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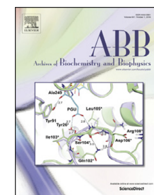

Calcium sensing receptor effects in adipocytes and liver cells: Implications for an adipose-hepatic crosstalk

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A B S T R A C T

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.

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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.

Table 1
Forward and reverse primer sequences for qPCR.

Target mRNA	Accession	Forward Primer (5′→ 3′)	Reverse primer (5′→ 3′)	Amplicon Length (bp)
CaSR	NM_001178065	GATGAGACAGATGCCAGTGC	AAAGAGGGTGAGTGCGATCC	135
PPARγ	NM_015869.4	AGCAAAGGCGAGGGCGATCTTG	GGATGGCCACCTCTTTGCTCTGC	149
LPL	NM_000237.2	TGGTGATCCATGGCTGGACGGT	AGCCCGCGGACACTGGGTAA	150
FAS	NM_004104.4	GGAAGCTGCCAGAGTCGGAGAACT	TGAGGGTCCATCGTGTGTGCCT	202
GPD	NM_005276.2	GGCTGAGGCCTTTGCGCGTA	ACTGGCTGGCCCTCGTAGCA	186
SREBP1c	NM_001005291.2	ACACAGCAACCAGAAACTCAAG	AGTGTGTCCTCCACCTCAGTCT	153
aP2	NM_001442.2	TGCAGCTTCCTTCTCACCTTGA	TCCTGGCCCAGTATGAAGGAAATC	256
IL6	NM_000600	CAATCTGGATTCAATGAGGAGAC	CTCTGGCTTGTTCCTCACTACTC	118
IL1β	NM_000576	GGACAAGCTGAGGAAGATGC	TCGTTATCCCATGTGTCGAA	120
TNFα	NM_000594.2	CCAGGCAGTCAGATCATCTTCTC	AGCTGGTTATCTCTCAGCTCCAC	150
GAPDH[a]	NM_002046	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT	71

[a] Reference control gene.

2.8. Western blot

After treatment, AT and HepG2 cells 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 1 mM sodium orthovanadate (Sigma), 1.5 μM pepstatin A (Sigma) and Complete® protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration of the lysate was determined by a method based on bicinchoninic acid (Pierce). Fifty μg 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 7.5%, 10% and 15% polyacrylamide gels (CaSR, P-Erk and aP2, respectively) and electrotransfered to polyvinylidene difluoride (PVDF) membranes using a buffer that contains 24 mM Tris, 194 mM glycine and 20% methanol. The immunoreaction was achieved by incubation of the membranes, previously blocked with a 3% skim milk solution in Tris-buffered saline (TBS) with 0.05% Tween 20 (Sigma), with an CaSR (6D4) antibody (Santa Cruz Biotechnology, Inc), Hsp90 antibody (Santa Cruz Biotechnology, Inc), p-ERK (E:4) and ERK 2 (K:23) antibodies (Santa Cruz Biotechnology, Inc), anti-human aP2 antibody (Cayman Chemical, Ann Arbor, MI) and anti-human β-Actin antibody (AC:15) (Santa Cruz, Inc). Detection of immune complexes was performed by incubation with peroxidase conjugated secondary antibodies, followed by incubation with the substrates of the enzyme. The images were digitized and density of the bands was quantified using Image J (National Institutes of Health, USA).

2.9. Statistical analysis

To compare parameters between controls and treated cells, variables were evaluated using Wilcoxon signed rank test and Friedman Test with Dunn's multiple comparisons when appropriate. Data are shown in the figures as means ± SEM, and a p-value less than 0.05 was considered significant.

3. Results

3.1. CaSR stimulation decreases adipocyte TG content

The effectiveness of adipose differentiation was verified by an increase in TG content as well as changes in cell morphology (Fig. 1A), in addition to the increase in expression of mature adipose cell markers as compared with non-differentiated control cells (not shown), as previously described [5]. The effect of 72 h exposure to cinacalcet was evaluated in LS14 adipocytes that were differentiated for 7 days (exposure: days 7–10). As shown in Fig. 1B, treatment with the calcimimetic decreased TG content by 20%.

