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Autophagy mediates calcium-sensing receptor-induced TNFα production in human preadipocytes

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ABSTRACT

Obesity is a major current public health problem worldwide due to the severe co-morbid conditions that this disease entails. The development of obesity-related cardiometabolic disorders is in direct association with adipose tissue inflammation that leads to its functional impairment. Activation of the Calcium-Sensing Receptor (CaSR) in adipose tissue contributes to inflammation and adipose dysfunction. Autophagy, a process of cell component degradation, is closely related to inflammation in many diseases, however, whether autophagy is associated with CaSR-induced inflammation remains unknown. Using LS14 and SW872 preadipose cell lines as well as primary human preadipocytes, we show that CaSR activation with the allosteric activator cinacalcet induces autophagosome formation. Cinacalcet-induced LC3II content elevation was precluded by knockdown of the CaSR and enhanced by CaSR overexpression, indicating a specific effect. Autophagy inhibition using 3-methyladenine prevented CaSR-induced TNFα production, indicating that autophagy contributes to CaSR-induced inflammation in human preadipocytes. Our results suggest that modulation of CaSR-induced autophagy is an attractive target in obese inflamed adipose tissue, to prevent the development of diseases triggered by adipose dysfunction. We describe a novel mechanism and possible new target to modulate and prevent adipose inflammation and hence the resulting disease-generating adipose tissue dysfunction.

1. Introduction

Obesity is a current worldwide problem involving high burden on health costs and decrease in quality of life due to co-morbidities such as insulin resistance, type 2 diabetes (T2D), cardiovascular and liver diseases as well as certain types of cancer [1]. These metabolic and cardiovascular obesity-linked disorders are a consequence of impaired adipose tissue functionality, where inflammation plays a key role [2]. The hallmark of dysfunctional adipose tissue is its impaired ability to properly store excess energy as triglycerides, particularly at the subcutaneous depot [3]. As a consequence, this lipid excess accumulates in ectopic depots such as the visceral area, which leads to M1 macrophage infiltration, proinflammatory cytokine secretion and insulin resistance [4]. Adipocyte progenitor cells, termed preadipocytes, comprise up to 50% of the stroma-vascular fraction from adipose tissue, and are increased in the obese visceral depot [5]. These cells are highly responsive to inflammatory conditions, upon which they secrete proinflammatory cytokines such as IL1β and TNFα that further contribute to obesity-associated comorbidities [6–9].

Previous work from our laboratory has shown that the G protein-coupled extracellular Calcium sensing receptor (CaSR) has a key role promoting adipose tissue dysfunction. CaSR activation in adipose cells is associated with functional alterations such as elevated visceral preadipocyte proliferation [10] and adipogenesis [11] as well as impaired lipid handling [12,13]. CaSR activation in vitro with the allosteric activator cinacalcet increases expression and secretion of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), IL-1ß and C—C motif chemokine ligand 2 (CCL2) [10,14]. However, the mechanisms involved in these actions have not been fully elucidated.

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Inflammation represents a disturbance in tissue homeostasis, and as such, it is associated with a number other cell responses aimed to prevent cell damage. Macroautophagy (herein called autophagy) is an adaptive mechanism, consisting in a "self-eating" process, which promotes the degradation of cell components in order to eliminate damaged structures and recycle metabolites [15]. This conserved cell function is orchestrated by several proteins, which trigger the formation of autophagosomes, which are vesicles that engulf target cell component and later fuse with lysosomes for degradation. Increasing evidence shows that autophagy and inflammation are closely interrelated in maintaining homeostasis [16]. Obesity has been associated with both TNFα and autophagy elevation in adipose tissue, particularly in subjects with insulin resistance or T2D [17–20]. However, the association between obesity-related autophagy and inflammatory processes in the context of visceral adipose tissue dysfunction has not been studied. Considering our previous work showing that CaSR activation in adipose cells contributes to its inflammatory status, the reported association between adipose tissue inflammation/dysfunction and autophagy, and recent data showing that CaSR elevates autophagy in several models including as cardiac fibroblasts, cardiomyocytes and kidney cells [21–23], our aim was to evaluate whether CaSR modulates autophagy in preadipose cells, and whether this is associated with CaSR-induced preadipocyte inflammatory factor expression, particularly TNFα.

2. Material and methods

2.1. LS14 cell culture and CaSR activation

The human preadipocyte cell lines LS14 (RRID:CVCL_DH87), derived from a metastatic liposarcoma [24] and SW872 (HTB-92, ATCC, Manassas, VA) from a fibrosarcoma, were used in these studies. The cells were grown in 10 cm plates with DMEM/Ham's F12 (1:1) (Cat. #D2906, Sigma-Aldrich, St Louis, MO) plus 10% fetal bovine serum (cat. #04-001-1A, Biological Industries, USA) and antibiotics (penicillin-streptomycin) at 37 °C and 5% CO_2. The medium was replaced every 2–3 days. For the experiments, the cells were seeded at 70–80% confluence in plastic culture plates. To activate the CaSR, we used the calcimimetic cinacalcet (cat. #S1260, Selleck Chemicals, Houston, TX), an allosteric CaSR ligand.

To assess autophagy flux, we used the autophagosome degradation inhibitor, chloroquine (Cat. #C6628, Sigma-Aldrich, St Louis, MO). 3-methyladenine (cat. #189490, Calbiochem, San Diego, CA) was used to inhibit the initial phase of the process (autophagosome formation).

2.2. CaSR silencing and overexpression

LS14 preadipocytes were detached using 0.25% trypsin, washed and resuspended in phosphate buffered saline (PBS). Electroporation was used to transfect small interfering RNAs (siRNA, 80 nM) with Gene Pulser Xcell System (BioRad, Hercules, CA) with two pulses of 90 V and 950 μF capacitance. Subsequently, cells were resuspended in fresh culture medium and the experiments were performed after 48 h. CaSR and non-targeting control siRNAs were obtained from Santa Cruz Biotechnology, Inc. (cat. #sc-47,741 CaSR, #sc-37,007 control, Dallas, TX). The overexpression of CaSR was carried out using a vector with the human sequence of CaSR bound to Flag-tag (hCaSR) that was previously generated [25]. The incorporation was obtained by electroporation as described above and the control cells (empty vector, EV) were transfected with the vector pcDNA3.1 (Invitrogen, CA).

