Calcium sensing receptor activation elevates proinflammatory factor expression in human adipose cells and adipose tissue

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A B S T R A C T

The proinflammatory status of adipose tissue has been linked to the metabolic and cardiovascular consequences of obesity. Human adipose cells express the calcium sensing receptor (CaSR), and its expression is elevated in inflammatory states, such as that associated with obesity. Given the CaSR’s association with inflammation in other tissues, we evaluated its role elevating the adipose expression of inflammatory factors. The CaSR activation by the calcimimetic cinacalcet (5 μM) in adipose tissue and in vitro cultured LS14 adipose cells elicited an elevation in the expression of the proinflammatory cytokines IL6, IL1β, TNFα, and the chemoattractant CCL2. This was in part reverted by SN50, an inhibitor of the inflammatory mediator nuclear factor kappa B (NFκB). Our observations suggest that CaSR activation elevates cytokine and chemokine production, partially mediated by NFκB. These findings support the relevance of the CaSR in the pathophysiology of obesity-induced adipose tissue dysfunction, with an interesting potential for pharmacological manipulation.

1. 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, other recent studies have shed light on a consistent proportion (about 30%) of obese subjects that seem to be metabolically healthy (Wildman et al., 2008). This phenomenon is of great interest, for it supports the concept that it is not the amount of fat present, but the biological characteristics 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 (Cifuentes et al., 2005). The receptor was originally described in 1993 as the main regulator of parathyroid hormone (PTH) secretion and circulating calcium concentrations (Brown et al., 1993). 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 (Ceglia et al., 2009), keratinocyte differentiation (Tu et al., 2004), tumor growth promotion or prevention (depending on the type of cancer) (Chakravarti et al., 2009), and insulin secretion from pancreatic islet beta cells (Gray et al., 2006), among others. The CaSR has been associated with inflammatory processes, both mediating an increase in proinflammatory factors (Abdullah et al., 2006; Wang et al., 2002), and responding to the presence of various cytokines by elevating its own expression (Canaff and Hendy, 2005; Canaff et al., 2008; Nielsen et al., 1997; Toribio et al., 2003). Our recent studies showed an elevation of its expression upon proinflammatory cytokine exposure in in vitro differentiated human primary adipose cells and the human differentiated adipose cell line LS14 (Cifuentes et al., 2010).
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 (Calabro et al., 2009; Kloting et al., 2010), we set out to study the effect of CaSR stimulation on the expression of inflammatory factors in human adipose cells. We also analyzed the contribution of signaling pathway involving key inflammatory mediators: nuclear factor kappa B (NFκB) in CaSR-induced adipose inflammatory state.

2. Materials and methods

2.1. LS-14 cell line culture and differentiation

Our studies used the preadipose cell line LS14, derived from a human metastatic liposarcoma, able to differentiate into lipid-laden adipocytes that express mature adipocyte genes (Hugo et al., 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/cm², 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.

2.2. Treatment of adipose cells

LS14 cells and differentiated adipocytes were exposed overnight to 5 μM of the calcimimetic cinacalcet or vehicle, according to our previous data (Cifuentes and Rojas, 2008) and other laboratory observations suggesting that such dose induces metabolic effects, without affecting cell viability. 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 μg/mL) (Calbiochem, Darmstadt, Germany) according to the manufacturer's recommendations, for 30 min. In additional experiments, cells were exposed to cinacalcet for 15 min and the decrease of the inhibitor kappa B alpha (IkBα) was evaluated by immunoblot (see below).

2.3. 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 the 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 s denaturing at 94 °C, 15 s annealing at 60 °C and 10 s 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^(-ΔΔCt). Results are expressed as expression ratio relative to GAPDH gene expression.

