabolic and cardiovascular disorders. As a consequence, knowing the mechanisms that promote adipose tissue inflammation becomes relevant in understanding, preventing and treating adipose tissue dysfunction.

Our laboratory reported the presence of the seven transmembrane domain, G protein-coupled calcium sensing receptor (CaSR) in human adipose cells (2). The receptor was originally described in 1993 as the main regulator of parathyroid hormone (PTH) secretion and circulating calcium concentrations (3). Different groups have later described its presence in other cell types, with many roles differing from that of calcium homeostasis, such as gastrin and gastric acid secretion (4), keratinocyte differentiation (5), tumor growth promotion or prevention (depending on the type of cancer) (6), and insulin secretion from pancreatic islet beta cells (7), among others. The CaSR has been associated with inflammatory processes, both mediating an increase in proinflammatory factors (8, 9), and responding to the presence of various cytokines by elevating its own expression (10-13). Our recent studies showed an elevation of its expression upon proinflammatory cytokine exposure in *in-vitro* differentiated human primary adipose cells and the human adipocyte cell line LS14 (14).

Given the association of the CaSR with proinflammatory processes, together with the known chronic low-grade inflammatory state in obese subjects associated with dysfunctional characteristics of adipose tissue (15, 16), we set out to study the effect of CaSR stimulation on the expression of inflammatory factors in human adipose cells. We also analysed the contribution of signalling pathway involving key inflammatory mediator nuclear factor kappa B (NFκB) in CaSR-induced adipose inflammatory state.

Materials and Methods

LS-14 cell line culture and differentiation

Our studies used the preadipose cell line LS14, derived from a human metastasic liposarcoma, able to differentiate into lipid-laden adipocytes that express mature adipocyte genes (La Pensee 2008; Hugo, 2006). Preadipose LS14 cells were seeded on plastic culture dishes (Nunc, Rochester, NY) and grown in DMEM/Ham's F-12 (1:1) medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS, Hyclone) and antibiotics (penicillin-streptomycin). For adipogenic differentiation, cells were seeded at a density of 35.000 cells/cm^2, serum-starved overnight and cultured in the same medium (serum-free), supplemented with the adipogenic cocktail consisting of 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1.7 μM insulin (Eli Lilly & Co., Mexico) and 0.25 μM dexamethasone (Sigma). The medium was replaced every 2-3 days.

Treatment of Adipose cells

LS14 cells and differentiated adipocytes were exposed overnight to 5 μM of the calcimimetic cinacalcet or vehicle. Upon experiment conclusion, cells were lysed with Trizol Reagent (Invitrogen, Carlsbad, CA) for RNA isolation. For the evaluation of the involvement of NFκB, cells were preincubated with the inhibitor of NFκB nuclear translocation SN50 (50 μM/mL) (Calbiochem, Darmstadt, Germany) for 30 minutes.

Isolation of total RNA, Reverse Transcription and Real-time PCR analysis

Total RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Contaminant DNA was removed by treating the samples with RNase-Free DNase set (Qiagen, Germany). The integrity of the RNA was checked by agarose gel electrophoresis whereas the purity was determined from the absorbance ratio (A260/A280). Total RNA was quantified by spectrophotometry (Biochrom WPA Biowave Spectrophotometer). Reverse transcription to cDNA was performed using 2 μg of RNA from each sample using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol.

Gene expression was assessed by real time PCR using a Light Cycler instrument (Roche, Germany). The reaction was performed using LightCycler®FastStart DNA Master SYBR Green I kit (Roche) and following manufacturers' protocol in a final volume of 20 μL. The cycle program consisted of an initial pre-incubation of 10 min at 95°C, then 40 cycles of 10 sec denaturing at 94°C, 15 sec annealing at 60°C and 10 sec extension at 72°C. All the reactions were performed in duplicate and positive and negative controls were included. The primer sets used (Table 1) were previously validated to give an optimal amplification and analysis of melting curves demonstrated specific single product for each gene primer. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔΔCt value was calculated by first subtracting each Ct value for the housekeeping control GAPDH from the Ct value for each gene of interest (ΔCt), and then subtracting the experimental control's ΔCt from the ΔCt value of each sample (ΔΔCt). Fold changes were finally determined by calculating $2^{-(\Delta\Delta Ct)}$. Results are expressed as expression ratio relative to GAPDH gene expression.

