Molecular and Cellular Endocrinology 501 (2020) 110654

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Calcium sensing receptor activation in THP-1 macrophages triggers NLRP3 inflammasome and human preadipose cell inflammation

Amanda D'Espessailles[a], Natalia Santillana[a], Sofía Sanhueza[a], Cecilia Fuentes[a], Mariana Cifuentes[a,b,c,*]

[a] Institute of Nutrition and Food Technology, University of Chile, El Líbano 5524, Macul, Casilla 138-11, Santiago, Chile
[b] Advanced Center for Chronic Diseases (ACCDiS), Facultad de Ciencias Químicas y Farmacéuticas & Facultad de Medicina, Universidad de Chile, Santiago, 8380492, Chile
[c] Center for Exercise, Metabolism and Cancer (CEMC), Facultad de Medicina, Universidad de Chile, Santiago, 8380492, Chile

ARTICLE INFO

Keywords:
Calcium sensing receptor
Preadipocytes
Macrophages
Crosstalk
NLRP3 inflammasome

ABSTRACT

Excess adipose tissue (AT) associates with inflammation and obesity-related diseases. We studied whether calcium-sensing receptor (CaSR)-mediated NLRP3 inflammasome activation in THP-1 macrophages elevates inflammation in LS14 preadipocytes, modeling deleterious AT cell crosstalk. THP-1 macrophages exposed to cinacalcet (CaSR activator, 2 μM, 4 h) showed elevated proinflammatory marker and NLRP3 inflammasome mRNA, pro-IL-1β protein and caspase-1 activity, whereas preincubation with CaSR negative modulators prevented these effects. The key NLRP3 inflammasome component ASC was silenced (siRNA) in THP-1 cells, and inflammasome activation was evaluated (qPCR, Western blot, caspase-1 activity) or they were further cultured to obtain conditioned medium (CoM). Exposure of LS14 preadipocytes to CoM from cinacalcet-treated THP-1 elevated LS14 proinflammatory cytokine expression, which was abrogated by THP-1 inflammasome silencing. Thus, CaSR activation elevates THP-1-induced inflammation in LS14 preadipocytes, via macrophage NLRP3 inflammasome activation. Modulating CaSR activation may prevent deleterious proinflammatory cell crosstalk in AT, a promising approach in obesity-related metabolic disorders.

1. Introduction

Obesity is a worldwide health problem, aggravated by the associated risk of developing cardiovascular disease, type 2 diabetes, hypertension and certain types of cancer, among other disorders. Adipose tissue (AT) is an important endocrine organ whose dysfunction has a critical role in obesity-induced metabolic alterations (Guzik et al., 2017; Vegiopoulos et al., 2017). In obesity, excess AT expansion can lead to increased secretion of proinflammatory cytokines such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), IL-6 and chemokines such as chemokine ligand 2 (CCL2), and relatively lower anti-inflammatory adipokines such as adiponectin. This proinflammatory secretion profile induces macrophage infiltration and further inflammation (Guzik et al., 2017; Kusminski et al., 2016), a phenomenon that is key in the pathogenesis of whole body metabolic alterations observed in obesity (Gerner et al., 2013). Macrophage infiltration in AT triggers a relevant paracrine communication (Sorisky et al., 2013). *In vitro*, macrophage-secreted factors induce adipocyte inflammation and insulin resistance (Permana et al., 2006), promote a profibrotic phenotype (Keophiphath et al., 2009), and impair adipogenesis (Lacasa et al., 2007), events that are associated with AT dysfunction, lipotoxicity and the systemic chronic low grade inflammation associated with obesity (Guzik et al., 2017; Sorisky et al., 2013).

Our laboratory has studied the activation of the extracellular calcium sensing receptor (CaSR) as one possible factor favoring AT dysfunction (Bravo-Sagua et al., 2016). The CaSR is a G protein-coupled receptor first cloned in 1993 as a regulator of parathyroid hormone secretion, with a crucial role maintaining circulating calcium homeostasis (Brown et al., 1993). This receptor is located in multiple other tissues, where it is able to respond to different stimuli (polyvalent cations, amino acids, ionic strength, pH) besides its main agonist Ca^{2+}, evidencing its unique ability to integrate and respond to multiple metabolic signals (Wellendorph et al., 2010). The CaSR is expressed in AT (Cifuentes et al., 2005), the human preadipose cell line LS14 (Cifuentes et al., 2012), human monocytes (Olszak et al., 2000) and THP-1 macrophages (Xi et al., 2010). Activation of CaSR mediates the expression

Abbreviations: AT, adipose tissue; CaSR, calcium sensing receptor; CoM, conditioned medium

* Corresponding author. INTA-Universidad de Chile, El Líbano 5524, Macul, Casilla 138-11, Santiago, Chile.
E-mail address: mcifuentes@inta.uchile.cl (M. Cifuentes).

https://doi.org/10.1016/j.mce.2019.110654
Received 18 June 2019; Received in revised form 13 November 2019; Accepted 13 November 2019
Available online 15 November 2019
0303-7207/

and secretion of several proinflammatory factors in primary and LS14 adipose cells (Cifuentes et al., 2012; Rocha et al., 2015), and mediates the expression and activation of the Nod-like receptor, pyrin domain-containing 3 (NLRP3) inflammasome in LS14 preadipocytes through ERK 1/2 signaling (D'Espessailles et al., 2018). Different roles have been described for the CaSR in numerous physiological processes involving gene expression, ion channel activity and cell fate, as well as diseases and conditions, including diabetes, Alzheimer's disease, infertility, cardiovascular disease, certain cancers and inflammation (Díaz-Soto et al., 2016; Riccardi and Kemp, 2012; Ward et al., 2012). An important and emerging aspect of CaSR pathophysiology is its ability to generate proinflammatory signaling and elevate its own expression upon proinflammatory conditions (Hendy and Canaff, 2016), thus generating a positive feedback loop that perpetuates inflammation.