2.4. Western blot analysis

Cells were homogenized at 4 °C in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 2 mM EGTA, 1% Triton X-100, 10% Glycerol) supplemented with complete protease inhibitors (Roche, Mannheim). Protein concentration of the lysate was determined by a method based on bicinchoninic acid (Pierce, Rockford IL). Fifteen to one hundred micrograms of protein were heat denatured in SDS-PAGE loading buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, and 40% glycerol, 20% 2-Mercaptoethanol). Proteins were electrophoresed on 8% (for CaSR) or 10% (for IkBα) polyacrylamide gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes using a buffer that contains 24 mM Tris, 194 mM glycine and 10% methanol. The immunoreaction was achieved by incubation of the membranes, previously blocked with a solution containing 4% BSA in Tris-buffered saline (TBS), with 0.05% Tween 20 (Sigma, St Louis, MO), with the corresponding antibody (mouse anti-human CaSR (Abcam Inc, Cambridge, MA), or rabbit anti-human IkBα (Cell Signaling Technology, Danvers, MA)) diluted 1:1000 in 3% BSA in TBS supplemented with 0.05% Tween 20. Detection of the immune complexes was performed with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove PA, USA) followed by an incubation with the enzyme substrates (ECL, Amersham Biosciences Piscataway, NJ, USA) and exposure to blue light sensitive films.

2.5. 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/m²; mean ± SD: 33.9 ± 4.6 kg/m²). Informed consent was
signed by the donors, and the protocol was approved by the Insti-
tutional Review Board at INTA, University of Chile and the Health
Service of Santiago. The adipose tissue was removed and trans-
ported 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 mm²), removing all vis-
ible 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 5 µM or vehicle for 48 h. In this case we al-
lowed a longer exposure period than that applied to cell cultures
(overnight) since the variable measured here was the secreted pro-
tein instead of the mRNA. 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).
Due to sample availability constraints and several cases where one
or more cytokine levels were undetectable before the treatment
with cinacalcet, we were unable to report all three cytokines for
the 12 adipose tissue donors.

2.6. Statistical analysis

To evaluate the fold-change in the expression of each cytokine,
nonparametric variables 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 cina-
calcet and those pretreated with the NFκB inhibitor SN50. Mann–Whitney test was used to compare CaSR expression in pre-
adipocytes and adipocytes. Data are shown in the figures as means
± SEM and a p-value less than 0.05 was considered significant.

3. Results

3.1. 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 both LS14 preadipose cells and those that
were in vitro differentiated to adipocytes for 7 days. As shown in
Fig. 1, overnight exposure to 5 µM cinacalcet was associated with
an increased expression of all inflammatory markers in adipocytes.

Fig. 1. CaSR stimulation elevates mRNA expression of proinflammatory factors. Quantitative real time PCR results of preadipose (left) and adipose differentiated (right) LS14 cells exposed overnight to 5 µM cinacalcet (Cina) or vehicle (control, CTL). Expression levels were evaluated in duplicate, normalized to each sample's GAPDH expression and then reported as 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.
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 \((1.9 \pm 0.6 \text{ versus } 44.0 \pm 21.2)\), 2-fold greater in adipocytes for CCL2 \((1.7 \pm 0.3 \text{ versus } 3.8 \pm 1.0)\) and 2-fold greater in preadipocytes for IL1\(\beta\) \((6.2 \pm 2.5 \text{ versus } 2.7 \pm 0.4)\). Interestingly, basal expression of the cytokines was much lower in differentiated compared with non-differentiated cells. Expression in differentiated adipocytes was 0.19 ± 0.04% of that in preadipocytes for IL6; 8.06 ± 1.92% for CCL2 and 29.27 ± 9.79% for IL1\(\beta\) \((n = 7, p < 0.05)\). The expression of the housekeeping gene between the two conditions remained similar. The lower expression of IL6 in differentiated cells is consistent with previous studies (Hugo et al., 2006).