Adipose tissue culture and cytokine secretion

Human omental fat was obtained from a total of 12 subjects (83% female, ages between 19 and 55 y) undergoing elective abdominal surgery, with a range of body mass index of 26.0-39.2 kg/m^2; mean ± SD: 33.9±4.6 kg/m^2). Informed consent was signed by the donors, and the protocol was approved by the Institutional Review Board at INTA, University of Chile and the Health Service of Santiago. The adipose tissue was removed and transported to the laboratory immersed in saline solution. The tissue was washed several times with Hanks Balanced Salt Solution (HBSS), and minced into small pieces (2-3 mm^2), removing all visible connective tissue, blood clots and vessels.

To evaluate the secretion of proinflammatory cytokines upon CaSR stimulation, 300 mg minced adipose tissue were incubated in 3 mL of DMEM:F12 medium, that was supplemented with the calcimimetic cinacalcet 5μM or vehicle for 48hrs. The medium was centrifuged at 5000xg for 5 min and stored at -80°C until the analysis was performed. Secretion of TNFα, IL6 and IL1β was determined in the conditioned medium by QuantiGlo ELISA kits (R&D Systems, Minneapolis MN).

Statistical Analysis

To evaluate the fold-change in the expression of each cytokine, nonparametric tests were evaluated using Wilcoxon signed rank test (to compare the effect of cinacalcet versus the vehicle control, normalized to 1). Wilcoxon matched pairs test was used to compare cytokine expression between cells treated with cinacalcet and those pretreated with the NFκB inhibitor SN50. Data are shown in the figures as means ± SEM and a p-value less than 0.05 was considered significant.

Results

Exposure to cinacalcet elevates adipose cytokine expression in human preadipose and adipose cells

We evaluated the effect of CaSR stimulation on the expression of IL6, CCL2 and IL1β in LS14 preadipose cells and cells that were in-vitro differentiated to adipocytes for 7 days. As shown in Figure 1, overnight exposure to 5μM cinacalcet was associated with an increased expression of all inflammatory markers in adipocytes and preadipocytes, although IL6 did not reach statistical significance for the latter (p=0.055).

When comparing the fold-change between preadipocytes versus adipocytes, the response is more than 20-fold greater in adipocytes for IL6 (1.9±0.6 vs 44.0±21.2), 2-fold greater in adipocytes for CCL2 (1.7±0.3 vs 3.8±1.0) and 2-fold greater in preadipocytes for IL1β (6.2±2.5 vs 2.7±0.4). Interestingly, basal expression of the cytokines was much lower in differentiated compared with non-differentiated cells, namely 0.19±0.04% for IL6; 8.06±1.92% for CCL2 and 29.27±9.79% for IL1b (n=7, $p<0.05$), while expression of the housekeeping gene between the cells remained similar. The lower expression of IL6 in differentiated cells is consistent with previous studies (17)

To evaluate whether the activation of the NFκB pathway was involved in the observed effect, we pre-incubated LS14 cells with SN50, which inhibits the translocation of the NFκB active complex into the nucleus. As depicted in Figure 2, our observations show that the effect of cinacalcet on IL6 and CCL2 is decreased by SN50, suggesting that the effect of CaSR activation on these two cytokines (but not IL1β although a trend p=0.06 was observed in adipocytes) acts in part by activating the NFκB pathway. Control experiments showed that there was no effect of SN50 alone on the expression of any of the cytokines (data not shown).

Cytokine secretion in adipose tissue explants

We evaluated the secretion of TNFα, IL6 and IL1β in medium conditioned by human adipose tissue explants that were cultured for 48 hrs with cinacalcet or control vehicle. Figure 3 shows that the secretion of proinflammatory cytokines increased by 174±33% for TNFα, 230±61 % for IL6 and 211±42% for IL1β upon exposure to the calcimimetic.

Discussion

We evaluated the effect of CaSR activation on the expression of inflammatory factors in the human adipocyte cell line LS14 and our observations were confirmed in human adipose tissue explants. Our findings suggest that CaSR activation is associated with an enhancement of the adipose proinflammatory environment. These observations, together with our previous findings of a greater CaSR expression upon exposure to obesity-associated proinflammatory cytokines, suggest that CaSR may participate in a positive feedback loop, enhancing inflammation and thus leading to adverse consequences for adipose and whole body metabolism.