The inflammasomes are signaling platforms assembled in response to numerous signals, which leads to activation of the cysteine protease caspase-1 and subsequent maturation and secretion of interleukins such as IL-1β and IL-18 (Schroder and Tschopp, 2010). The NLRP3 inflammasome responds to metabolic stress signals present in obesity, such high glucose (Zhou et al., 2010a,b), saturated fatty acids (Reynolds et al., 2012) and oxidative stress (Zhou et al., 2010a,b). Accordingly, the NLRP3 inflammasome has been associated with obesity-related chronic inflammation, insulin resistance and diabetes (Rheinheimer et al., 2017). To assemble the inflammasome, NLRP3 interacts with the adapter protein apoptosis-associated speck-like protein (ASC), which has a caspase activation recruitment domain (CARD) that binds to the CARD domain of procaspase-1. The ensuing self-cleavage of procaspase-1 activates caspase-1 and subsequently converts pro-IL-1β and pro-IL-18 into their mature secreted forms (Stienstra et al., 2011). In 2012, Rossol et al. reported the activation of NLRP3 inflammasome after CaSR stimulation in primary human monocytes and the human monocyte cell line THP-1 (Rossol et al., 2012), findings that were consistent with those of Lee et al. (G.-S. Lee et al., 2012) in mouse bone marrow-derived macrophages. Later, Liu et al. (2015) confirmed that CaSR stimulation upregulated NLRP3 inflammasome components and induced proteolytic processing and IL-1β secretion in M1-polarized (proinflammatory) THP-1 macrophages.

It has been proposed that local paracrine interactions within dysfunctional AT generate a positive feedback loop that perpetuates the inflammatory condition (Guzik et al., 2017; Lacasa et al., 2007), particularly in the hypoxic and high glucose environments associated with obesity (Avila-George et al., 2017; Peshdary et al., 2016). In this context, infiltrated proinflammatory macrophages may produce signals that trigger a proinflammatory phenotype in preadipocytes. Previous research suggests that the activation of the NLRP3 inflammasome may be relevant mediating and/or enhancing this effect, as its activation has been linked with dysfunctional inflammation of the AT and the development of diseases associated with obesity (Rheinheimer et al., 2017). We aimed to study whether the specific positive allosteric CaSR modulator cinacalcet mediates the activation of NLRP3 inflammasome in human THP-1 macrophages, and if this in turn has an effect elevating inflammatory marker expression in the human preadipocyte cell model LS14, as an *in vitro* dysfunctional cell communication model that would further exacerbate AT inflammation.

2. Material and methods

2.1. Cell line culture

THP-1. THP-1 cells (ATCC Cat# TIB-202, RRID:CVCL_0006) were purchased at ATCC (Manassas, VA, USA) and grown in RPMI medium (Sigma, St Louis, MO, USA) with 10% fetal bovine serum (FBS). THP-1 monocytes were differentiated into macrophages with 100 nM PMA (phorbol miristate acetate, Sigma) for 24 h. Cells then were washed with phosphate-buffered saline solution (PBS) and fresh medium without FBS was used. After an overnight period, when indicated, cells were exposed for 3 h to 100 ng/ml lipopolysaccharide (LPS, (Sigma)) to enhance their proinflammatory phenotype. After washing the cells to remove LPS, cells were incubated with fresh RPMI with 2,5% FBS and pretreated for 40 min with pharmacological CaSR inhibitors (1 μM NPS2143 (Tocris Bioscience, Bristol, UK) or 10 μM calhex 231 (Sigma)) and/or the calcimimetic cinacalcet (Selleckchem, Houston, TX, USA) 2 μM for 4 h. As these agents are allosteric CaSR modulators, the experiments were performed in presence of physiological calcium concentrations (1 mM), achieved by supplementing RPMI medium (that contains 0.4 mM Ca^{2+}) with 0.6 mM $CaCl_2$. This supplementation had no effect on any of the variables evaluated. After treatment, THP-1 cells were either lysed (for PCR or Western Blot) or gently and thoroughly washed with PBS to remove all pharmacological agents and then incubated with fresh medium for 24 h to obtain the conditioned medium (CoM) to be used to stimulate LS14 preadipocytes (see below).

