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Calcium sensing receptor effects in adipocytes and liver cells: Implications for an adipose-hepatic crosstalk

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ABSTRACT

The calcium sensing receptor (CaSR) is expressed in human adipose cells, and its activation may associate with adipose tissue (AT) dysfunction. We evaluated whether CaSR stimulation influences adipocyte triglyceride (TG) and fatty acid binding protein 4 (aP2) content, and hepatocyte TGs and proinflammatory cytokine expression. The effect of the calcimimetic cinacalcet on TGs (fluorimetry), lipogenic genes (qPCR) and aP2 (immunoblot) was evaluated in LS14 adipocytes or AT. In the human HepG2 hepatic cell line, we assessed CaSR expression and cinacalcet effect on TGs and lipogenic and proinflammatory genes. CaSR activation decreased adipocyte TG content by 20% and the expression of GPD and LPL by 34% and 20%, respectively. Cinacalcet increased aP2 protein expression by 60%. CaSR expression was shown in HepG2 cells and human liver samples. Cinacalcet-treated HepG2 cells in the presence of oleic acid exhibited a19% increased TG content. No changes were observed in the expression of lipogenic genes in HepG2 cells, however there was a 50%–300% elevation in the expression of proinflammatory cytokines. CaSR activation in adipocytes may associate with decreased TG storage ability and increased aP2. Hepatic CaSR stimulation may elevate steatosis and proinflammatory factors. We propose that CaSR may contribute to obesity-associated hepatic metabolic consequences.

1. Introduction

The main role of adipose tissue (AT) is based on its ability to safely store excess energy as triglycerides (TG). The synthesis of TGs needs the coordinated regulation of glucose availability, appropriate insulin sensitivity and fatty acids, as well as the action of transcription factors and enzymes that coordinate the final assembly of fatty acyl CoAs onto the glycerol backbone. From a whole-body perspective, the inability of adipose cells to properly handle incoming fatty acids is expected to make excess lipids available to all other tissues in the circulation, resulting in ectopic fat deposition and toxicity in metabolically relevant organs, such as the liver [1].

The CaSR is a seven transmembrane domain protein expressed in numerous tissues and organs, including human adipose cells [2]. We have previously shown that CaSR activation is associated with elevated expression of inflammatory factors in adipocytes and preadipocytes [3,4]. We also showed that CaSR stimulation elevates visceral preadipocyte adipogenic differentiation [5], however the ability of differentiated adipose (or other) cells to handle TGs or fatty acids upon CaSR activation has not been studied.

From the observations discussed above, our laboratory has proposed a role for CaSR activation on AT dysfunction. With excess proinflammatory signaling in AT, impaired TG storage is expected [6], thus increasing the availability of circulating free fatty acids to organs that are ill-suited for their disposal [7]. As a relevant potential pathophysiological process, hepatic steatosis is a key factor associated with obesity-related metabolic impairment [8].

The fatty acid binding protein 4 (FABP4/aP2) is an intracellular protein responsible for binding and transporting free fatty acids inside the cell. Recently, its presence was described in human plasma, and the evidence suggests that circulating aP2 levels may be linked with obesity-associated comorbidities [9,10] as well as hepatic inflammation and fibrosis [11]. In addition, AT dysfunction and inflammation in obesity increase levels of aP2 in the tissue and plasma [9,12].

In the present study, we aimed to evaluate *in vitro* the effect of

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CaSR activation on TG accumulation in cell models of human adipocytes and hepatocytes, namely LS14 and HepG2. We also assessed whether activation of the CaSR increases adipose aP2 content and promotes inflammation in hepatic cells, suggesting novel mechanisms by which this receptor may have local and peripheral dysfunctional effects.

2. Methods

2.1. LS14 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 [13]. 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) and antibiotics (penicillin-streptomycin) at 37 °C in a controlled atmosphere incubator (5% CO_2). 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., Indianapolis, IN) and 0.25 μM dexamethasone (Sigma). The medium was replaced every 2–3 days.