The low-grade inflammatory state that characterizes obesity has been proposed to be key in the dysfunction of adipose tissue and the development of several of the negative health consequences of this disease (18, 19). The elevation in the production of proinflammatory cytokines and chemokines such as TNFα, IL1β, IL6 and CCL2 leads to adverse effects like local and systemic insulin resistance, together with local activation of signaling pathways and mechanisms that will increase and perpetuate inflammation. The source of the different adipose secretory products has been the focus of many different studies. Whether adipocytes, preadipocytes, infiltrated immune cells (particularly macrophages) or other cells

in adipose tissue are more relevant in the secretion of a specific factor becomes important when considering that the cellular composition of obese "sick" compared with healthy functional adipose tissue may be quite different. Infiltration of inflammatory macrophages and an altered ratio of preadipose to mature adipose cell content due to an impaired differentiation process (which, in turn, can be induced by the proinflammatory environment) will alter the secretory profile according to each cell type's secretion pattern. Our approach was to study the effects on the main adipose cells, i.e., preadipocytes and differentiated adipocytes in the context of CaSR effects on tissue inflammation.

The proinflammatory cytokine TNFα, whose expression is increased in the adipose tissues of experimental animal models of obesity and type 2 diabetes (20), is mainly produced by macrophages in adipose tissue (21), with a central role in adipose dysfunction. It has been reported that the relative release of TNFα from adipocytes is very low (2%) compared with that of whole adipose tissue (22). In our expression experiments in isolated adipose and preadipose cells, its expression was too low (in many cases undetectable) to be able to report the effect of cinacalcet, however in whole adipose tissue, we could observe an effect of the calcimimetic. This could be the consequence of the stimulation of non-adipose cells such as monocyte-derived macrophages, which have also been shown to express functional CaSR (23, 24).

Among the many roles that have been attributed to the CaSR in different cell types, the receptor has been described to produce (8, 9) and be regulated by (10-13) inflammatory mediators. Given this association of CaSR with inflammation on one hand, and the proinflammatory character of the obese state on the other, we are interested in the role that the CaSR plays in obesity-related inflammation. In previous work, we studied the effect of elevated obesity-associated cytokines, modulating the expression of the receptor in human adipocytes. We reported that exposure to IL1β, TNFα or IL6 upregulates CaSR expression in human adipocytes, and that secretory products of adipose tissue explants elicit this same effect, notably more strongly as the adipose tissue donor has a larger BMI (14). Results from the present study add complexity to this scenario. We now report that the activation of the CaSR leads to an elevation of the inflammatory factors, which is expected to have a number of deleterious consequences to adipose tissue functionality. The elevation of the chemokine CCL2 in preadipocytes and adipocytes is expected to enhance the recruitment of monocytes, leading to an increase in the infiltration of differentiated inflammatory macrophages. The likely elevated expression of CaSR in inflamed obese adipose tissue is thus expected in turn to maintain a positive feedback loop worsening the inflammatory status.

The response to CaSR stimulation increasing inflammatory factor secretion was greater in adipocytes than preadipocytes. This different responsiveness to CaSR stimulation may have interesting physiological implications. First, it is relevant to note that we observed that basal cytokine expression was considerably lower in adipocytes, particularly for IL6 (less than 1% of that of preadipocytes) and CCL2 (8%) and also, albeit not so substantially, for IL1β (30%). This is consistent with previous reports, showing greater inflammatory profile in preadipose cells than in cells after adipogenic differentiation (25, 26). It is interesting that while cytokine and chemokine expression of preadipocytes is already at a greater basal level, adipocytes can be induced to increase their expression; the case is particularly marked for IL6. In this scenario, we can speculate that the activation of CaSR would target *in-vivo* both preadipocytes and adipocytes (and likely, infiltrated macrophages as well) to elevate overall inflammatory factor production in adipose tissue. We evaluated whether the effects observed in isolated adipose cells were consistent with what happens at the whole tissue level. Experiments using human adipose tissue explants support the findings, showing an increase in the secretion of the inflammatory factors. Although we did not measure its release in adipose tissue explants, it is expected that CCL2 secretion will be elevated as well.

