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Obesity-associated proinflammatory cytokines increase calcium sensing receptor (CaSR) protein expression in primary human adipocytes and LS14 human adipose cell line ☆

Mariana Cifuentes [a,*], Cecilia Fuentes [a], Pamela Mattar [a], Nicolas Tobar [a], Eric Hugo [b], Nira Ben-Jonathan [b], Cecilia Rojas [a], Jorge Martínez [a]

[a] Institute of Nutrition and Food Technology (INTA), Universidad de Chile, Chile
[b] Department of Cancer and Cell Biology, College of Medicine, University of Cincinnati, USA

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ABSTRACT

Obesity-associated health complications are thought to be in part due to the low-grade proinflammatory state that characterizes this disease. The calcium sensing receptor (CaSR), which is expressed in human adipose cells, plays an important role in diseases involving inflammation. To assess the relevance of this protein in adipose pathophysiology, we evaluated its expression in adipocytes under obesity-related pro-inflammatory conditions. As in primary adipose cells, we established that LS14, a recently described human adipose cell line, expresses the CaSR. Differentiated LS14 and primary adipose cells were exposed overnight to cytokines typically involved in obesity-related inflammation (interleukin (IL)1β, IL6 and tumor necrosis factor (TNF)α). The cytokines increased CaSR abundance in differentiated adipocytes. We incubated LS14 cells with medium previously conditioned (CM) by adipose tissue from subjects with a wide range of body mass index (BMI). Cells exposed to CM from subjects of higher BMI underwent a greater increase in CaSR protein, likely resulting from the greater proinflammatory cytokines secreted from obese tissue. Our observations that proinflammatory factors increase CaSR levels in adipocytes, and the reported ability of CaSR to elevate cytokine levels, open new aspects in the study of obesity inflammatory state pathophysiology, providing a potential novel therapeutic prevention and treatment target.

Introduction

The study of the low-grade chronic inflammatory state associated with obesity is of great interest, since it may represent a link between the obese state and its health complications such as cardiovascular disease and insulin resistance [1,2]. Research over the past few years, has shown that excess adipose tissue may become a source of inflammatory factors that alter normal lipid metabolism and tissue function [3,4]. In obese subjects, there is monocyte-derived macrophage infiltration in adipose tissue, which occurs in direct relation to adipocyte size [5]. These macrophages, in addition to adipocytes and stromal preadipocytes, are an important source of proinflammatory factors such as tumor necrosis factor alpha (TNFα),[1] interleukin (IL)1β and IL6 [6]. The elevated expression of these cytokines in obese adipose tissue contributes to defects in insulin signalling, systemic insulin resistance and cardiovascular complications [7,8].

NFκB is a cytoplasmic transcription factor whose activation is implicated in a number of conditions and diseases related to overnutrition including insulin resistance and diabetes [9–11]. A small molecule, the inhibitor kappa B alpha (IκBα) binds to NFκB, retaining it in the cytoplasm in the quiescent state. Inflammatory cytokines trigger the stimulation of IκB kinase (IKKB) that phosphorylates IκBα, which can no longer bind and inactivate NFκB. The free NFκB translocates into the nucleus and activates the transcription of genes involved in inflammation, including IL1β, TNFα and IL6 [10]. It has been suggested that activation of NFκB by factors related to overnutrition and excess adipose tissue is the final common inflammatory pathway linking obesity and metabolic syndrome and other obesity-related comorbidities [9,11].