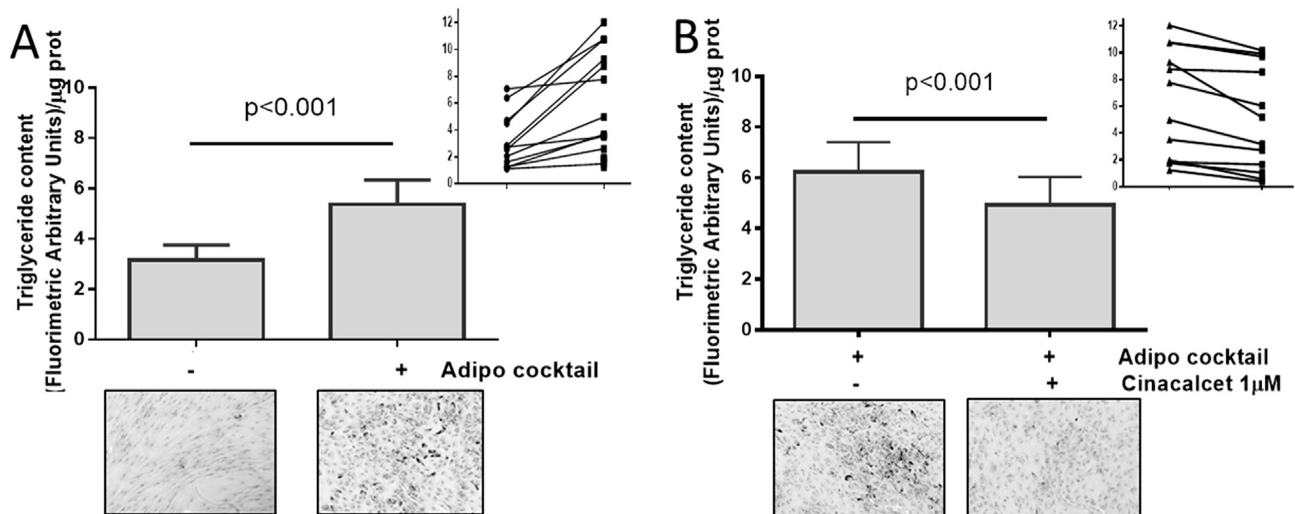

Fig. 1. Triacylglycerol content in LS14 adipocytes. **A**. Validation of LS14 differentiation evaluated by TG content and cell morphology. LS14 cells cells after 10 days of exposure to the adipogenic cocktail compared to vehicle-treated non-differentiated control cells. Bars represent mean ± SEM for n = 12 independent experiments. **B**. Effect of 1 μM cinacalcet for 72 h in seven-day differentiated adipocytes (days 7–10). Data are expressed as mean ± SEM, n = 12 experiments. TG content was evaluated by fluorimetry using Nile Red. P values: Wilcoxon matched-pairs signed rank test. The respective insets show the plot for each individual experiment. Below each bar are shown representative photographs of oil Red O staining after 10 days of differentiation, 10× magnification.

3.2. Cinacalcet downregulates adipocyte expression of lipogenic genes

The expression (mRNA) of lipid handling factors was evaluated in LS14 cells treated with cinacalcet 1 μM for 24 h. Treatment with the calcimimetic decreased the expression of the lipogenic enzymes glycerol 3 phosphate dehydrogenase (GPD) and lipoprotein lipase (LPL) by 34% and 20%, respectively, without affecting the *de novo* lipogenic enzyme fatty acid synthase (FASN) or the master regulator of adipocyte metabolism PPARγ (Fig. 2).

3.3. Cinacalcet increases aP2 content in human adipose tissue

Based on the proposed link between CaSR activation and obesity-associated AT dysfunction, together with recent evidence involving aP2 in obesity-associated pathogenesis [17], we evaluated the effect of CaSR activation on aP2 mRNA expression and protein content in human LS14 adipocytes and AT. As shown in Fig. 3A, aP2 mRNA expression was elevated upon CaSR stimulation with cinacalcet in LS14 adipocytes. To support this finding, we initially performed a dose-response experiment in a single human omental AT explant, and observed the expected dose-dependent increase (Fig. 3B) in aP2 protein content. Subsequently, exposure of AT explants from other seven donors to 2 μM cinacalcet showed that CaSR stimulation elevated aP2 by 60% (Fig. 3C).