Fain and Madan (2005) observed that half of the elevation of this chemokine was the result of endogenous TNFα and IL1β, both of which were indeed elevated in our adipose tissue explants exposed to cinacalcet.

In search for the intracellular mechanisms activated by the CaSR that lead to the inflammatory response, we evaluated the activation NFκB. In its cytoplasmic latent, inactive form, the small inhibitor kappa B alpha (IκBα) molecule binds to NFκB. The cellular stimulation elicited by inflammatory factors activates the cytoplasmic enzyme IκB kinase that phosphorylates IκBα, after which the phosphorylated IκBα can no longer bind and inactivate NFκB. The molecule is thus released from the complex and metabolized. The free NFκB can then translocate into the nucleus and activate the transcription of genes involved in inflammation. The human CaSR gene promoter region contains functional kappa-B response elements to the NFκB pathway (11) and activation of the CaSR was shown to elicit the translocation of NFκB to the nucleus (27). Our observations after preincubating the cells with the NFκB nuclear translocation inhibitor SN50, allow us to suggest that the CaSR-dependent elevation of IL6 and CCL2 in preadipose and adipose cells is in part (60-70% for IL6 and 30-40% for CCL2) mediated by NFκB.

The CaSR is expressed in numerous cells and organs, with a remarkable number of roles and versatility in its functions. After first describing the presence of the CaSR in human adipose cells (2), increasing evidence is being collected to establish its physiological role in this tissue. We observed an antilipolytic effect of its stimulation in isolated human adipocytes (28), which has been recently confirmed in the adipocyte cell line SW872 by He et al. (29). These authors observed that the CaSR-elicited decline in lipolysis was associated with decreased cyclic AMP, protein kinase A activity, hormone sensitive lipase and adipose triglyceride lipase, all of which are key players in the lipolytic process. We also recently established that cytokines that are typically present in inflamed adipose tissue from obese subjects elicit an elevation in the expression of the receptor in human adipose cells (14). Our present observations showing that CaSR stimulation in turn elevates proinflammatory cytokine expression, illustrates the complexity of the role of the receptor in adipose tissue. Our ongoing research is focused on understanding more of this interesting protein in adipose biology and potentially, its role in the pathophysiological aspects of obesity.

In summary, we have shown in LS14 and human adipose tissue explants that the calcimimetic cinacalcet elicits a proinflammatory response. We provide evidence that NFκB translocation to the nucleus is in part required for the effect of CaSR activation, which supports the involvement of the proinflammatory signaling activation. This alteration is expected to progressively impair adipose tissue function and ultimately lead to peripheral insulin resistance and cardiovascular risk. Knowledge of the specific events at which CaSR downstream signaling acts on inflammation is relevant, as different therapeutic targets may emerge. With an extensive number of substances regulating its expression and activity, and numerous intracellular signaling pathways, the CaSR has an increasingly recognized role as an integrator of local signals to regulate diverse cell and tissue functions (Riccardi 2009, Magno 2011). For a highly complex endocrine organ such as adipose tissue, the presence and function of the CaSR may be of great physiological relevance, and this study provides the rationale for future research in this important aspect of adipose pathophysiology.

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Abbreviations

CaSR	calcium sensing receptor
CCL2	CC chemokine ligand 2
cDNA	complementary deoxyribonucleic acid
DMEM:F12	Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12
ELISA	enzyme-linked immunosorbent assay
GAPDH	gliceraldehyde 3-phosphate dehydrogenase
HBSS	Hanks Balanced Salt Solution
IL1β	interleukin 1 beta
IL6	interleukin 6
IκBα	inhibitor-Bα
NFκB	nuclear factor kappa B
PTH	parathyroid hormone
RNA	ribonucleic acid
TNFα	tumor necrosis factor alpha