The calcium sensing receptor (CaSR) is a seven transmembrane domain, G-protein coupled receptor, originally described in 1993 as the main regulator of PTH secretion and circulating calcium concentrations. Subsequently, different groups have described many roles that differ from calcium homeostasis, namely gastrin and gastric acid secretion [12], keratinocyte differentiation [13], tumor

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* Corresponding author. Address: INTA–Universidad de Chile, El Líbano 5524, Macul, Casilla 138-11, Santiago-Chile, Chile. Fax: +56 2 221 4030.
E-mail address: mcifuentes@inta.cl (M. Cifuentes).
[1] *Abbreviations used:* TNFα, tumor necrosis factor alpha; IL, interleukin; IκBα, inhibitor kappa B alpha; IKKB, IκB kinase; CaSR, calcium sensing receptor; HBSS, Hanks balanced salt solution; PVDF, polyvinylidene difluoride; TBs, Tris-buffered saline; BMI, body mass index.

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growth promotion or prevention (depending on the type of cancer) [14], and insulin secretion from pancreatic islet beta cells [15], among others. In addition, CaSR activity is modulated by a large list of substances and biochemical conditions such as organic multivalent cations [16], pH [17], ionic strength [18] and certain L-aminoacids [19], further suggesting several other roles for the receptor. Our laboratory reported the presence of the CaSR in human adipose cells [20] and an antilipolytic effect of its stimulation in isolated mature human adipocytes [21]. The receptor has been also linked to inflammatory states, both with an elevation in cytokine production upon its stimulation [22,23] as well as an upregulation of its expression when exposed to an inflammatory environment [24–26].

Given the association of CaSR with inflammatory states on one hand, and the proinflammatory character of the obese state on the other, we sought to study the role of elevated cytokines that are described in the obese adipose tissue milieu, affecting the expression of the receptor in human adipocytes. We speculated that, as seen in other cell types, exposure to elevated cytokine levels would upregulate CaSR expression in human adipocytes. Given the ability of the receptor to elevate cytokine expression, this upregulation may result in an enhanced proinflammatory environment in obese adipose tissue that will sustain the metabolic and cardiovascular complications of the disease.

Materials and methods

LS14 cell line culture and adipogenic differentiation

Our studies used the recently-generated preadipose cell line LS14, derived from a human metastasic liposarcoma, able to differentiate into lipid-laden adipocytes that express mature adipocyte genes [27,28].

Preadipose LS14 cells were seeded on plastic culture dishes (Nunc, Rochester, NY) and grown in DMEM/Ham's F12 (1:1) medium (Sigma) supplemented with 10% fetal bovine serum 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, 1.7 μM insulin (Eli Lilly & Co., SA de CV, Mexico) and 0.25 μM dexamethasone (Sigma, St. Louis, MO). The medium was replaced every 2–3 days and the experiments were performed after 7–9 days of exposure to the adipogenic conditions.

Isolation of human preadipocytes

Human omental fat was obtained from 18 subjects (28% male) undergoing elective abdominal surgery, with a wide range of body mass index (24.0–53.2 kg/m^2). To the best of our knowledge, there is no evidence suggesting that gender may influence CaSR expression. 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. Adipocytes were isolated using a method based on Rodbell [29], by incubation of the tissue with 1 g/l collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37 °C for 60 min with continuous mixing. The resulting cell suspension was filtered through sterile gauze, and floating adipocytes were removed. The remaining digest was centrifuged at 800*g* for 10 min, the supernatant was discarded, and the cell pellet was resuspended and seeded in DMEM:F12 (1:1) supplemented with 10% FBS and antibiotics (penicillin–streptomycin).

Primary cell culture and adipogenesis

Cells were grown with the above-mentioned medium at 37 °C in a controlled atmosphere incubator. At passages 3–4, cells were seeded at 35,000 cells/cm^2 and exposed to the differentiation cocktail after FBS removal overnight. The adipogenic medium consisted of DMEM:F12 (1:1) supplemented with 1 μM human insulin (Eli Lilly), 0.25 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). The medium was replaced every 2–3 days and the experiments were performed after 7–10 days of exposure to the adipogenic conditions. Adipose differentiation could be easily assessed by observing the presence of cytoplasmic lipid droplets under the microscope, which coincides with the expression of mature adipocyte proteins such as LPL, adiponectin and aP2, that are absent before differentiation (data not shown).