2.3. Primary preadipocyte isolation and culture

Human omental fat was obtained from a total of 8 subjects (87.5% female, age between 16 and 60 years) undergoing elective abdominal surgery, with a range of body mass index (BMI) between 21.7 and 41.3 kg/m^2; and a mean ± SD BMI of 32,9 ± 6,3 kg/m^2. 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. Adipose tissue was removed and transported to the laboratory immersed in saline solution immediately after surgery. The isolation of human adipose tissue-derived (primary) preadipocytes was based on Rodbell's method as we described previously [11]. After passage 3 in culture with DMEM:F12 and 10% FBS, preadipocytes were seeded and 24 h later, switched to 2.5% FBS and exposed to cinacalcet.

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

We evaluated gene expression by qPCR. Briefly, cells were lysed with TRIzol® (cat. #15596018 Invitrogen, CA) and total RNA was isolated using PureLink™RNA mini kit (cat. #12183018A, Invitrogen, CA), according to the manufacturer's instructions. Reverse transcription was achieved using the high-Capacity cDNA Reverse Transcription kit (cat. #4368813, Applied Biosystems, CA) and qPCR was performed using the SYBR®FAST qPCR kit (cat. #4385612, Applied Biosystems, CA) with thermal conditions of 20-s preincubation at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Gene expression was calculated using the model described by Pfaffl [26], using *GAPDH* as reference gene. The sequences of PCR primers are shown in Table 1.

2.5. TNFα secretion

To evaluate the secretion of TNFα upon CaSR stimulation, LS14 conditioned medium was obtained after 70–80% confluent cells by 24 h exposure, centrifuged at 5000 × *g* for 10 min and the supernatant stored at −80 °C until the analysis was performed. The content of TNFα was determined using the QuantiGlo ELISA kit (Cat. #QTA00B, R&D Systems, Minneapolis, MN).

2.6. Western blot analysis

Cells were homogenized at 4 °C in NP40 lysis buffer (150 mM NaCl, 50 mM Tris base, pH 8.0, and 1% NP40) and supplemented with 1 mM sodium orthovanadate (cat. #S6508, Sigma-Aldrich, St Louis, MO), 1.5 μM pepstatin A (cat. #P5318, Sigma-Aldrich, St Louis, MO) and Complete® protease inhibitor cocktail (cat. #11836153001, Roche,

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

Target mRNA	Accession number	Forward Primer (5′ → 3′)	Reverse primer (5′ → 3′)
CaSR	NM_001178065	GATGAGACAGATGCCAGTGC	AAAGAGGGTGAGTGCGATCC
ATG5	NM_001286106.1	AACTGAAAGGGAAGCAGAACCA	CCATTTCAGTGGTGTGCCTTC
ATG7	NM_006395.2	CGTTGCCCACAGCATCATCTTC	CACTGAGGTTCACCATCCTTGG
BEC-1	NM_003766.4	GGCTGAGAGACTGGATCAGG	CTGCGTCTGGGCATAACG
LC3A	NM_032514.3	CCAGCAAAATCCCGGTGAT	TGGTCCGGGACCAAAAACT
LC3B	NM_022818.4	ACCATGCCGTCGGAGAAG	GGTTGGATGCTGCTCTCGAA
TNFα	NM_000594.2	CCAGGCAGTCAGATCATCTTCTC	AGCTGGTTATCTCTCAGCTCCAC
GAPDH	NM_002046	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT