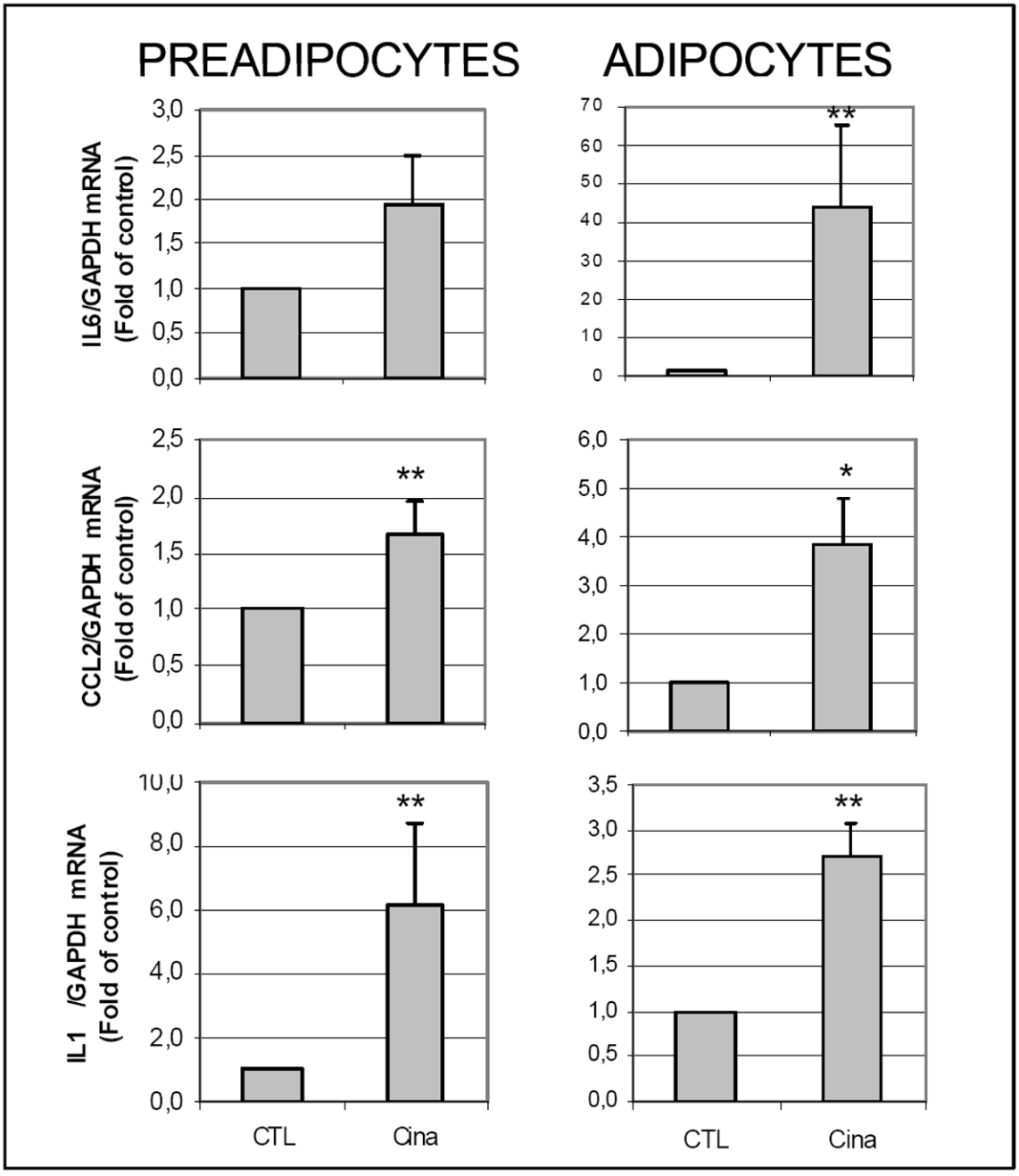

Figure 1.

CaSR stimulation elevates mRNA expression of proinflammatory factors. Quantitative PCR results of preadipose (left) and in-vitro differentiated (right) LS14 cells exposed overnight to 5 μM cinacalcet (Cina) or vehicle (control, CTL). Expression levels were normalized to each sample's GAPDH expression and then reported as a fold of control (mean ± SEM). *p<0.05 **p<0.01, Wilcoxon Signed Rank test evaluating the difference from the vehicle control, normalized to 1; n=8 independent experiments.