Cytokine treatment

Differentiated adipocytes (7–10 days) were exposed to either vehicle (culture medium) or 2 ng/ml IL1β (Sigma), 10 ng/ml IL6 (Sigma) or 20 ng/ml TNFα (Sigma), based on previous studies of both CaSR expression in parathyroid cells [24–26] and cytokine treatment in *in vitro*-differentiated 3T3-L1 cells [30] and primary human adipose cells [31]. After an overnight treatment, cells were lysed for protein analysis.

Conditioned media experiments

Minced adipose tissue was obtained as described above, and cultured in DMEM:F12 (1:1) at 37 °C in a controlled atmosphere incubator. After 48 h, the conditioned medium was removed, centrifuged at 1500*g* for 5 min and kept at −80 °C until performing the experiments.

Differentiated LS14 cells were exposed overnight to a mix containing 75% of the medium that had been conditioned by whole adipose tissue, and 25% of fresh DMEM:F12 (1:1) 4×. The latter was added so that the cells had access to a final 1× concentration of fresh nutrients.

Western blot analysis

Differentiated adipocytes were homogenized at 4 °C in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 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% 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 a mouse anti-human CaSR monoclonal antibody (Abcam Inc, Cambridge, 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. The intensity of the bands was quantified in digitalized film images using Image J Software (National Institutes of Health, USA).

Statistical analysis

Results are presented as means ± SEM. Differences between means were analyzed using 1-way ANOVA and Tukey's post hoc test. Statistical significance was considered at $p < 0.05$.

Results

Expression of CaSR in the LS14 cell line

We have previously reported the CaSR expression in primary human adipocytes and preadipocytes [20]. Consistent with the close similarity of the novel human-derived adipose cell line LS14 to primary adipose cells already reported, the CaSR protein is present in both preadipocytes and adipose-differentiated cells.

Exposure to proinflammatory cytokines elevates CaSR abundance in adipocytes

LS14 preadipocytes were exposed to the adipogenic stimulus for 7–9 days. Differentiated cells were treated with either vehicle (CTL), or the cytokines IL1β, TNFα or IL6 overnight, and the abundance of the CaSR protein was evaluated by Western Blot, as indicated in Materials and methods. As shown in Fig. 1, cytokine exposure is associated with an elevation of CaSR protein. To assess whether this phenomenon also occurs in primary human *in vitro-differentiated* adipocytes, we analyzed CaSR expression after cytokine stimulus in cells purified from human omental fat (Fig. 2). The differentiated adipocytes showed a similar pattern of cytokine stimuli compared to LS14 cell line.

Adipose tissue-conditioned media from obese subjects elevates CaSR expression

Once established that CaSR expression in adipose cells responds to proinflammatory stimulus, we further evaluated whether adipose tissue secreted factors, most likely reflecting a greater proinflammatory state in obese patients, had an effect on CaSR expression. To this end, we assessed the capacity of media conditioned by adipose tissue from subjects with a wide range of BMIs to stimulate CaSR expression in differentiated LS14 cells. Fig. 3 shows that in subjects with a greater BMI, media conditioned by their adipose tissue exerts a larger increase in CaSR expression in LS14 cells.

To address whether our most consistently effective cytokine, TNFα, was involved in the observed effect, we performed a subset of experiments before and after immunoprecipitating TNFα out from the conditioned media. Briefly, 10 micrograms of anti-human TNFα (R&D system, Minneapolis, MN) were incubated overnight with 200 μl of previously hydrated Protein A Sepharose CL-4B (Sigma, St. Louis). After this, one ml of media conditioned by adipose tissue from a subject of BMI 53 kg/m^2 was incubated twice with