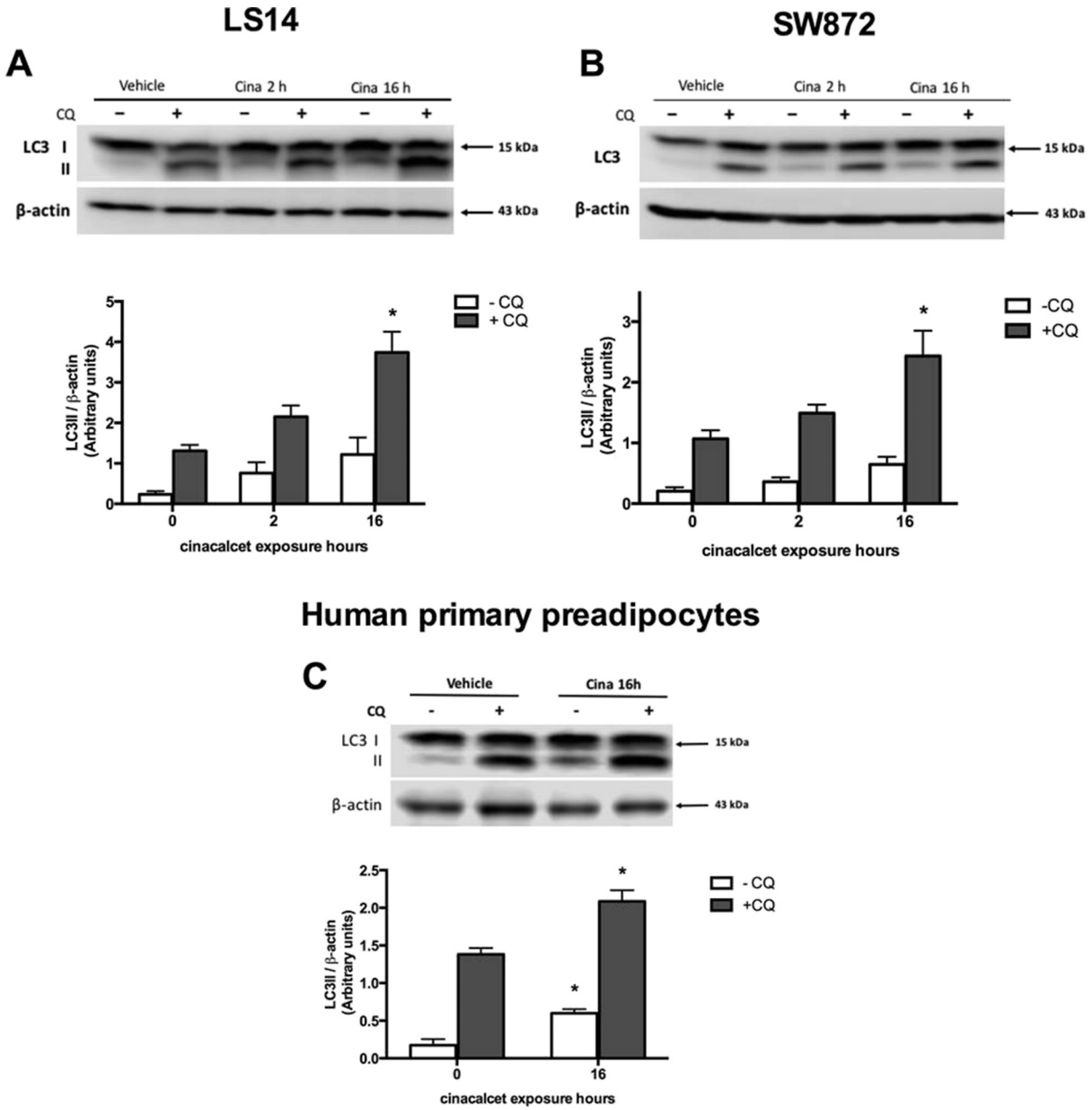

Fig. 1. Cinacalcet increases autophagy flux in (A) LS14, (B) SW872 and (C) primary human preadipocytes. Cells were exposed to vehicle or cinacalcet 2 μM for 2 and 16 h (A and B) or 16 h (C), either alone (white bars) or adding CQ 20 μM (gray bars) for the last 3 h. Each panel shows a representative image of LC3II and β-actin (internal loading control) and the respective densitometry analysis. Bars represent mean ± SEM of 5–7 independent experiments. *$p < 0.05$ vs. all other conditions (A and B) and vs. the respective vehicle condition without cinacalcet (C), two-way ANOVA and Tukey's post hoc test for multiple comparisons.

Basel, Switzerland). The lysate was centrifuged (12,000 *g*, 15 min) and protein concentration was determined by a bicinchoninic acid-based assay (cat. #23277, Pierce, Rockford, IL). The lsysate was heat-denatured at 100 °C for 5 min in SDS-PAGE loading buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol and 20% 2-mercaptoethanol). Twenty-five to 30 μg were loaded in 10% or 15% polyacrylamide gels and electrophoresed at 80–120 V by 90 min. Electrotransferring to nitrocellulose 0,22 μm (cat. #10600001, Amersham™ Protran®, Munich, Germany) or polyvinylidene difluoride (PVDF) 0,45 μm (cat. #88518, Thermo scientific, IL) membranes was performed in a buffer containing 24 mM Tris, 194 mM glycine and 20% methanol at 90 V for 1–1.5 h. Membranes were blocked in 5% nonfat milk in Tris-buffer saline (TBS) and the immunoreaction was then performed by 16 h incubation with primary antibody (LC3 Cell Signaling cat. #2775, 1:500; SQSTM1/p62 Novus Biological cat. #H00008878 1:1000; β-actin Santa Cruz cat. #47778, 1:3000; CaSR Santa Cruz cat. #47741, 1:250) dissolved in TBS with 0.05% tween 20 (cat. #7949, Sigma, St Louis, MO) and 5% BSA. Membranes were incubated with peroxidase-conjugate secondary antibody (Goat anti-Rabbit cat. #2004 and Goat-anti Mouse cat. #2005, Santa Cruz Biotechnology, Santa Cruz, CA) followed by an incubation with the enzyme substrate ECL (cat. #20-500-500A and 20–500-500B, Biological Industries, Cromwell, CT) and scanned in C-DiGit Blot Scanner (LI-COR). The images were analyzed with ImageJ software (NIH, Bethesda).

2.7. Immunofluorescence study

To examine LC3 puncta pattern, we seeded LS14 cells in 24-well plates with cover glass, and experiments were performed at 60–70% confluence. At experiment conclusion, cells were washed twice with cold PBS and permeabilized/fixed with 100% methanol at −20 °C for 10 min. Cells were then washed and blocked by 2 h incubation with BSA