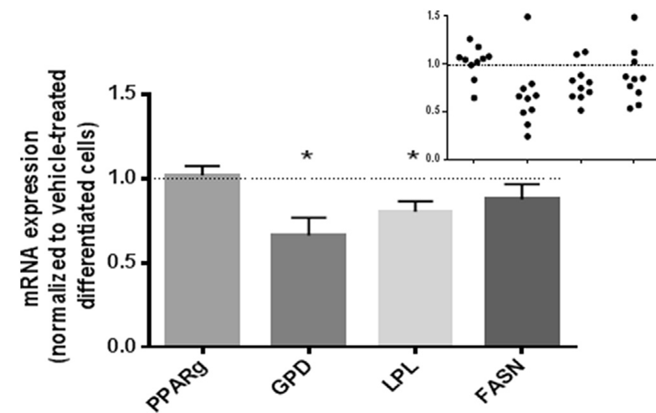

Fig. 2. mRNA expression of factors associated with lipid handling in cinacalcet-treated cells. Seven-day differentiated LS14 adipocytes were treated with cinacalcet 1 μM for 24 h. Bars represent mean ± SEM for each gene versus its own vehicle-treated control, normalized to 1 and represented by the dotted line. *P < 0.05 vs 1, Wilcoxon Signed Rank Test, n = 10 independent experiments. The inset shows the corresponding raw data.

3.4. Effect of CaSR stimulation in HepG2 hepatocytes

In the context of our model of CaSR-induced AT dysfunction, we next evaluated the possible impact on hepatocytes, which are also relevant in obesity-related disorders. Given the role of liver steatosis determining metabolic and cardiovascular disorders associated with obesity [18], we chose the oleic acid-loaded HepG2 cell model [19,20] to test the effects of CaSR stimulation in the context of an elevated free-fatty acid environment. We first verified that the receptor is expressed in HepG2 cells, and Fig. 4A shows the presence of CaSR transcripts that are the same size as those amplified in the positive control from parathyroid tissue. In addition, to ascertain the potential relevance of our findings, we evaluated the expression in samples of human hepatic tissue and found that the receptor is expressed in human liver (Fig. 4A, lower panel). As shown in the figure, these preliminar observations suggest that livers from obese patients may express higher levels of CaSR. To our knowledge, this is the first report documenting the presence of the CaSR in human hepatic cells. To support these findings, the expression of CaSR protein was verified in HepG2 cells by Western blot (Fig. 4B) confirming the presence of a band of the same size as that present in lysates from Caco-2 cells, used as positive control. Receptor activation was confirmed by the elevation of ERK phosphorylation upon exposure to the agonists cinacalcet and spermine (Fig. 4C), as previously shown in LS14 preadipocytes [4].

3.5. CaSR stimulation increases TG content in oleate-exposed HepG2 cells

In order to evaluate lipid handling in HepG2 cells under an elevated free fatty acid environment, we exposed the cells to 0.6 mM oleic acid. This condition was chosen given that oleic acid is more steatogenic but less apoptotic than palmitic acid in hepatocytes [19,21]. After establishing in viability essays that this concentration does not affect cell survival (Fig. 5A), we evaluated the effect on TG accumulation. We observed that the TG content increased by 93% when 0.6 mM oleic acid was added to the culture medium (Fig. 5B). Treatment of oleic acid-exposed HepG2 cells with the calcimimetic cinacalcet elevated TG content by 19% (Fig. 6).