LS14. The human preadipose cell line LS14 (RRID:CVCL_DH87), is derived from a metastasic liposarcoma, and 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, USA) and grown in DMEM:F-12 (1:1) medium (Sigma) supplemented with 10% FBS and antibiotics (penicillin-streptomycin) at 37 °C in a controlled atmosphere incubator (5% CO_2). The medium was replaced every 2–3 days. LS14 preadipocytes were exposed for 24 h with CoM from treated or non-treated THP-1 cells. CoM was added in 1:1 ratio with fresh DMEM:F-12 medium to provide cells with an adequate nutrient environment. Fresh DMEM:F-12 and RPMI exposed to the culture atmosphere without cells for 24 h were used in ratio 1:1 as vehicle conditions for control cells. LS14 fresh medium (DMEM:F-12) was used in preliminary experiments as a second control to evaluate possible effects of the RPMI:DMEM:F-12 medium mixture and no effects were observed (not shown). At experiment conclusion, LS14 preadipocytes were lysed to analyze proinflammatory cytokine gene expression.

2.2. ASC silencing

ASC-specific and non-targeting control small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The siRNAs were transfected at a final concentration of 40 nM. THP-1 monocytes were pelleted at 150 g for 5 min, washed and resuspended in PBS. Transfection with siRNAs was performed by electroporation using the Gene Pulser Xcell System (BioRad, Hercules, CA, USA) with a pulse of 90 V and 950 μF capacitance. Cells were then resuspended in warm culture medium with 10% FBS, seeded, and allowed to grow overnight. THP-1 monocytes were then washed and 100 nM PMA was used for 24 h to induce differentiation into macrophages. After differentiation, the cells were washed and treated as described above.

2.3. Isolation of total RNA, Reverse Transcription, and qPCR analysis

Cultured cells were lysed with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and RNA was extracted using the E.Z.N.A.®Total RNA Kit I (OMEGA Bio-tek, Norcoss, GA, USA) according to the manufacturer's indications. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). mRNA expression was evaluated using the Step-one Real-time PCR System using the SYBR® FAST qPCR Kit (Applied Biosystems). The thermal cycling conditions consisted of a 20 s pre-incubation at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The results were normalized by the reference gene GAPDH and the expression was calculated using the Pfaffl model (Pfaffl, 2001). Sequences for PCR primers are shown in Supplementary Table S1.

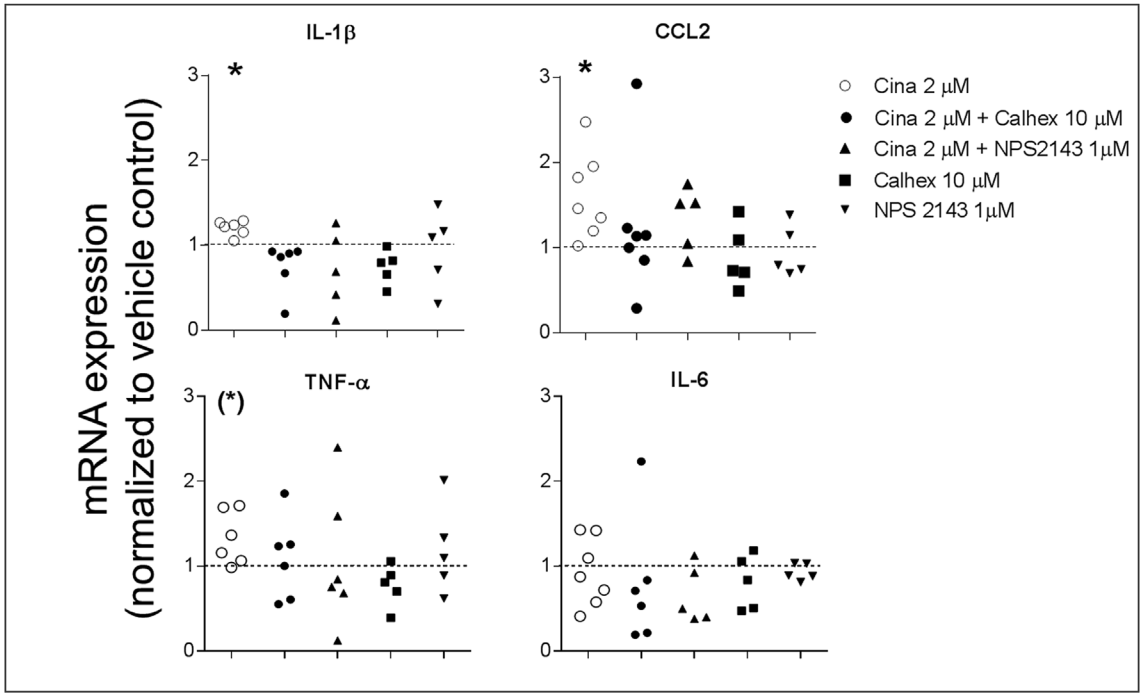

Fig. 1. CaSR stimulation in LPS-treated THP-1 macrophages increases mRNA expression of proinflammatory cytokines. LPS-stimulated (100 ng/ml, 3 h) THP-1 macrophages were exposed to cinacalcet (cina) 2 μM for 4 h after pre-treatment with vehicle, 10 μM calhex 231 or 1 μM NPS 2143 (all in 1 mM final Ca^{2+}concentration) for 40 min. mRNA expression of *IL-1β, CCL2, TNF-α* and *IL-6* was evaluated by qPCR (n = 6), using each sample's GAPDH expression as the internal reference gene, and reported as fold of control untreated cells (value of 1, represented by the dotted line). Dots represent each independent experiment. *P < 0.05, (*) = 0.06 for the difference versus control (untreated) value 1, Wilcoxon Signed Rank Test.