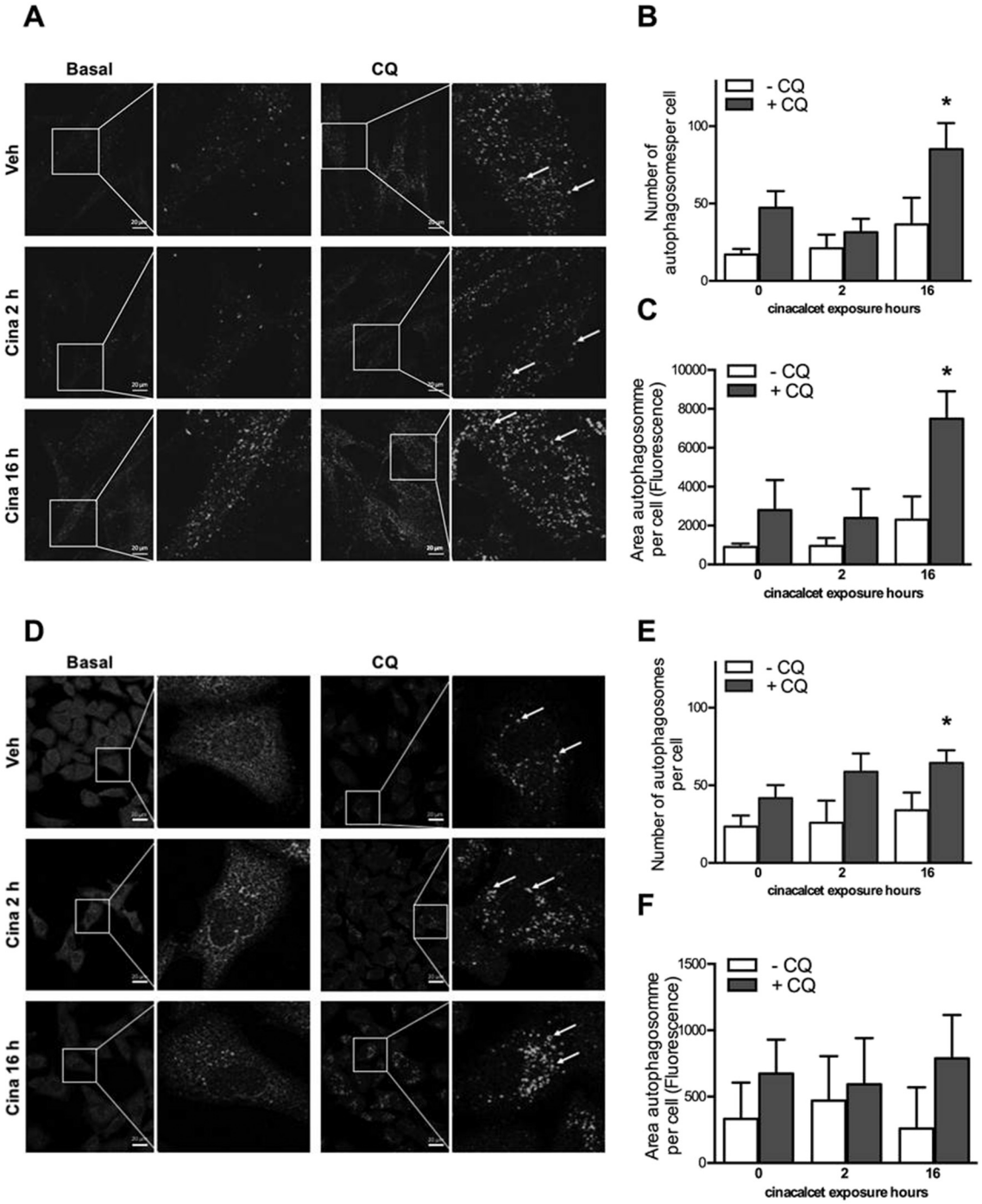

Fig. 2. Cinacalcet increases autophagosome number in (A–C) LS14 and (D-F) SW872 preadipocytes. The cells were treated with cinacalcet 2 μM for 2 and 16 h, either alone (white bars) or adding CQ 20 μM (gray bars) for the last 3 h. (A and D) Immunofluorescence images representative of each condition (Scale bar: 20 μm.), the arrows in the enlarged squared areas to the right show individual autophagosomes. The graphs depict the quantification of (B and E) autophagosome number per cell (LC3 stain dots), and (C and F) autophagosome fluorescent area per cell. Bars represent mean ± SEM of 4–5 independent experiments. *$p < 0.05$ vs all other conditions, two-way ANOVA and Tukey's post hoc test for multiple comparisons.

5% in PBS at 4 °C. The immunodetection was performed by 16 h incubation with primary antibody, followed by two washes with PBS and 2 h incubation with AlexaFluor® 488-conjugated secondary antibody (cat. #A11008, Life Technologies, CA) and Hoechst 33342 (cat. #H3570, Molecular Probes-Invitrogen, Carlsbad, CA). Immunofluorescence images were taken with a Carl Zeiss LSM700 confocal microscope and analyzed with ImageJ software.

2.8. Statistical analysis

We used non-parametric statistics that do not have any distributional assumption, given the low reliability of normality testing for small sample sizes used in this work [27]. To compare parameters between controls and treated cells, variables were evaluated using Wilcoxon match-paired signed rank test, Friedman Test or 2-way ANOVA Test with Tukey's multiple comparisons post hoc, when appropriate. Data are shown as means ± SEM, and a p-value < 0.05 was considered