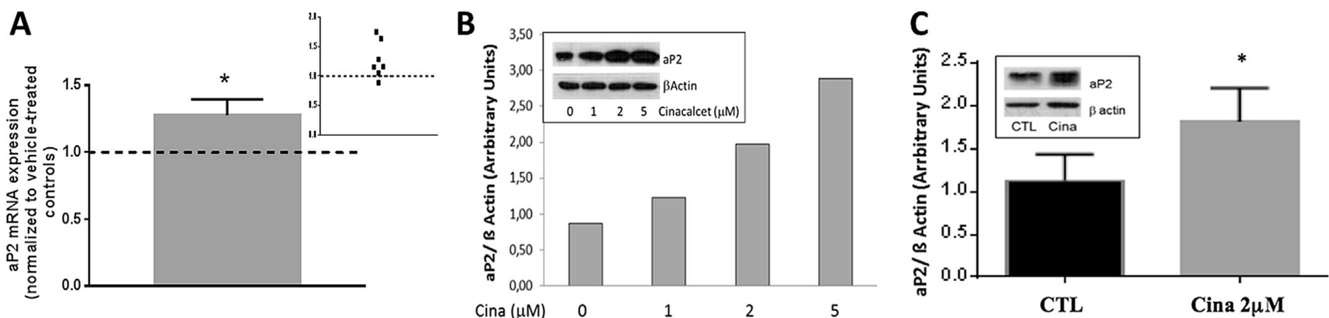

Fig. 3. CaSR activation elevates aP2 content in human adipose cells. **A)** aP2 mRNA expression in LS14 adipocytes exposed for 16 h to 2 μM cinacalcet. The expression of aP2 was evaluated by qPCR, using each sample's GAPDH expression as the reference gene, and then reported as fold of vehicle-treated cells (value of 1, represented by the dotted line). The bar represents mean ± SEM for n = 7 independent experiments and the inset depicts the individual values. P < 0.05 for the difference from 1, Wilcoxon Signed Rank Test. **B)** Increasing doses of cinacalcet (Cina) were evaluated for aP2 protein by Western blot in lysates from one human AT explant. **C)** Human AT explants were treated with vehicle (CTL) or 2 μM Cina for 24 h. The aP2 content was evaluated by Western blot in AT lysates. The bars in C are mean ± SEM (samples from n = 7 independent donors), where *p < 0.05. Wilcoxon matched-pairs signed rank test. The insets show representative blots for the respective experiments.