2.4. Western blot

THP-1 cells were homogenized at 4 °C in lysis buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM MgCl2, 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, Basilea, Switzerland). Protein concentration of the lysate was determined by a method based on bicinchoninic acid (Pierce, Rockford, IL, USA). 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 15% polyacrylamide gels and electrotransfered to polyvinylidene difluoride (PVDF) membranes using a buffer containing 24 mM Tris, 194 mM glycine and 20% methanol. The immunoreaction was achieved by incubation of the membranes, previously blocked with a 5% BSA solution in Tris-buffered saline (TBS) with 0.05% Tween 20 (Sigma) with anti CaSR (Santa Cruz Biotechnology Cat. #47741, RRID:AB_626774), anti-IL-1β (Santa Cruz Biotechnology Cat# sc-7884, RRID:AB_2124476) and anti-caspase-1 (Abcam Cat# ab108362, RRID:AB_10858984) antibodies. β-Actin (AC:15) (Santa Cruz Biotechnology Cat# sc-69879, RRID:AB_1119529) was used as internal control. Detection of immune complexes was performed by incubation with peroxidase-conjugated secondary antibodies, followed by incubation with the substrates of the enzyme using the gel documentation system C-DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA). The images were digitized, and the band densities were quantified using UN-SCAN-IT Graph Digitizer Software (RRID:SCR_013725) (Silk Scientific Inc., USA).

2.5. Caspase-1 activity

Caspase-1 activity was measured in cultured THP-1 cells with Caspase-Glo® 1 Inflammasome Assay (Promega Corporation, Madison, WI, USA) by bioluminescence following the manufacturer's instructions.

2.6. Statistical analysis

To compare parameters between controls and treated cells, variables were evaluated using the non-parametric Wilcoxon signed rank test, Wilcoxon matched-pairs signed rank test, and Kruskal-Wallis with Dunn's post-hoc test, as appropriate. Data are shown as individual experiments in the figures, and a p-value less than 0.05 was considered significant.

3. Results

3.1. PMA and LPS exposure elevate proinflammatory cytokine expression in THP-1 cells

Our first approach was to characterize the expression of CaSR and proinflammatory cytokines as well as NLRP3 inflammasome components upon PMA-differentiation of THP-1 monocytes, with or without LPS (100 ng/ml, 3 h) in our hands. CaSR was expressed in undifferentiated THP1 cells however at a lower level than in PMA-differentiated and LPS-exposed THP1 macrophages (Supplementary Fig. S1). As expected, differentiation of THP-1 monocytes into adherent macrophages elevated *IL-1β* mRNA expression almost 600-fold. Moreover, LPS treatment exacerbated this response an additional 80%, reaching almost 1000 times the monocyte levels (Supplementary Fig. S2). At a lower scale, PMA-differentiation increased mRNA expression of *CCL2* (~12-fold), *TNF-α* (~5-fold) and *IL-6* (~15-fold) compared to undifferentiated THP-1, and LPS treatment consistently elevated this response by 100–200%. LPS treatment did not induce an increase of the mRNA expression of the NLRP3 inflammasome components.

3.2. CaSR stimulation increases inflammatory marker expression and NLRP3 inflammasome activation in LPS-treated THP-1 cells

After confirming the expected increase in mRNA expression of

inflammatory factors in PMA-differentiated THP-1 cells, we studied the effect of CaSR activation with 2 μM cinacalcet (in 1 mM final Ca^{2+}concentration) for 4 h on the expression of proinflammatory cytokines and NLRP3 inflammasome components. Under these conditions, cinacalcet did not affect mRNA expression of the evaluated cytokines (*IL-1β, TNF-α, CCL2* and *IL-6,* Supplementary Fig. S3A) nor the NLRP3 inflammasome markers pro-IL-1β (Supplementary Fig. S3B) or pro-CASP-1 (Supplementary Fig. S3C).

To evaluate the effect of CaSR activation in activated THP-1 macrophages, we pre-exposed the cells to LPS (100 ng/ml, 3 h) before the treatment with the CaSR positive modulator. As depicted in Fig. 1, CaSR stimulation in LPS-treated THP-1 macrophages increased mRNA expression of *IL-1β* and *CCL2* ($p < 0.05$, $n = 7$), with a trend towards the same effect for *TNF-α* ($P = 0.06$, $n = 6$). No effect was observed on IL-6 expression upon CaSR activation. Pre-exposure of LPS-treated THP-1 macrophages to the pharmacological CaSR inhibitors calhex-231 or NPS2143 abolished these effects, confirming the specificity of the involvement of the CaSR. The inhibitors had no effects *per-se* on the expression of these factors in THP-1 macrophages.

To further evaluate the inflammatory effect of CaSR activation on LPS-treated THP-1 macrophages, we analyzed whether cinacalcet treatment affects the expression of the NLRP3 inflammasome components. We observed an increase in the expression of *NLRP3* and *ASC* mRNA ($P < 0.05$) compared with vehicle-exposed cells (dotted line, Fig. 2). Pharmacological inhibition of CaSR abolished these increments. CaSR stimulation did not induce variation of *CASP-1* expression in this model.