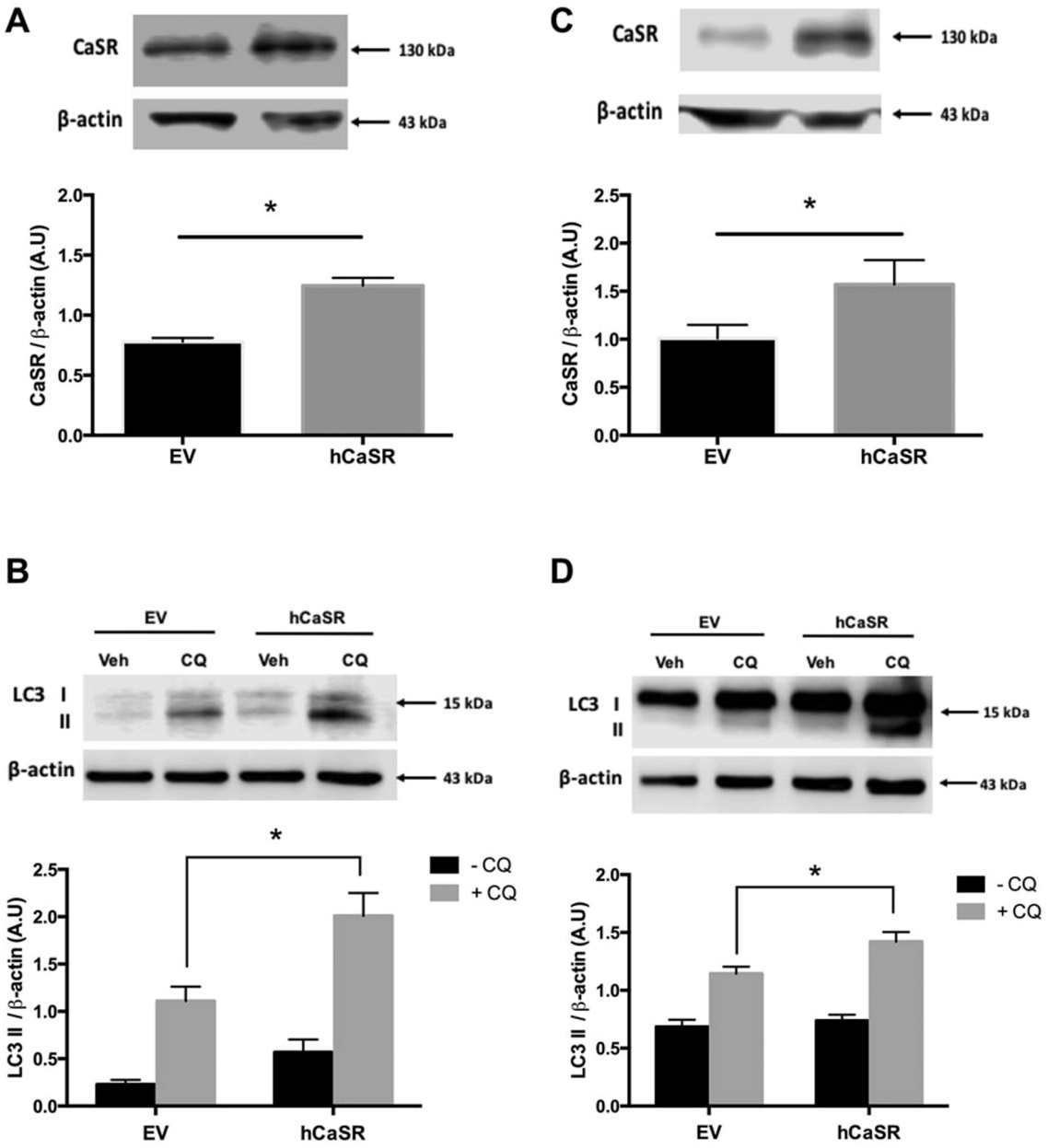

Fig. 3. CaSR overexpression enhances cinacalcet effect increasing LC3II content. Efficacy of CaSR gene overexpression (hCaSR) versus empty vector (EV) elevating CaSR protein expression in (A) LS14 and (C) SW872 preadipocytes. LC3II content in (B) LS14 and (D) SW872 cells overexpressing CaSR as compared with the EV-transfected controls exposed to vehicle or CQ 20 μM (3 h). Representative Western blot images showing LC3II and β-actin (internal loading control) are depicted above each graph showing the densitometry analysis of 6–8 independent experiments. Bars represent mean ± SEM and $*p < 0.05$, Wilcoxon matched-pair test (A, C) or two-way ANOVA and Tukey's post hoc test for multiple comparisons (B, D). AU: arbitrary units.

significant.

3. Results

3.1. Cinacalcet stimulates autophagy in human preadipocyte cell lines and primary human preadipocytes

To evaluate the effect of CaSR activation on autophagy in preadipocytes, we exposed two different human preadipocyte cell lines (LS14 and SW872) as well as human primary preadipocytes to the calcimimetic cinacalcet. Autophagy flux was determined by assessing LC3II accumulation after exposing the cells to the lysosome function inhibitor chloroquine (CQ), which prevents autophagosome degradation (higher LC3II indicates elevated autophagy flux). Cells were exposed to cinacalcet for 2 and 16 h, based on the observed effect shown by Kaizuka et al. [28] in Hela cells (2 h) and our previous results of the proinflammatory response at 16 h in LS14 cells [10,14]. We observed that 16 h of cinacalcet exposure in CQ-treated cells increased LC3II levels by 164% for LS14, 40% for SW872, and 37% for primary preadipocytes (Fig. 1).

To confirm the effect of CaSR activation on autophagosome formation, we evaluated autophagy flux by imaging autophagy vacuoles number and area after exposing the cells to cinacalcet for 16 h, adding CQ during the last 3 h. Greater number of LC3-containing dots and an increase in the fluorescent area per cell were observed upon cinacalcet treatment (Fig. 2).