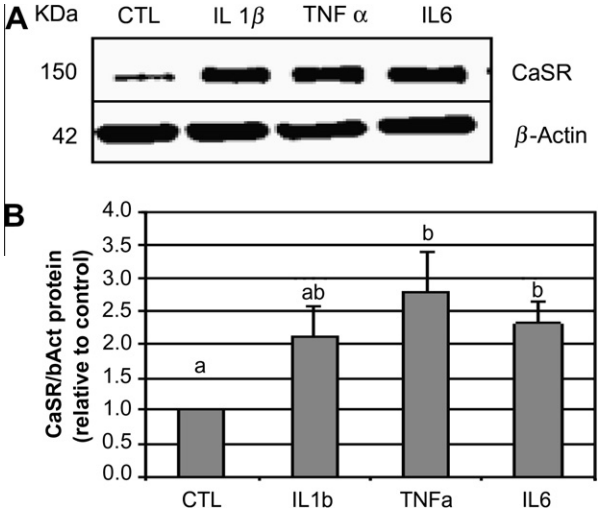

Fig. 2. Proinflammatory cytokines elevate CaSR abundance in human adipocytes. (A) Representative blot of *in vitro* differentiated human adipocytes exposed to vehicle (CTL), IL1β, TNFα or IL6 overnight. (B) Quantification of CaSR/βActin protein from experiments in cells from seven different subjects for each treatment. Bars with different letters denote significant differences $p < 0.05$, ANOVA and Tukey's post hoc test.

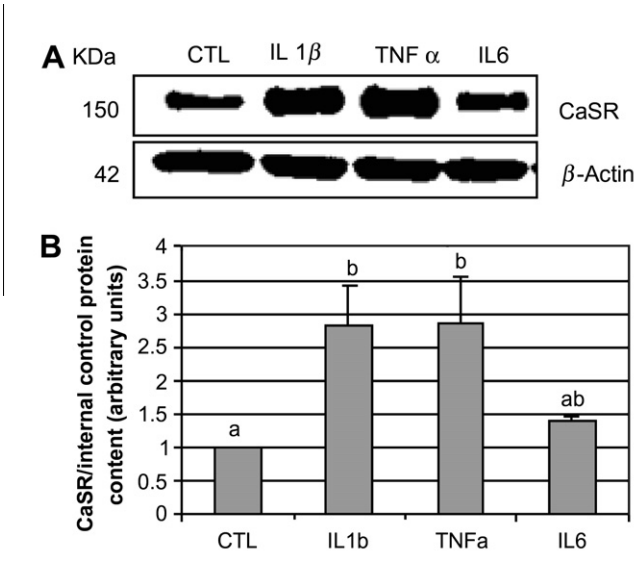

Fig. 1. Cytokine exposure induces the elevation of CaSR abundance in the LS 14 cell line. (A) Representative blot of *in vitro* differentiated LS14 cells exposed overnight to vehicle (CTL), IL1β, TNFα or IL6 as described in Materials and methods. (B) Quantification of CaSR/βActin protein from four to seven independent experiments. Bars with different letters denote significant differences $p < 0.05$, ANOVA and Tukey's post hoc test.

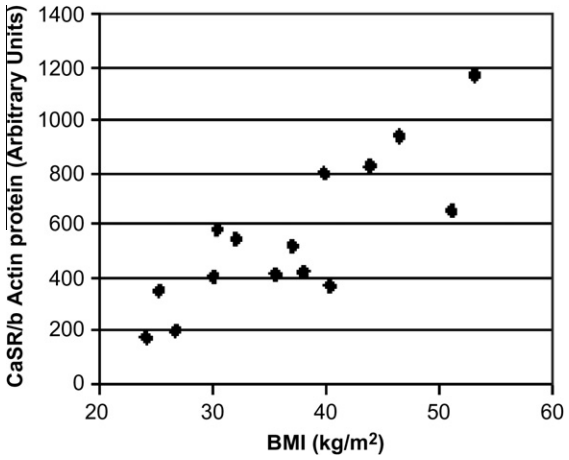

Fig. 3. Factors secreted by adipose tissue from subjects with greater BMI elicit a larger effect on CaSR protein abundance. Differentiated LS14 cells were exposed overnight to medium that was conditioned by adipose tissue from subjects with different BMI. Each dot represents the expression (normalized with βActin as an internal control) of the cells treated with conditioned medium. $R^2 = 0.67$ $P = 0.0002$, $n = 15$.