We then evaluated the effect of cinacalcet on inflammasome NLRP3 activation in THP-1 cells with or without LPS pre-exposure. As shown in Fig. 3, exposure to the CaSR positive modulator in "naïve" (not LPS-exposed) THP-1 cells did not affect caspase-1 activity (an indicator of NLRP3 inflammasome assembly). However, in cells whose inflammatory response was enhanced by LPS pre-exposure cinacalcet elevated caspase-1 activity by 45% ($P < 0.05$).

To verify the specific role of the NLRP3 inflammasome on the induction of caspase-1 activity by cinacalcet in THP-1 cells, we used siRNA to silence the expression of its key component ASC. A ~60% reduction in ASC mRNA expression after siRNA transfection was confirmed by qPCR (Supplementary Fig. S4). Consistent with our previous

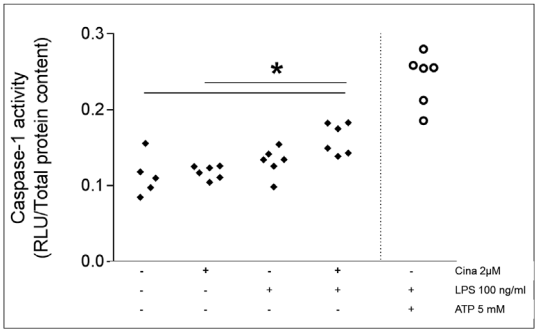

Fig. 3. CaSR stimulation increases caspase-1 activity in LPS-treated THP-1 macrophages. THP-1 macrophages were treated with cinacalcet (cina) 2 μM (in 1 mM final Ca^{2+}concentration) for 4 h with or without LPS pretreatment. Caspase-1 activity was evaluated in cultured cells by a bioluminescence assay. Dots represent each independent experiment ($n = 6$), *$P < 0.05$ for the difference between groups, Kruskal-Wallis with Dunn's post-hoc test. ATP (5 mM, 4 h) was used as positive control for NLRP3 inflammasome activation (open circles, not included in the statistical analysis). RLU = relative luminescence units.

observations, in control silencing experiments (with a random siRNA sequence, siCTL), LPS-treated THP-1 cells exposed to cinacalcet 2 μM, showed an elevation in caspase-1 activity (Fig. 4). This elevation was abolished in cells that were transfected with siRNA against ASC, confirming previous observations (Lee et al., 2012) and thus indicating a key involvement of the NLRP3 inflammasome.

3.3. Effect of cinacalcet-mediated NLRP3 inflammasome activation in THP-1 macrophages on inflammatory marker expression in LS14 preadipocytes

After showing that cinacalcet exposure in LPS-treated THP-1 cells enhances proinflammatory cytokine expression and NLRP3 inflammasome activity, we sought to evaluate whether media conditioned by THP-1 cells under these stimuli exert a proinflammatory effect on other metabolically relevant cell type in obesity, such as preadipocytes, using the human preadipose LS14 cell line. THP-1 cells were exposed to the different experimental conditions and CoM was collected according to the protocol described in materials and methods for 24 h after

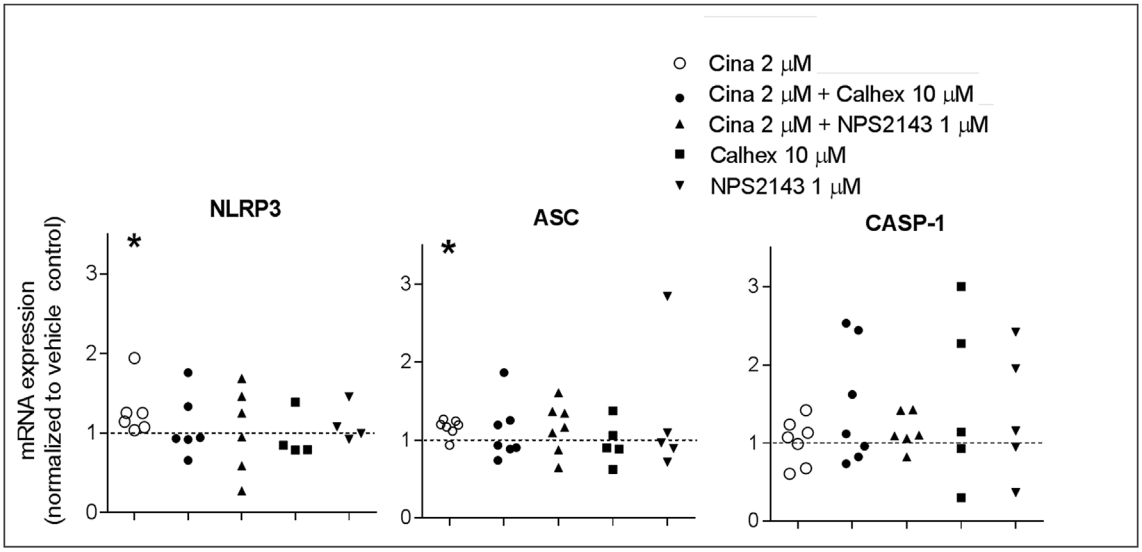

Fig. 2. CaSR stimulation in LPS-treated THP-1 macrophages increase mRNA expression of NLRP3 inflammasome components. THP-1 macrophages were treated with 10 μM calhex 231 or 1 μM NPS 2143 for 40 min before treatment with cinacalcet (cina) 2 μM (1 mM final Ca^{2+}concentration) for 4 h. mRNA expression of *NLRP3, ASC* and *CASP-1* was evaluated by qPCR ($n = 6–7$), using each sample's GAPDH expression as the internal control gene, and reported as fold of untreated cells (value of 1, represented by the dotted line). Dots represent each independent experiment. *$P < 0.05$ for the difference versus control value, Wilcoxon Rank Test.

