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

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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 calcimimatic 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.

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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).

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-κBα; NFκB, nuclear factor kappa B; PTH, parathyroid hormone; RNA, ribonucleic acid; TNFα, tumor necrosis factor alpha.

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Table 1
Forward and reverse primer sequences for real time PCR.

Target mRNA	Accession	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
CCL2	NM_002982	ACTGAAGCTCGCACTCTCGCCT	CTGAGCGAGCCCTTGGGGAATG
IL1β	NM_000576	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG
IL6	NM_000600	CAATCTGGATTCAATGAGGAGAC	CTCTGGCTTGTTCCTCACTACTC
GAPDH[a]	NM_002046	TCAACGACCACTTTGTCAAGCTCA	GCTGGTGGTCCAGGGGTCTTACT

[a] Housekeeping control.

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 mediator 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 metastasic 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/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.

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 (IκBα) 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 $2^{-(\Delta\Delta Ct)}$. 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 IκBα) polyacrylamide gels and electrotransfered 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 IkB (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

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