the immobilized antibody. The supernatant resulted of this procedure was used to stimulate CaSR expression in LS14 cells as already described. As shown in Fig. 4 (inset A), immunoprecipitation of TNFα significantly reduced the presence of the cytokine in the conditioned medium. Exposure of LS14 cells to this medium decreased by 30% the expression of CaSR protein as compared with the untreated conditioned medium. These observations together support that a greater expression of CaSR in adipocytes may be induced by cytokines present in adipose tissue from obese subjects.

NFκB activity modulates CaSR expression in LS14 cells

To evaluate whether the NFκB pathway is involved in the cytokine-elicited elevation of the CaSR expression in LS14 cells, we used the cell-permeable inhibitor SN50 (Calbiochem). This peptide contains the nuclear localization sequence of the transcription factor NFκB p50 and inhibits the translocation of the active NFκB complex into the nucleus. Fig. 5 shows that preincubation with SN50 inhibits the stimulatory effect of IL1β, TNFα and IL6 on the expression of CaSR in LS14 cells. Cells exposed to IL1β, TNFα and IL6 after pre-treatment with 50 μg/ml SN50 showed 61 ± 10%, 56 ± 19% and 66 ± 9% of the expression of cells without SN50 exposure, respectively.

Discussion

Obesity, particularly visceral or central obesity, has been described as a low-level, chronic inflammatory state in adipose tissue. Research over the past few years has strongly suggested that the development of many obesity-associated complications, such as type 2 diabetes and cardiovascular disease is directly associated with this inflammatory state [32,33]. Adipocytes from obese patients, particularly those that have undergone hypertrophy, show an altered secretion profile of biologically active molecules [34]. This, together with a significant macrophage infiltration in obese adipose tissue, contribute to the modified secretion pattern [5,35], increasing the presence of proinflammatory cytokines such as TNFα, IL1β and IL6 [3,36]. Our work showing cytokine-induced increased levels of the inflammatory mediator CaSR in adipocytes represents a novel finding. Since an enhanced expression of the CaSR is expected to elevate the secretion of proinflammatory factors [22,23], our findings possibly illustrate a positive feedback mechanism that perpetuates the inflammatory state in adipose tissue.

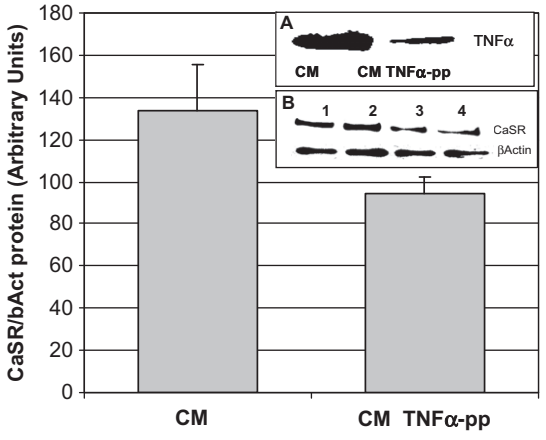

Fig. 4. Western blot showing the decrease in the effect of conditioned medium from a subject of BMI 53 kg/m^2 after immunoprecipitation (pp) of TNFα. Anti-human TNFα antibody was used to immunoprecipitate TNFα from the conditioned medium before adding to LS14 cells, as in experiments as shown in Fig. 3. The reduction in TNFα content in the medium is shown in inset A. Inset B shows the blot of experiments performed in duplicate (lanes 1–2: untreated conditioned medium (CM); lanes 3–4 immunoprecipitated medium). As compared with CM, the pp reduced by 30% the expression of the CaSR.