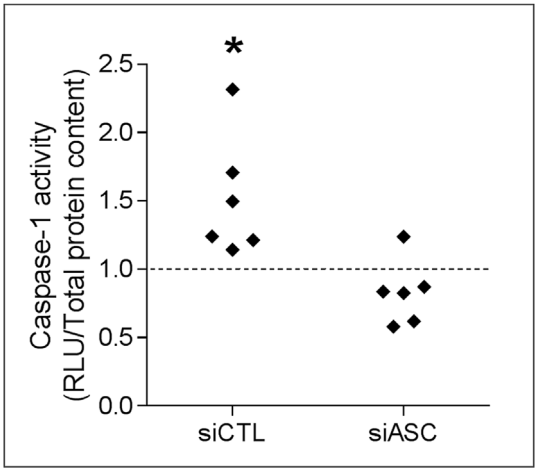

Fig. 4. *Asc* silencing in THP-1 macrophages suppresses the effect of cinacalcet elevating caspase-1 activity. Caspase-1 activity (luminiscence, n = 6) was measured in LPS-activated THP-1 macrophages exposed to cinacalcet (2 μM, 4 h) or vehicle. mRNA silencing of *ASC* (siASC) or control (scrambled sequence, siCTL), was performed 48 h prior to the experiments. Dots represent the cinacalcet/vehicle value for each independent experiment. *P < 0.05 for the differences compared to the respective vehicle-treated cells. (value of 1, dotted line), Wilcoxon signed Rank test. RLU = relative luminescence units.

concluding the treatments. As expected, THP-1 macrophage CoM elevated mRNA expression of proinflammatory cytokines in LS14 preadipocytes (Supplementary Fig. S5). Notably, THP-1 CoM from cinacalcet-exposed THP-1 cells elevated *CCL2*, *IL-6, TNF-α* and *IL-1β* mRNA expression (P < 0.05) beyond the effect of the CoM itself (dotted line, Fig. 5). Interestingly, CoM from THP-1 cells where ASC expression was silenced (siASC, Fig. 5) abolished the effect of cinacalcet, strongly suggesting that cinacalcet-induced production of inflammatory mediators in THP-1 macrophages is mediated by NLRP3 inflammasome activation. As shown in Fig. 6, media that was conditioned by THP-1 cells pre-exposed to the specific CaSR negative allosteric modulator calhex 231 before and during cinacalcet treatment was not able to exert the same proinflammatory expression profile in LS14 cells as compared to CoM prepared by cinacalcet-exposed THP-1 cells, further supporting a role for CaSR activation in THP-1 cells.

4. Discussion

The results from the present work show that exposure of LPS-activated THP-1 macrophages to the CaSR activator cinacalcet induces mRNA expression of the proinflammatory factors IL-1β and CCL2, as well as the NLPR3 inflammasome components NLRP3 and ASC, together with the activation of the NLPR3 inflammasome. Our observations suggest that CaSR activation in THP-1 macrophages induces the secretion of factors that elevate the expression of inflammation markers in LS14 preadipocytes. Interestingly, our results indicate that this process depends on the cinacalcet-induced activation of the NLRP3 inflammasome within the macrophages. The present findings support previous reports of CaSR-induced proinflammatory effects in monocytes, macrophages and THP-1 cells (Xi et al., 2010, G.-S. Lee et al., 2012; Rossol et al., 2012; Liu et al., 2015), however to the best of our knowledge, this is the first report using the calcimimetic cinacalcet.

Consistent with our findings, Lacasa et al. (2007) showed that human primary preadipocytes exposed to medium conditioned by macrophages (*in vitro*-differentiated or isolated from human AT) showed a dysfunctional phenotype, evidenced by a proinflammatory response in addition to impaired adipogenesis (Lacasa et al., 2007). The authors proposed TNF-α as the main macrophage secretion product responsible for the proinflammatory effects in preadipocytes. Our results indicate that the proinflammatory effect of CaSR-stimulated macrophage CoM on LS14 preadipocytes is dependent on CaSR-induced NLRP3 inflammasome activation, whose main secretion product related to obesity pathophysiology is IL-1β (Rheinheimer et al., 2017). It is