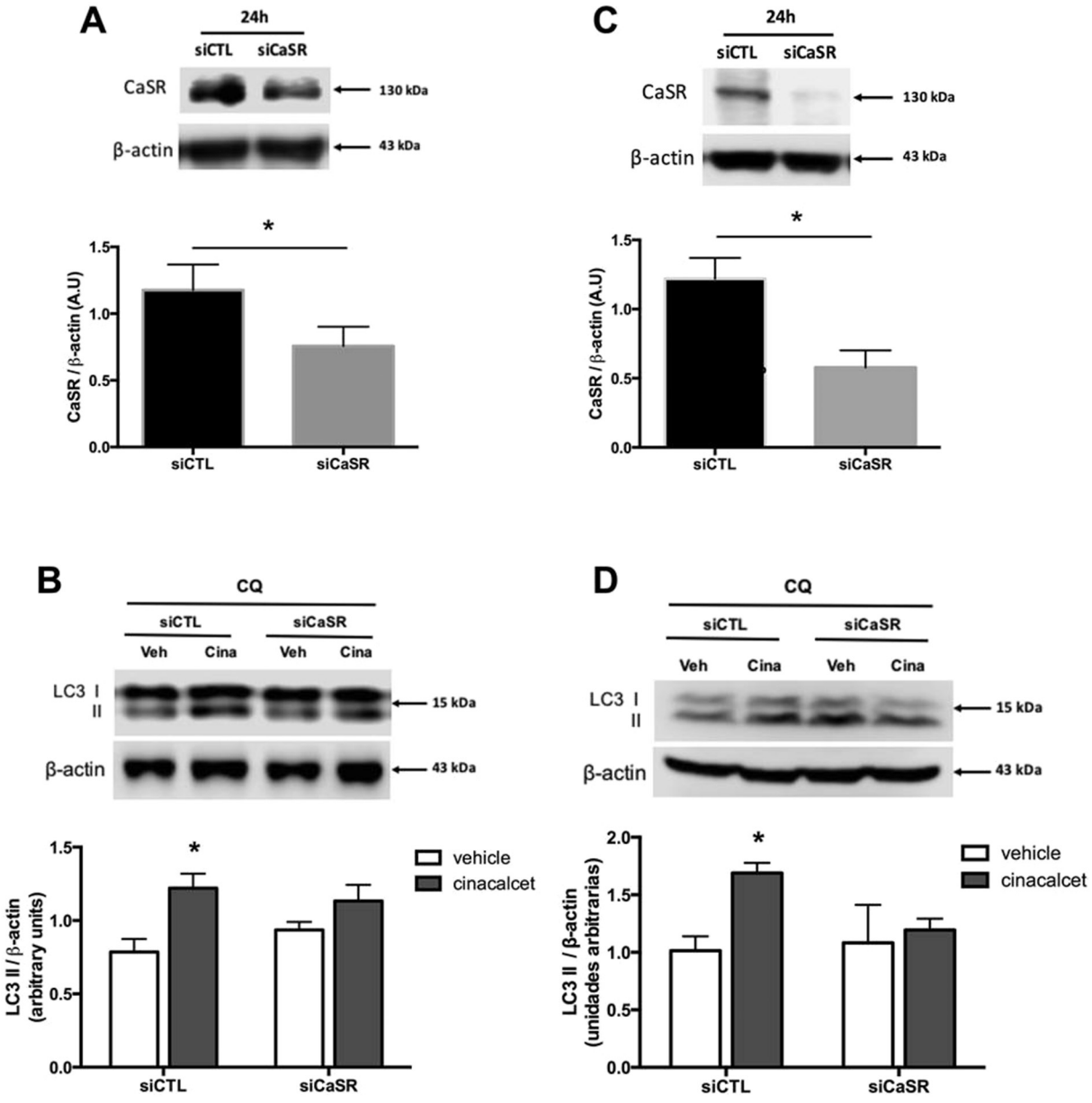

Fig. 4. CaSR silencing prevents cinacalcet effect increasing LC3II content in LS14 and SW872 preadipocytes. Efficacy of CaSR gene silencing (siCaSR) versus control siRNA (siCTL) reducing protein expression in (A) LS14 and (C) SW872 cells. A representative Western blot image is shown. Representative images showing LC3II and β-actin (internal loading control) content in (B) LS14 and (D) SW872 cells exposed to cinacalcet 2 μM (16 h) and CQ 20 μM (last 3 h). Under each image the densitometry analysis of 7 and 5 (A and C) and 5 (B, D) independent experiments is shown. Bars represent mean ± SEM and $*p < 0.05$ Wilcoxon matched-pair test (A, C) or vs. the respective vehicle-treated control, two-way ANOVA and Tukey's post hoc test for multiple comparisons (B, D).

3.2. CaSR mediates cinacalcet-induced increase on autophagy flux

To confirm that cinacalcet-induced autophagy elevation is mediated by CaSR activation, we evaluated LC3II protein levels in calcimimetic-treated LS14 and SW872 preadipocytes after CaSR overexpression and silencing. Results in control experiments (cells transfected with empty vector (EV) or scrambled sequence siRNA for overexpression and silencing, respectively) were consistent with previous results with cinacalcet increasing LC3II content. CaSR overexpression enhanced such response (Fig. 3), whereas the effect was reverted in the CaSR-silenced cells (Fig. 4), confirming that cinacalcet is acting through CaSR activation.

3.3. CaSR activation stimulates autophagy-related gene expression in LS14 preadipocytes

To address whether the effects of cinacalcet on autophagy involve changes in autophagy-related gene expression, we used qPCR to assess mRNA changes in response to cinacalcet in LS14 preadipocytes. As shown in Fig. 5A, cinacalcet increased the expression of *Atg5, LC3b*, *Atg7* and *Beclin-1*. To verify that this effect occurred through CaSR activation, we downregulated CaSR expression (using siRNA) and evaluated *Atg7* and *Bec1*, where cinacalcet showed the largest effect (Fig. 5A). We observed that CaSR silencing prevents cinacalcet-induced elevation in *Atg7* and *Bec1* gene expression (Fig. 5B and C).

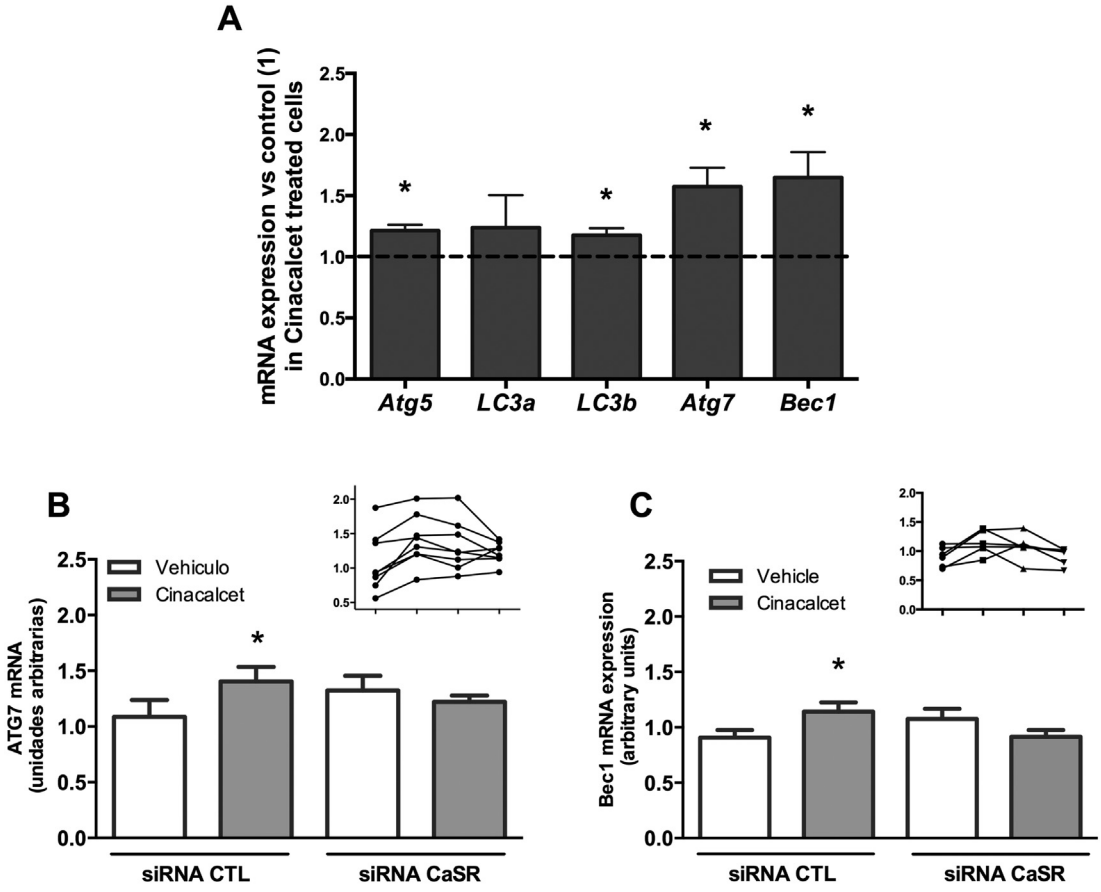

Fig. 5. CaSR activation increases autophagy gene expression in LS14 preadipocytes. Autophagy-related gene expression was assessed after 16 h exposure to cinacalcet 2 μM by qPCR. (A) mRNA expression in cinacalcet-treated cells, presented as fold of vehicle-treated controls (dotted line, value of 1), $n = 7$ independent experiments, $^*p < 0.05$ for the difference from 1, Wilcoxon signed rank test. (B) Atg7 and (C) Bec1 gene expression was evaluated in LS14 cells transfected with siRNA targeted to human CaSR (siRNA CaSR) or a scrambled sequence (siRNA CTL), and exposed (16 h) to cinacalcet 2 μM. The insets show the individual data for 6–8 independent experiments, $^*p < 0.05$ vs. the respective vehicle-treated control, Friedman Test. Bars represent mean ± SEM and GAPDH was used as the reference control gene.