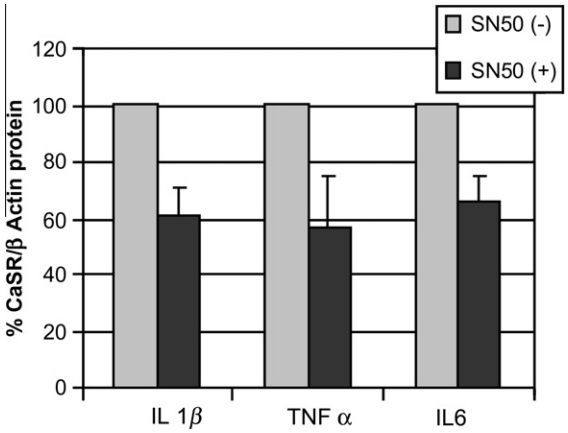

Fig. 5. NFkB inhibition with SN50 (black bars, SN50 (+)) decreases the cytokine-elicited elevation in CaSR protein abundance in LS14 cells. For comparison purposes, the vehicle control (SN50 (−)) for each cytokine (gray bars) is normalized to 100%.

The CaSR was first described in 1993 as a G-protein coupled receptor playing an essential role in calcium homeostasis in bovine parathyroid cells [37]. Since then, an increasing number of studies showing that other cell types from the gastrointestinal tract, nervous system, pancreas and skin (among others) functionally express the receptor, have revealed its involvement in numerous physiological conditions. One of these novel functions is its participation in inflammatory events, which was initially discovered due to the hypocalcemia present in critically ill patients, particularly those with sepsis and burns [38,39]. These patients show high levels of circulating proinflammatory cytokines such as IL1β, IL6 and TNFα. In an animal model, severe burn-induced hypocalcemia was associated with a 50% increase in CaSR expression in the parathyroid gland [40]. Further evidence has supported the elevation of CaSR in the presence of IL1β, IL6 and TNFα in different cell models and tissues [24,26].

The intracellular mechanisms reported, involve the NFκB route, which agrees with the presence of NFκB response elements in the human CaSR promoter [26,41]. Our observations are consistent with these previous reports, suggesting that the NFκB pathway is involved in cytokine-elicited elevation of CaSR expression in human adipocytes. Our observation suggesting that NFkB signalling mediates IL6 effect is intriguing. This pathway has been described in TNFα and IL1β signalling; however IL6 is known to exert its effects through JNK/STAT3. Nevertheless, and consistent with our results, an interaction between STAT3 and NFκB has been reported. A recent study in MCF10A cells observed a decrease in the effects elicited by IL6 upon NFκB inhibition [42]. In addition, a molecular cross-talk between the STAT3 and NFκB pathways has been described in several recent reports [43,44]. The activation of NFκB by IL6 in adipose cells may represent a novel finding that requires further investigation.

Biological activity of TNFα is a relevant example of dependence of NFκB, whose activity elicits the upregulation of the expression of many genes related to inflammation, such as IL6 and IL1β [45–47]. Our data, showing that the effect of TNFα on CaSR expression was consistently higher that those observed with IL1β and IL6, may reflect the TNFα-dependent upregulation of IL1β and IL6 expression and the consequent additive effects of the three cytokines on CaSR expression.