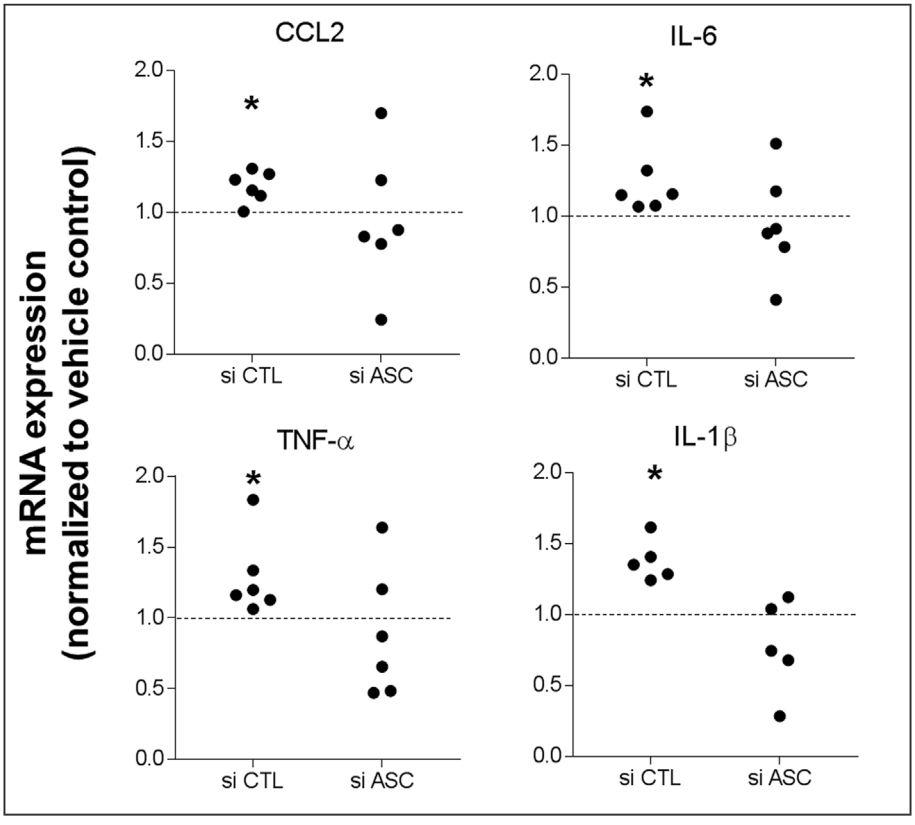

Fig. 5. Conditioned medium (CoM) from cinacalcet-treated THP-1 cells expressing NLRP3 inflammasome elevates proinflammatory cytokine expression in LS14 cells. mRNA expression of *CCL2, IL-1β, TNF-α* and *IL-6* in LS14 preadipocytes exposed for 24 h to CoM from LPS-activated NLRP3 inflammasome-expressing (siCTL) or NLRP3 inflammasome-silenced (siASC) THP-1 macrophages treated with 2 μM cinacalcet (in 1 mM final Ca^{2+} concentration), expressed as fold from the respective vehicle-treated siCTL or siASC THP-1 cells (dotted line, value of 1). mRNA expression in LS14 preadipocytes was evaluated by qPCR using GAPDH as the internal reference gene. Dots represent each independent LS14 CoM exposure experiment (n = 5–6). *P < 0.05 for the differences vs. exposure to vehicle-treated THP-1 CoM (value 1), Wilcoxon Signed Rank Test.