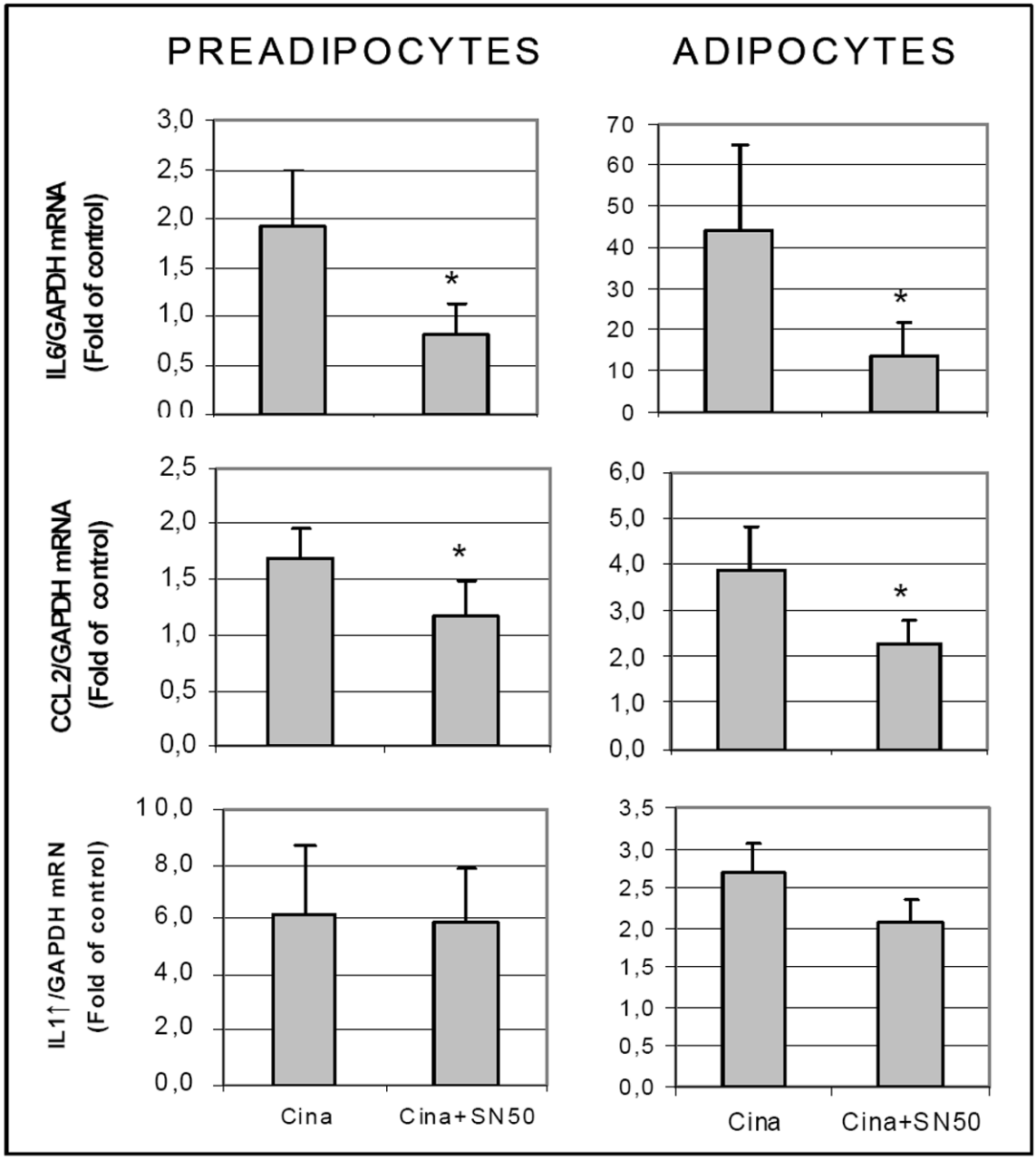

Figure 2.

Inhibition of NFκB pathway partly reverses CaSR-induced elevation of IL6 and CCL2 expression in adipose cells. Preadipose (left) and adipose differentiated (right) LS14 cells pre-exposed to the NFκB nuclear translocation inhibitor SN50 (50 μg/mL), were compared with those exposed with cinacalcet (Cina) alone overnight. Bars represent mean ± SEM of the fold change from not treated control cells (normalized to 1) in cytokine expression from 6 independent experiments evaluated by real-time PCR. *$p<0.05$ versus Cinacalcet, Wilcoxon matched pairs test.