3.4. Autophagy mediates cinacalcet-induced of TNFα production

Based on our previous evidence that CaSR activation increases TNFα secretion [14], we evaluated whether this effect is related to CaSR-induced autophagy in LS14 preadipocytes. The effect of autophagy inhibition with 3MA (an inhibitor of the initiation phase of autophagy) on TNFα gene expression and secretion was assessed in LS14 preadipocytes. As shown in Fig. 6A, exposure to 3MA, reverts cinacalcet-induced stimulation of *TNFα* expression. Consistently, the expected elevation of TNFα secretion in cinacalcet-exposed LS14 preadipocytes was reverted when autophagy was inhibited (Fig. 6B). Treatment with 3MA alone did not affect *TNFα* expression or secretion (insets in 6A and 6B), and for every experiment, the inhibition of autophagy was confirmed by levels of LC3II/βactin by Western blot (data not shown). In CaSR-overexpressing cells, the inhibition of TNFα secretion by 3MA was exacerbated (Fig. 6C), with a 4-fold response as compared with the 1.6-fold inhibition observed in the native cells. Control experiments were performed to verify that the exposure to 3MA inhibits autophagy, as shown by a decrease in LC3II (Fig. 6D).

4. Discussion

The present work shows that CaSR activation stimulates autophagy in human preadipocytes. In addition, our data support a role for autophagy mediating CaSR-induced TNFα expression/secretion, thereby suggesting that autophagy induction is involved in adipose inflammation. These findings are in line with the emerging concept of a close link between autophagy and inflammation [16], where autophagy is viewed as a mechanism involved in maintaining homeostatic balance by modulating inflammatory responses.

Among the cellular components of adipose tissue, particularly the visceral depot, preadipocytes constitute one relevant source of proinflammatory factors. Dysregulation of their secretory and differentiation capacities are particularly relevant in conditions of obesity and insulin resistance [7,29,30]. Our previous work established that CaSR activation stimulates human preadipocyte proinflammatory expression [10,14], and the current observations provide evidence for a possible mechanism, showing that cinacalcet-induced stimulation of preadipocyte autophagy mediates TNFα production.

To our understanding, few studies have explored the role of CaSR in the modulation of autophagy. A recent work by Chi et al. showed that CaSR activation induces autophagy and contributes to fibrosis in cardiac fibroblasts [21]. Liu et al. reported that CaSR activation stimulated autophagy in neonatal rat cardiomyocytes, whereas in rats with induced cardiac hypertrophy, treatment with the CaSR inhibitor Calhex 231 decreased markers of autophagy and ameliorated cardiac hypertrophy [23]. Additionally, a recent study showed that cinacalcet increases autophagy with nephroprotective effects in human glomerular endothelial cells and animal murine podocytes [22]. To the best of our knowledge, our results are the first to describe CaSR as modulator of autophagy in adipose cells.

Interestingly, a recent report described cinacalcet as a novel autophagy modulator [28], and the observations are interpreted as cinacalcet acting as an autophagy inhibitor. The authors showed that