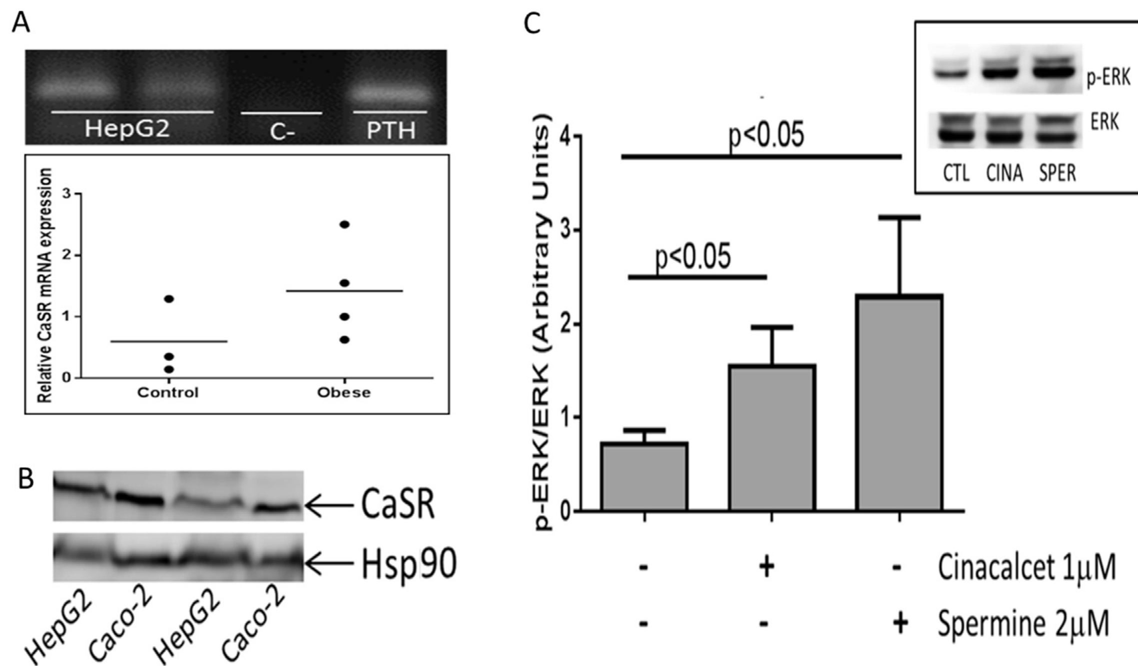

Fig. 4. CaSR expression in human hepatic cells. A. Photograph of the mRNA abundance (PCR products) of CaSR in HepG2 cells, negative control (no cDNA added, C-) and positive control (human parathyroid cDNA, PTH). Below are shown preliminary results of human adult hepatic samples expressed based on nutritional status (obese versus non obese). **B**. Western blot using a human CaSR antibody showing the corresponding bands in HepG2 and the positive control Caco-2 cells using Hsp90 as loading control. **C**. One-hour exposure to cinacalcet 1 μM or spermine 2 μM elevates phosphoERK/total ERK content in HepG2 cells. The graph shows the data of n = 8 experiments, with bars representing media ±SEM. P values: Friedman test. The inset shows a representative Western blot.

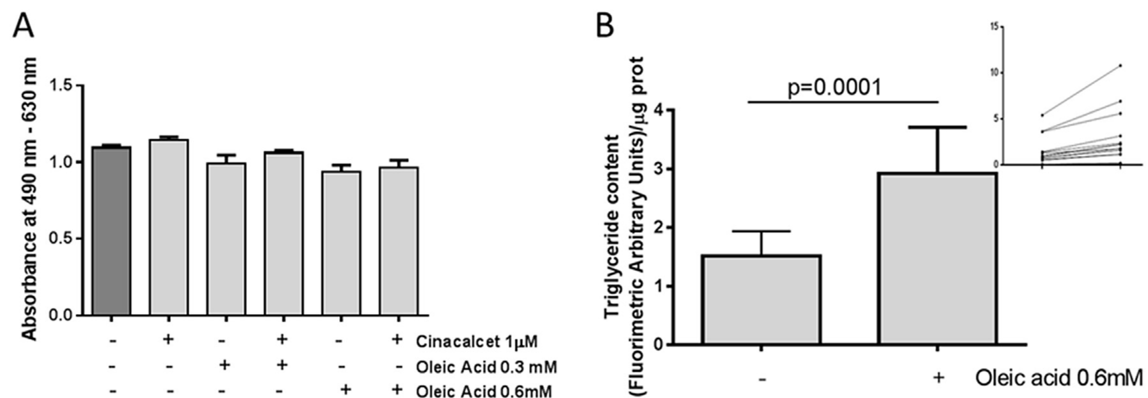

Fig. 5. Effects of oleic acid exposure in HepG2 cells. **A**. Cell viability (MTS assay) after 72 h exposure to cinacalcet (Cina) 1 μM, oleic acid 0.3 mM and 0.6 mM, and their combinations. Bars represent means ± SEM of the absorbance at 490 nm (minus 630 nm background subtraction) performed in triplicate (n = 1 experiment), and no treatment differed more than 15% versus vehicle-treated control (darker bar). **B**. Effect of 72 h exposure to 0.6 mM oleic acid added to the culture medium on TG content in HepG2 cells. Depicted p value is Wilcoxon matched-pairs signed rank test. Bars represent means ± SEM n = 14 independent experiments. The inset shows the same data presented as a dot plot with lines depicting each individual experiment.

3.6. Cinacalcet has no effect on the expression of lipogenic genes in oleic acid-exposed HepG2 cells

Given that we observed that CaSR stimulation elevated TG content after 72 h in HepG2 cells exposed to 0.6 mM oleic acid, we evaluated the possible changes in the expression of relevant lipogenic genes in hepatocytes (PPARγ, SREBP1c and FAS). Taking into consideration that gene expression would take place earlier than our observations, and to be consistent with the above experiments in adipocytes, we evaluated the effect at 24 h of cinacalcet exposure. Cinacalcet had no effect on the expression of these genes in HepG2 cells exposed to oleic acid (data not shown).