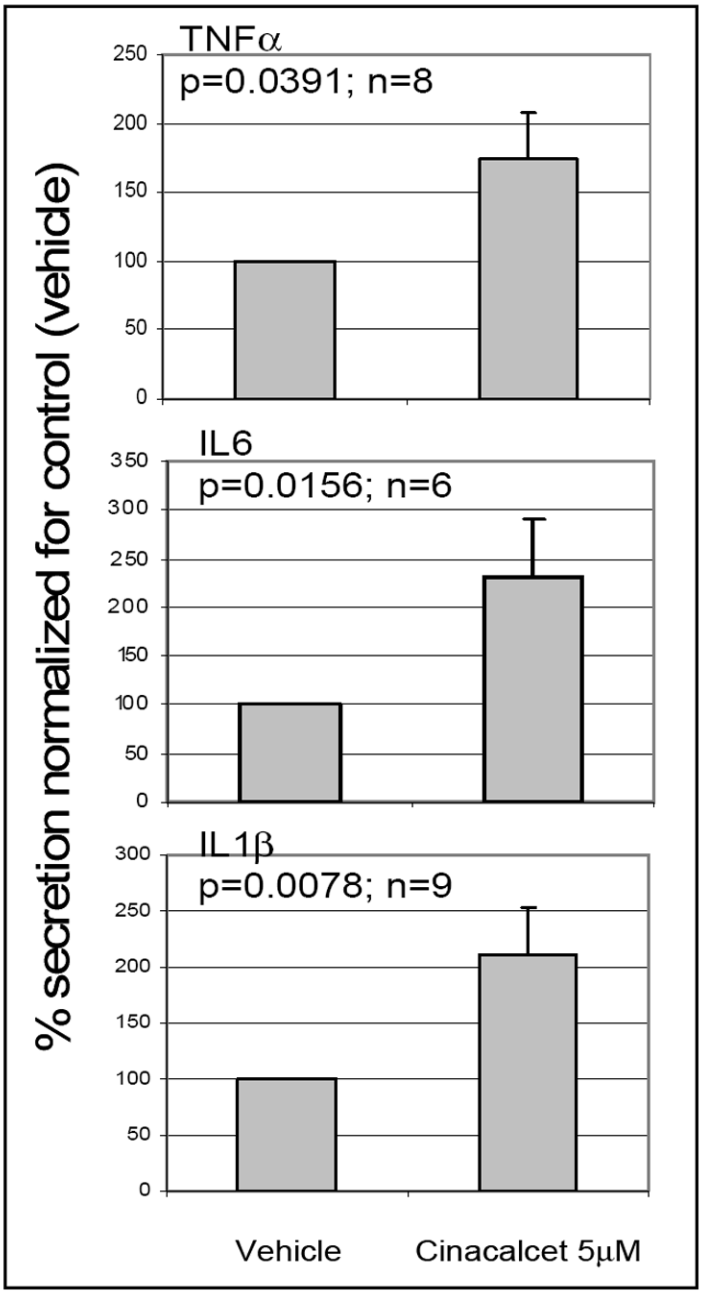

Figure 3.

CaSR stimulation elevates cytokine secretion. TNFα, IL6, and IL1β secretion, measured in media conditioned by adipose tissue explants, is shown as a percentage of the respective vehicle-treated control conditions (100%). P values for Wilcoxon Signed Rank test evaluating the difference from the 100% value are shown for each cytokine. The number of adipose tissue samples evaluated for each cytokine is indicated in the figure.

Table 1

Forward and reverse primer sequences for qPCR

Target mRNA	Forward Primer (5'→ 3')	Reverse primer (5'→ 3')
CCL2	ACTGAAGCTCGCACTCTCGCCT	CTGAGCGAGCCCTTGGGGAATG
IL1β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG
IL6	CAATCTGGATTCAATGAGGAGAC	CTCTGGCTTGTTCCTCACTACTC
GAPDH[1]	TCAACGACCACTTTGTCAAGCTCA	GCTGGTGGTCCAGGGGTCTTACT

[1]housekeeping control