To evaluate whether the activation of the NF\(\kappa\)B pathway was involved in the observed effect, we pre-incubated LS14 cells with SN50, which inhibits the translocation of the NF\(\kappa\)B active complex into the nucleus. As depicted in Fig. 2, our data 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 IL1\(\beta\)), although a trend \( p = 0.06 \) was observed in adipocytes, acts in part by activating the NF\(\kappa\)B pathway. Control experiments showed that there was no effect of SN50 alone on the expression of any of the cytokines (data not shown). Further evidence supporting the involvement of the NF\(\kappa\)B pathway in the effect of cinacalcet, was obtained by evaluating the decrease in I\(\kappa\)B\(\alpha\) after exposing cells to the calcimimetic. Immunoblot experiments showed that 15 min of exposure to cinacalcet decreased I\(\kappa\)B\(\alpha\) content by 48 ± 15% \((\text{mean} \pm \text{SEM}, p < 0.05 \text{ for the difference from the control})\).

**Fig. 2.** Inhibition of NF\(\kappa\)B pathway partially inhibits CaSR-induced elevation of IL6 and CCL2 expression in adipose cells. Preadipose (left) and adipose differentiated (right) LS14 cells exposed to the NF\(\kappa\)B nuclear translocation inhibitor SN50 (50 \(\mu\)g/mL) before overnight treatment with cinacalcet (Cina, 5 \(\mu\)M), were compared with those exposed to Cina alone. Bars represent mean ± SEM of the fold change in cytokine expression compared to untreated control cells (normalized to 1) from six independent experiments evaluated in duplicate by quantitative real time PCR. *\( p < 0.05 \) versus Cina, Wilcoxon matched pairs test.

**Fig. 3.** CaSR stimulation decreases inhibitor kappa B alpha (I\(\kappa\)B\(\alpha\)) expression. Representative immunoblot showing the effect of 5 \(\mu\)M cinacalcet (Cina) on the content of I\(\kappa\)B\(\alpha\) compared with vehicle control (CTL). The inset depicts a positive control experiment showing the effect of exposure to TNF\(\alpha\) (10 ng/mL) for 15 min.
value of 100%, Wilcoxon Signed Rank test, \( n = 6 \). Fig. 3 shows a representative immunoblot with these results. The figure inset shows that the effect of cinacalcet followed the same direction as the positive control used (exposure to the inflammatory agent TNF\(\alpha\) (R&D Systems) 10 ng/mL for 15 min).

3.2. CaSR expression in preadipocytes and adipocytes

In order to evaluate whether the different response to CaSR stimulus may be due to differences in CaSR expression in adipocytes versus preadipose cells, we compared the CaSR protein content in untreated LS14 preadipocytes and cells exposed to the adipogenic stimulus for 7 days. The results depicted in Fig. 4 show that the expression in LS14 preadipocytes is eight times greater than that in differentiated adipocytes.

3.3. Cytokine secretion in adipose tissue explants

We evaluated the secretion of TNF\(\alpha\), IL6 and IL1\(\beta\) in medium conditioned by human adipose tissue explants that were cultured for 48 h with cinacalcet or control vehicle. Fig. 5 shows that the secretion of proinflammatory cytokines increased by 1.7-fold for TNF\(\alpha\), 2.3-fold for IL6 and 2.2-fold for IL1\(\beta\) upon exposure to the calcimimetic.

4. Discussion

We have shown that the activation of the CaSR in preadipocytes and differentiated adipocytes of the human adipocyte cell line LS14, leads to an elevation of the proinflammatory cytokines IL1\(\beta\), and IL6, and the chemokine CCL2. These observations were consistent with data obtained from experiments with adipose tissue explants. We have also provided evidence suggesting that the inflammatory response triggered by CaSR is mediated at least in part by NFkB activation (i.e. nuclear translocation).