3.7. Cinacalcet elevates the expression of proinflammatory cytokines in HepG2 cells

After observing no changes in lipogenic enzyme expression despite the elevation in HepG2 TG content, and based on the possible effect of inflammation-induced elevation in CD36 fatty acid transporter activity [22], we assessed whether cinacalcet had an effect of HepG2 expression of the proinflammatory factors IL6, IL1β and TNFα. As shown in Fig. 7, cinacalcet elevated the mRNA content for the three cytokines by approximately 300%, 50% and 245%, respectively.

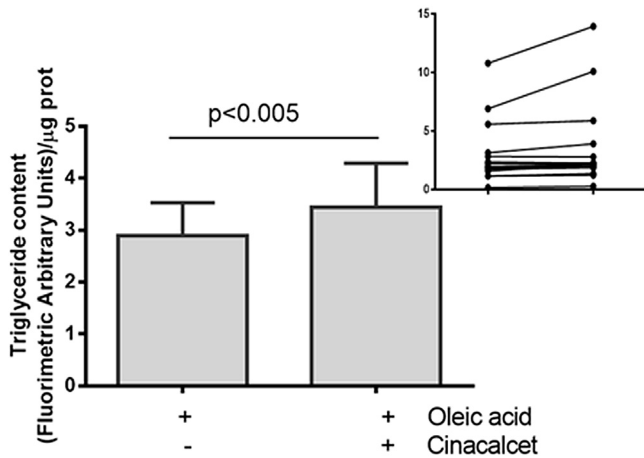

Fig. 6. Effect of CaSR activation on HepG2 cells exposed to 0.6 mM oleic acid. 1 µM cinacalcet was added for 72 h before evaluating TG content fluorimetrically (Nile Red). Bars represent mean ± SEM n = 17 independent experiments. Depicted p value corresponds to Wilcoxon matched-pairs signed rank test. The inset shows the same data presented as a dot plot to illustrate differences in each individual experiment.