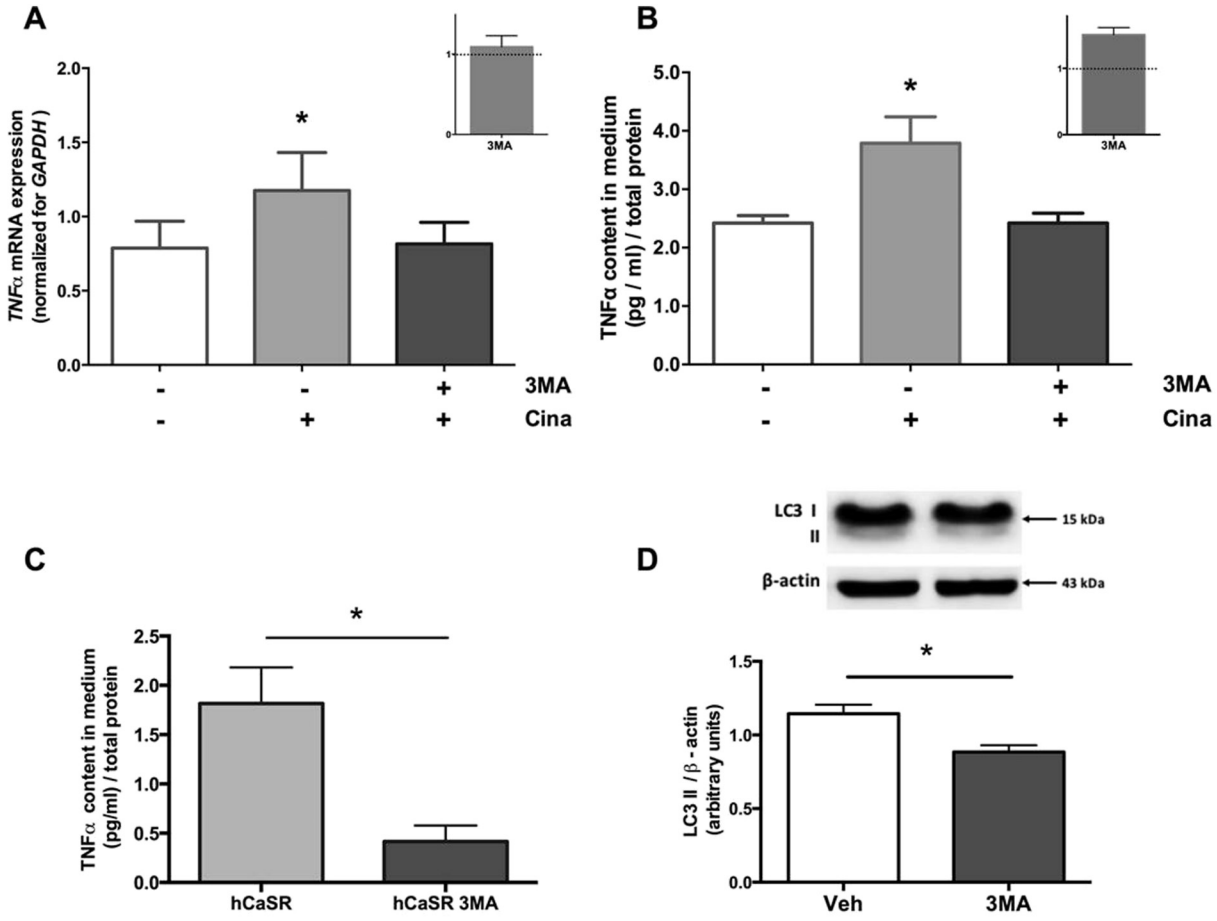

Fig. 6. Autophagy inhibition abolishes the effect of cinacalcet elevating TNFα expression and secretion in LS14 preadipocytes. Cells were exposed for 16 h to 2 μM cinacalcet with or without pre-exposure (1 h) to 5 mM 3-methyladenine (3MA). (A) TNFα mRNA expression was evaluated by qPCR and (B) TNFα protein secretion in the medium recovered after 24 h by ELISA. The insets depict control experiments showing no effect of 3MA alone on TNFα mRNA ($n = 6$) or protein ($n = 4$). The dotted line represents the control untreated cells normalized to 1. (C) TNFα secretion in CaSR-overexpressing cells with or without 3MA exposure. (D) Control experiments to verify that 1 h exposure to 5 mM 3MA inhibits autophagy, as shown by a decrease in LC3II using βactin as the loading control. Bars represent mean ± SEM. *$p < 0.05$, Friedman Test (A, B); or Wilcoxon matched-pairs signed rank test (C, D).

cinacalcet exposure induces the formation of GFP-LC3 puncta under starvation conditions, which could actually be consistent with our results. Kaizuka's observations together with our results suggest that cinacalcet may be activating autophagosome formation; however autophagic flux is not increasing accordingly, as evidenced by elevated LC3II levels in cinacalcet-treated cells without CQ. This phenomenon could actually contribute to the deleterious consequences of CaSR-induced autophagy elevation as autophagosomes are not being cleared, thus not completing autophagy "cleaning" process.

The role of autophagy is highly tissue- and cell-specific, and this is particularly relevant in the context of the different organs involved in the development of obesity-related diseases. Hepatic autophagy is downregulated in obesity, and suppression of autophagy results in defective insulin signaling in hepatic cell models and livers of intact mice [31]. In pancreatic β cells, autophagy deficiency may be deleterious, particularly in a context of metabolic stress such as obesity [32]. In skeletal muscle, exercise-related beneficial effects on insulin resistance (a key defect in T2D) and whole-body glucose homeostasis may be associated with autophagy elevation [33]. There is scarce evidence regarding autophagy (dys) regulation in adipose tissue in obesity, however most studies agree that autophagy is up-regulated, particularly in obese insulin resistant individuals [17,19,20].

It is relevant to distinguish autophagy that is activated to offset obesity-related cellular stress signals, as a homeostatic mechanism promoting cell protection and survival, from pathologically-altered autophagy as a consequence of other external stimuli, reflecting obesity-associated tissue dysregulation, even contributing to adipose dysfunction. Research in this direction shows that autophagy upregulation in obese visceral adipose tissue occurs in parallel with elevated inflammation markers such as TNFα [17,20]. Addressing the "chicken-or-egg" question, our data are consistent with those of Crisan et al., where autophagy inhibition abrogated TNFα expression and secretion in human peripheral blood mononuclear cells [34], and Jia et al. [35], where TNFα-induced apoptosis was inhibited by autophagy blockage in a human leukemic cell line. Consistent with this idea, Kovsan et al. [17] stated that elevated autophagy precedes obesity associated morbidity, whereas Kosacka et al [20] proposed that induction of adipose tissue autophagy contributes to increased adipose tissue macrophage infiltration, an event that is considered key in adipose inflammation and dysfunction. Our present results showing that autophagy stimulation mediates CaSR-induced TNFα production are consistent with those recently published by Kosacka et al. [36], where inhibition of autophagy with LY294002 in visceral adipocytes from obese rats with metabolic syndrome downregulated TNFα expression. On the other hand, Jansen et al [19] reported that autophagy inhibition with 3MA in adipose tissue from obese individuals increased the expression and secretion of several proinflammatory factors. However, the use of 3MA in adipose tissue that contains lipid-laden differentiated adipocytes, is complicated by the fact that 3MA is a potent protein kinase A activator and hence a pro-lipolytic agent [37]. Free fatty acid production upon lipolytic

stimulus in adipocytes is expected to generate a lipotoxic and proinflammatory environment [38], so 3MA could be elevating proinflammatory factors through an autophagy-independent mechanism. Our studies are not confounded by the use of 3MA since preadipocytes do not contain lipid droplets and do not express the lipolytic machinery.

To date, most of the research has focused on the role of proinflammatory factors such as TNFα modulating autophagy [16], however less attention has been paid to the effect of dysregulated autophagy elevating inflammation. Our studies propose that stimulated autophagy after CaSR activation mediates an increase in the production of proinflammatory cytokine TNFα. The concept of autophagy as an upstream regulator of cytokine production is supported by several studies [34,35], which highlights the relevance of focusing on events that trigger excess autophagy elevation, other than physiological cues. As seen in other pathophysiological conditions such as oxidative stress, excess autophagy activation may lead to cell death [39]. In this work, we used the specific CaSR allosteric activator cinacalcet as a first approach to study the effects of CaSR activation in preadipocyte autophagy. Our findings showing that CaSR elevates preadipocyte autophagy and that this process may mediate TNF-α secretion should be confirmed in future studies in other adipose cell types, in whole adipose tissue explants and using physiological agonist candidates to be present in obesity-associated inflammatory conditions.

5. Conclusion

This study presents the novel concept that CaSR activation stimulates autophagy in preadipocytes, which in turn mediates the elevation of TNFα production. These findings contribute to emerging data that link CaSR activation to autophagy, and provide new concepts to understand and target the mechanisms of adipose cell inflammation. Future research must focus on the numerous possible endogenous agonists that may be locally acting on CaSR to induce adipose autophagy and inflammation.

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Author contributions

PM, RBS, NT and CF performed experiments, PM, RBS, GB and MC analyzed data, whereas PM, RBS, NT, RT, GB, SL and MC participated in study design, data discussion and manuscript preparation.

Conflict of interest

The authors declare no conflict of interests/financial disclosure statement.

Transparency document

The Transparency document associated this article can be found, in online version.

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