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 (Guilherme et al., 2008; Tilg and Moschen, 2008). The elevation in the production of proinflammatory cytokines and chemokines such as TNF\(\alpha\), IL1\(\beta\), 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 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 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 (Cifuentes et al., 2005), further evidence is being collected to establish its physiological role in this tissue. We observed an antipolytic effect of its stimulation in isolated human adipocytes (Cifuentes and Rojas, 2008), which has been recently confirmed in the adipocyte cell line SW872 by He et al., who observed a decreased expression or activity in key players of the lipolytic process (He et al., 2011). In other cell types, the receptor has been described to produce (Abdullah et al., 2006; Wang et al., 2002) and be regulated by (Canaff and Hendy, 2005; Canaff et al., 2008; Nielsen et al., 1997; Toribio et al., 2003) inflammatory mediators. Given this association of CaSR with inflammation and the proinflammatory characteristics of the obese state, our interest is focused on 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 (Cifuentes et al., 2010). Results from the present study add complexity to this scenario.

CaSR stimulation was induced by cinacalcet and not the main physiological agonist Ca2+ because the latter is expected to have multiple other effects in cell function and make the interpretation of results more difficult than using the more specific calcimimetic agent. Noteworthy, cinacalcet requires the presence of Ca2+ to be effective, since it acts as an allosteric modulator via increasing the sensitivity to extracellular calcium (which is at a concentration of 1 mM in our culture medium), shifting the concentration-response curve of the receptor towards the left (Nemeth et al., 2004).

The increase in inflammatory factor secretion in response to CaSR stimulation was greater in adipocytes than preadipocytes, which may have interesting physiological implications. First, it is interesting that our immunoblot experiments showed that CaSR expression is 8-fold higher in preadipocytes than in differentiated adipocytes, so the greater response would not be explained by receptor abundance. Also, it is relevant that we observed a considerably lower basal cytokine expression in adipocytes, particularly for IL6 (less than 1% of that of preadipocytes) and CCL2 (8%) and also, for IL1β (30%). This is consistent with previous reports, showing greater inflammatory profile in preadipose cells than in differentiated adipocytes (Mack et al., 2009; Poulain-Godefroy and Froguel, 2007). It is interesting that while basal cytokine and chemokine expression of adipocytes is lower than in preadipocytes, our data suggests that the former can be induced to increase their expression, particularly for IL6.

Given the above-discussed 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. Since monocyte-derived macrophages have also shown to express functional CaSR (Olszak et al., 2000; Yamaguchi et al., 1998), it is possible that infiltrated macrophages explain the effect of the calcimimetic elevating TNFα in whole adipose tissue, whereas it was too low (below the detection limit of our method, data not shown) in adipose and preadipose cells. Although we did not measure CCL2 release in adipose tissue explants, it is expected that its secretion will be elevated as well. Fain and Madan (2005) observed that elevation of this chemokine was partially 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. The cellular stimulation elicited by inflammatory factors activates a cytoplasmic kinase that phosphorylates IkB, after which it can no longer bind NFκB and is degraded. The free NFκB can then translocate into the nucleus and activate the transcription of genes involved in inflammation. On the other hand, activation of the CaSR was been shown to elicit the translocation of NFκB to the nucleus (Mentaverri et al., 2006). Consistent with this, our observations after preincubating LS14 cells with the NFκB nuclear translocation inhibitor SN50 and the decrease in IkB upon cinacalcet treatment suggest that the CaSR-dependent elevation of IL6 and CCL2 in preadipose and adipose cells is in part mediated by NFκB.

In summary, we have shown in LS14 cells and human adipose tissue explants that the calcimimetic cinacalcet elicits a proinflammatory response. If verified in vivo, 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. For a highly complex endocrine organ such as adipose tissue, the presence and function of a versatile protein such as the CaSR may be of great physiological relevance. The CaSR has an increasingly recognized role as an integrator of local signals to regulate diverse cell and tissue functions (Magno et al., 2011; Riccardi et al., 2009), and this study provides the rationale for future research in this important aspect of adipose pathophysiology.

5. Conclusions

Our findings suggest that CaSR activation is associated with an enhancement of the adipose proinflammatory environment. These observations, together with our previous findings of increased CaSR expression upon exposure to obesity-associated proinflammatory cytokines, suggest that CaSR may participate in a positive feedback loop enhancing inflammation, which can lead to adverse consequences for adipose and whole body metabolism. 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.

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References