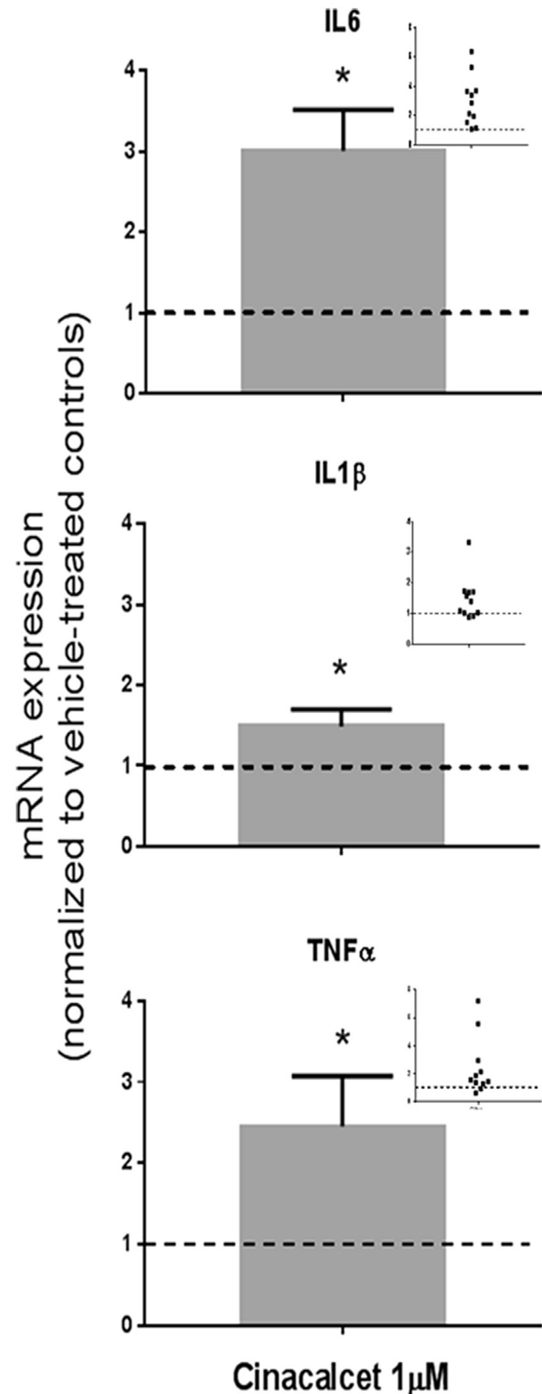

Fig. 7. CaSR activation elevates the expression of proinflammatory cytokines in HepG2 cells. Twenty-four hour exposure to cinacalcet elevated mRNA expression of IL6, IL1β and TNFα in HepG2 cells, evaluated by qPCR, using each sample's GAPDH expression as the reference gene. The bars represent the fold of vehicle-treated cells (mean ± SEM) and each inset shows the same data presented as a dot plot to illustrate differences in each individual experiment. *$p < 0.05$, Wilcoxon signed rank test evaluating the difference from vehicle-treated cells (normalized to 1 and represented by the dotted line); n = 11 independent experiments.

4. Discussion

With the aim of exploring the occurrence of a CaSR-induced AT dysfunction, and considering a possible impact of this phenomenon on metabolically relevant tissues that are particularly affected in obesity, we studied whether CaSR affects TG accumulation in adipocytes and hepatocytes. Our data show that activation of CaSR decreases TG content in differentiated LS14 adipocytes, and in the context of an elevated oleic acid environment, increases TGs in HepG2 cells. This scenario, together with CaSR-induced elevated aP2 in AT, suggest that CaSR stimulation may not only generate AT dysfunction, as we have previously proposed [4,23], but may also directly and indirectly affect peripheral organs such as the liver. This would occur via altered TG handling in AT, possibly elevating free fatty acid availability, and higher circulating levels of aP2. The latter has been shown to increase gluconeogenesis in liver and proposed to contribute to obesity-related conditions such as insulin resistance, diabetes mellitus, atherosclerosis, hypertension, and cardiac dysfunction [6,9,17].

Gene expression analysis showed that cinacalcet treatment in differentiated adipocytes decreases the lipogenic enzymes GPD and LPL. LPL hydrolyzes circulating chylomicrons and very low density lipoproteins to make fatty acids available to enter the adipocyte [24]. GPD on the other hand, provides the glycerol backbone necessary for fatty acid esterification into the TG. The decrease in the expression of these factors would thus be consistent with a concerted decrease in overall entry and storage of fatty acids in the adipocyte. Supporting a suppression of lipogenic pathways in conditions of dysfunctional fat, one study in omental AT explants from adult females, showed lower expression of GPD and LPL in women with obesity and impaired insulin sensitivity as compared with those with normal weight [25]. Another study showed lower expression of lipogenic enzymes in AT in obese versus lean humans, and this was concomitant with greater hepatic lipogenesis [8], also consistent with our observations. In our study, FASN and PPARγ remained unchanged, indicating that the effect of cinacalcet is specific to certain transcripts, and not an overall shutdown of lipid metabolism gene expression. It is well known that PPARγ acts as a transcription factor of a number of genes expressed in mature adipocytes and is considered the master regulator of adipose differentiation and metabolism [26]. However, our results showed lower LPL and GPD gene expression after cinacalcet exposure with no concomitant change in the expression of PPARγ. It is important to consider that PPARγ activity is regulated by post-translational modifications such as phosphorylation [26], so the absence of changes in mRNA expression does not rule out a role of the transcription factor in these events.