2.2. HepG2 cell line culture

The HepG2 cell line (American Type Culture Collection, Manassas, VA) derived from a human liver hepatocellular carcinoma was used for these experiments. They are considered an adequate model for human hepatocytes since they show morphological (epithelial-like) and functional differentiation in culture, secreting a variety of plasmatic proteins [14]. The cells were maintained in Minimum Essential Medium (Sigma) supplemented with 10% FBS and antibiotics (penicillin-streptomycin) at 37 °C in a controlled atmosphere incubator (5% CO_2).

2.3. Cell viability

Cell viability was assessed in subconfluent HepG2 cells by the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, WI, USA), according to the manufacturer's instructions in an ELx808 microplate reader (BioTek Instruments, Inc. VT, USA). In brief, HepG2 cells were seeded at 6000 cells/cm^2 in 96-well plates and exposed to the experimental conditions for 72 h with daily medium change. At the end of the incubation period, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) compound solution was added to the media (100 μl per well), and absorbance was recorded after 3 h at 490 nm (and 630 nm to subtract background absorbance). Cell viability is expressed in arbitrary units as the absorbance at 490 nm minus 630 nm.

2.4. Human hepatic tissue

Complementary DNA was obtained from human liver samples taken at the time of bariatric surgery or cholecystectomy as described in a previous study [15]. Patients were separated in two groups depending of the presence or absence of obesity as defined by a body mass index greater than 30 kg/m^2. These samples were evaluated for the presence of CaSR transcripts by qPCR (see below).

2.5. Triglyceride content

To assess TG content, cells were treated with 0.25% trypsin solution at 37 °C for 2 min or until de cells were detached from the culture dish. The trypsin was then inactivated with culture medium containing FBS to a final concentration of 10%. Cells were sedimented by centrifugation at 800*g* for 20 min and then incubated at room temperature for 5–10 min in 1 μg/mL Nile Red (Sigma) solution in PBS. Fluorescence was evaluated using 485 nm excitation and 572 nm emission filters (Synergy 2 fluorimeter, BioTek Instruments). Results are normalized by the protein content measured using the bicinchoninic acid method (Pierce, Rockford IL). In order to rule out that normalization was confounding the results, we also evaluated normalization for number of cells, and the results were unchanged (data not shown).

2.6. Isolation of total RNA, reverse transcription and qPCR analysis

Cultured cells were lysed with Trizol® reagent (Invitrogen, Carlsbad, CA) and RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen) according to the manufacturer's indications. Reverse transcription was performed (25 °C for 10 min followed by 2 h at 37 °C and 5 min at 85 °C) using the high Capacity cDNA Reverse Transcription kit (MultiScribe™ MuLV reverse transcriptase, 50 U/μL, Applied Biosystems, Carlsbad, CA). mRNA expression was evaluated using the Step-one Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR® FAST qPCR Kit (Applied Biosystems). The thermal cycling conditions consisted of a 20 s preincubation at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The results are normalized by the reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and the expression was calculated using the Pfaffl model [16] determining the quantification cycle (Cq) within the log phase of the amplification curve. Every reaction includes a set of no-template controls (one for each primer pair), that show very high Cqs (above 35) or undetected amplification. The reference gene GAPDH was chosen since it consistently shows a stable expression under our experimental conditions. In preliminary studies, the alternative reference gene beta-actin showed a similar pattern, however due to a larger variability, GAPDH was selected as the sole reference gene. As an internal quality control for each experiment, cDNA is discarded or RNA re-extracted in experiments where GAPDH expression is too low or differs by more than 1 Cq across conditions. To verify the presence of CaSR transcripts in hepatic cells, PCR products were resolved by electrophoresis in a 1.5% agarose gel in 0.04 M Tris acetate and 0.001 M EDTA buffer and stained with ethidium bromide. Sequences for PCR primers are depicted on Table 1. All primers are intron-spanning, to prevent or identify amplification of genomic DNA.

2.7. Human omental adipose tissue

Human omental fat was obtained from 8 subjects (75% female) undergoing elective abdominal surgery, with a body mass index of 34.6 ± 5.5 kg/m^2 (mean ± SD) and aged 37.9 ± 11.8 years. 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 tissue was washed with Hanks Balanced Salt Solution, cleaned and minced into small pieces (2–3 mm^2) and cultured in DMEM/Ham's F-12 (1:1) medium (Sigma) supplemented with antibiotics (penicillin–streptomycin) at 37 °C in a controlled atmosphere incubator.