We studied the cytokines IL1β, TNFα and IL6 due to the relevance in human adipose tissue inflammatory state and its negative metabolic and vascular consequences [3,48,49], and also the evidence that supports a role of these three cytokines elevating CaSR expression or promoter activity in other models [24,26]. *In vitro* studies in human adipose tissue have suggested that endogenous release of IL1β and TNFα upregulates IL6 production [50]. Elevated IL6 secretion by adipose tissue in obesity is reflected in greater circulating levels of the cytokine; however this is not the case for TNFα. Nonetheless, *in vitro* experiments show that adipose tissue from subjects with greater fat mass elevate TNFα production considerably more than IL6 and IL1β [51]. This fact, underscores the relevance of some specific factors as paracrine signals within adipose tissue. It is difficult to speculate on the local physiological concentrations of cytokines within obese adipose tissue, which will depend on secretion and clearance capacity of the individual. The cytokine concentrations that we used in these studies were based on previous literature and are higher than reported circulating levels. Among the few studies that have evaluated interstitial concentrations in adipose tissue, the three cytokines have shown to be greatly elevated locally (10–1000-fold) as compared with circulating levels [52,53].

Interindividual variability and insufficient material are some of the downsides when working with primary cell cultures. Part of our experiments were performed in the novel human adipose cell line LS14 [28], and the findings were validated in primary human *in vitro*-differentiated adipocytes. To our knowledge, this is the first report showing that, like primary human adipose cells, LS14 cells express the CaSR before and after adipose differentiation. LS14 cells behaved like primary adipocytes in all our experiments, confirming previous reports showing that they constitute a reliable model for human adipose cells [27].

To test if adipose tissue from obese patients produces and releases soluble factors that increase the abundance of CaSR, we incubated differentiated LS14 cells with medium that had been previously conditioned by omental adipose tissue from subjects with a wide range of BMI (24–53 kg/m^2). Consistent with the response that we observed to the individual synthetic cytokines, CaSR expression increased upon exposure to the conditioned media in a direct correlation with the tissue donor's BMI, suggesting that there may be an enhanced activity of the receptor in the adipose tissue of an obese subject. It has been shown that adipose tissue from individuals with greater BMI secretes larger amounts of proinflammatory cytokines [3], however we cannot rule out the possibility that other kind of active component from the conditioned media may exert the observed effect. We approached this issue by performing a subset of experiments before and after immunoprecipitating TNFα out of the conditioned medium, and the observations suggest that this cytokine is indeed involved in the observed effect. The LS14 cell line was used here as a neutral assay tool to avoid the interindividual variability in cells derived from primary cultures from patients with different BMI. Performing this type of experiment with adipose cells from a single individual would have been a real challenge (mainly to obtain enough cells) and the results more difficult to interpret.

Because multiple and diverse agents are able to modulate CaSR activity, the receptor has been considered an integrator environmental signals to regulate diverse cell and tissue functions [54]. Given adipose tissue's wide and variable range of secretory products, this concept becomes very important in relation to obesity pathophysiology. The observed effect of cytokines on CaSR expression in adipocytes may be of great relevance for understanding and targeting treatments for this disease. In light of the ability of the CaSR to induce the expression of inflammatory mediators [22,23], a positive feedback may ensue, thus exacerbating and perpetuating the inflammatory condition in the obese adipose tissue. Upon cytokine-elicited NFκB activation and the induction in CaSR expression, an increased overall CaSR activity can be expected, with more binding sites available for activation by the multiple factors present in the adipose tissue microenvironment [55]. In light of our previous work showing that, in human isolated mature adipocytes, CaSR activation elicits an inhibition of basal lipolysis [21], together with the observation that it enhances lipogenesis in differentiated cells (unpublished data), it is possible to speculate that the receptor is an important part of a network of events that maintain the altered state of the tissue. A decreased triglyceride turnover and an elevated proinflammatory environment in conditions of continuous excess energy input, as occurs in obesity, is an ideal scenario for aggravating the pathological condition.

By proposing the participation of the CaSR as a possible (albeit not unique) link between obesity and inflammation, we may be looking at a pharmacological target for breaking, at least in part, this vicious circle. This would imply providing a means to dissociate obesity from some of its complications. Further studies are needed to explore the potential of manipulating this pathway to help in attenuating the metabolic and cardiovascular consequences of the chronic, low-grade inflammatory condition in obesity.

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