The expression of CaSR has been described in a large number of cells and tissues. However, to the best of our knowledge, no reports have been published in human hepatic cells and only one in rats, where the CaSR agonist $GdCl_3$ generates an increase in cytosolic calcium and bile flux [27]. In addition, CaSR expression was reported in the BRL cell line derived from buffalo rat liver cells, where its activation elevated intracellular calcium through the Gq-PLC-IP3 pathway [28]. The present study shows the presence of the CaSR in the human HepG2 cell line, its response (ERK phosphorylation) to two different well-known CaSR activators (the CaSR-specific calcimimetic cinacalcet and the CaSR agonist spermine), and an effect elevating TG content in conditions of high oleic acid and the expression of proinflammatory cytokines. As an important initial contribution, we have also documented the presence of the CaSR in human hepatic tissue in a small number of samples, with an interesting preliminary suggestion that there may be an influence of the nutritional status on its expression (i.e. obese subjects may express more CaSR), which will require further analysis.

In addition to the effect on lipid handling, the present study established that CaSR stimulation elevates aP2 content in adipose cells. Considering the recently recognized role of aP2 as novel adipocyte endocrine signal [10], these findings may have important potential implications at the hepatic level. Circulating aP2 levels are increased in patients with obesity, metabolic syndrome, T2D and hyperlipidemia, and deficiency of aP2 in both genetic and diet-induced animal models results in reduced insulin levels and increased insulin sensitivity [9,10]. At the hepatic level, recombinant aP2 stimulates glucose production and gluconeogenic activity in primary hepatocytes *in vitro* and in lean mice *in vivo,* and neutralization of secreted aP2 reduces the altered phenotype [17]. In addition, treatment with an aP2 inhibitor reduced liver TG content, SCD-1 and FASN gene expression [29]. These data suggest a relevant role of AT-secreted aP2 in the development of metabolic alterations.

To emulate an environment of excess free-fatty acid availability, HepG2 cells were treated with oleic acid. Hepatic steatosis in humans involves oleic and palmitic acid accumulation [30], however, it has been observed that oleic acid induces greater TG accumulation with less apoptotic effects than palmitic acid in hepatocyte cell culture [19]. In addition, it has been reported that oleic acid induces morphological changes similar to those described in steatotic hepatocytes [20]. Under our oleic acid-treated conditions, TG content was elevated, which is consistent with other studies [19], and CaSR activation increased this response. This observation prompted us to evaluate the expression of liver lipogenesis-related genes; however, cinacalcet had no effect on the expression of lipogenic enzymes, indicating that other pathways must play a role in the observed increase of TG content. CaSR activation is associated with an inflammatory state in several cell types; including AT [3,31,32]. We previously observed that cinacalcet treatment in human AT explants elevate IL1β secretion [3] and it has been reported that this cytokine elevates hepatic steatosis in response to high-fat diet in mice [6]. The present observations suggest that CaSR-induced inflammation also occurs locally at the hepatic level, since cinacalcet elevated IL6, IL1β and TNFα expression in HepG2 cells. Interestingly, a number of studies have observed that hepatic inflammatory stress contributes to TG accumulation and non-alcoholic fatty liver disease progression, and may also mediate our results [33]. In HepG2 cells, treatment with TNFα and IL6 increases the hepatic fatty acid translocase CD36, which plays an important role in hepatic fatty acid transport facilitating the uptake of long-chain fatty acids through mTOR phosphorylation [22,34]. The cinacalcet-induced elevation in TG content under elevated oleic acid conditions observed in our study may be due to an elevation in CD36 activity level, stimulated by the (also CaSR-induced) inflammatory response.

Our observations may have important implications if replicated at the whole body level. A reduced adipocyte capacity to safely store fat as TGs may result in excess available circulating fatty acids that will reach other organs, causing the well-described phenomenon of lipotoxicity. The aP2 role as an adipokine in this context would be an additional novel and relevant mediator in the effects of CaSR activation contributing to the metabolic comorbidities associated with obesity. Furthermore, besides the signals resulting from the direct effects of CaSR on AT, the present work opens a new aspect of CaSR-induced pathophysiology and inter-organ cross-talk. We have described the presence of CaSR and a direct effect of CaSR stimulation in human hepatic cells, a model of great relevance in whole body metabolism and obesity-related alterations. Thus, CaSR-induced alterations in aP2, adipose and hepatic TG handling as well as proinflammatory factor elevation, may represent important new and potential targets in the fight against the metabolic and cardiovascular consequences of obesity.

Disclosure statement

The authors have nothing to disclose.

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