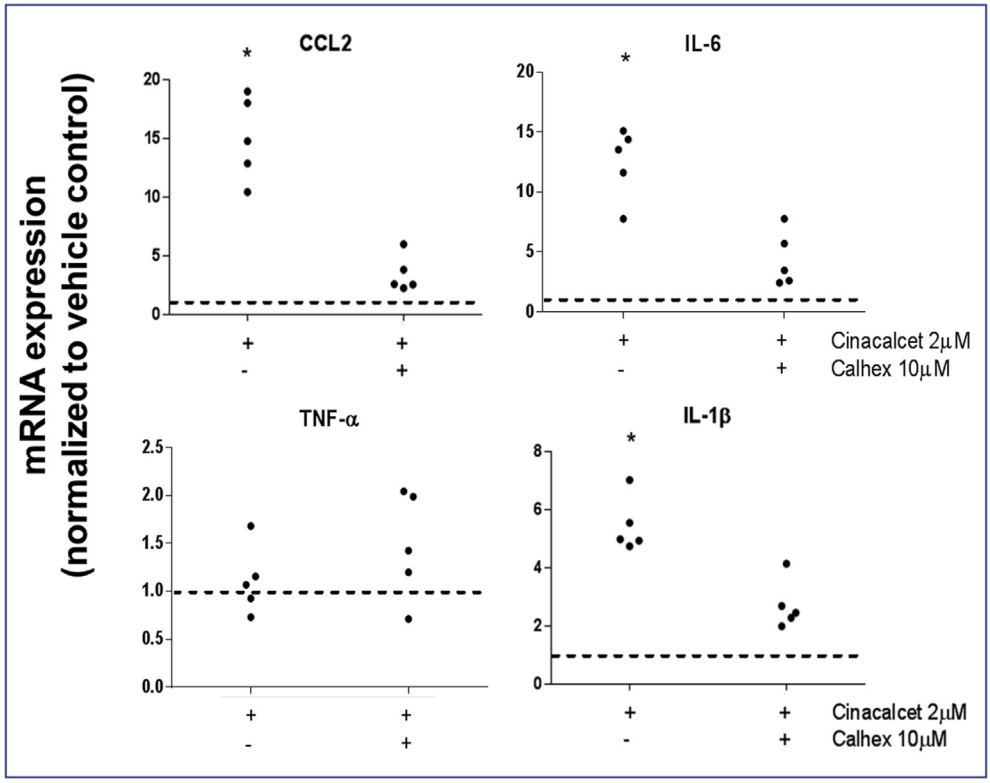

Fig. 6. Exposure of THP-1 cells to the negative allosteric CaSR modulator Calhex 231 abolishes the effect of the CoM elevating proinflammatory cytokine expression in LS14 preadipocytes. mRNA expression of *CCL2, IL-6, TNF-α* and *IL-1β* in LS14 preadipocytes exposed for 24 h to CoM from LPS-activated THP-1 macrophages previously treated with 2 μM cinacalcet for 4 h (in 1 mM final Ca^{2+} concentration) or cinacalcet in addition to 40 min pre-exposure to the negative alosteric modulator calhex 231 (10 μM). mRNA expression in LS14 preadipocytes was evaluated by qPCR using GAPDH as the internal reference gene. Dots represent each independent LS14 CoM exposure experiment (n = 5). *P < 0.05 denotes the difference from exposure to the non-conditioned RPMI-DMEM:F-12 control mixture (same proportion as for the CoM-exposed cells), represented by the dotted line on value 1, Friedman Test and Dunn's multiple comparison for the three conditions (non-conditioned medium-cinacalcet-cinacalcet + calhex).

possible that this CaSR-induced factor acts in concert with TNF-α in THP-1 CoM, which is consistent with the fact that CaSR stimulation was able to increase the effect of THP-1 CoM on LS14s beyond an already large effect of the CoM from vehicle-treated THP-1 cells.

We and others have described a functional role of the CaSR in adipose cells influencing different aspects of AT physiology and promoting inflammation (Bravo-Sagua et al., 2016; Y. H. He et al., 2012; Y. He et al., 2013). Although we do not yet fully understand the physiological role of CaSR in AT, it may be relevant in the pathophysiological context of obesity. As in other inflammation scenarios, CaSR-induced inflammatory response may initially be part of the homeostatic response to the metabolic challenge of energy surplus. However, upon continuous positive energy balance in obesity, the adaptive capacity is surpassed, leading to deleterious chronic inflammation and tissue dysfunction, resulting in metabolic alterations such as insulin resistance (Caputo et al., 2017). Under dysfunctional AT conditions, inflammation and cell damage or death may induce high local extracellular calcium concentrations (Colella et al., 2016; Kaslick et al., 1973), which may exacerbate CaSR activation. Other endogenous CaSR agonists are likely elevated in obese states, such as polyamines (Codoñer-Franch et al., 2011), making CaSR a suitable novel target in obesity-related AT inflammation and dysfunction.

Negative allosteric modulators of the CaSR (calcilytics) have shown *in vitro* to be promising agents to treat inflammatory-related diseases (J. W. Lee et al., 2017; Zeng et al., 2016), and recent studies have highlighted their potential as therapeutic tools in allergic asthma (Penn, 2015; Yarova et al., 2015). Riccardi and colleagues have proposed that endogenous CaSR agonists such as polycations, whose levels are increased with inflammation and airway hyper-responsiveness, may be activating the CaSR and thus triggering airway hyper-responsiveness and inflammation (Yarova et al., 2015). Calcilytics were initially developed for osteoporosis therapy, however they failed in clinical trials due to lack of efficacy. These trials showed that calcilytics are safe and well tolerated, and they are currently being considered for other purposes such as hypocalcemic conditions, asthma and pulmonary arterial hypertension (Nemeth et al., 2018). Our work provides the rationale to consider an additional potential application for calcilytics in the context of the low-grade inflammation characteristic of AT dysfunction, thus representing a tool to prevent the ensuing obesity-related cardiometabolic disorders.

The present work evaluated NLRP3 activation upon CaSR stimulation in THP-1 cells exposed to LPS in order to enhance their inflammatory phenotype. Our experiments aimed to model obesity conditions, where activated proinflammatory ("M1") macrophages infiltrate AT (Guzik et al., 2017). Under these circumstances, the CaSR activator cinacalcet induced an increase in THP-1 inflammatory marker expression and inflammasome activation beyond the effect of LPS stimulation. In addition, our data indicate that CaSR activation in THP-1 cells is able to elevate the ability of their CoM to induce a proinflammatory phenotype in preadipose cells. Previous studies have reported that macrophage CoM prevents adipogenesis and elevates preadipocyte proliferation (Sorisky et al., 2013). Even though the present study did not evaluate these parameters, a putative scenario of lower adipogenesis and higher proliferation, would yield an excess proinflammatory preadipocyte population within AT, thus aggravating the inflammatory condition. This evidence, together with our previously-reported direct effect of CaSR activators elevating the chemokine CCL2 in LS14 and human primary preadipocytes (both of which express the CaSR) (Cifuentes et al., 2012; Rocha et al., 2015), which is expected to enhance macrophage recruitment within dysfunctional AT, suggests that in the pathophysiological condition within obese AT, CaSR activation may induce an additional detrimental macrophage-preadipocyte crosstalk.

Overall, the CaSR-dependent NLRP3 inflammasome activation in human THP-1 macrophages shown in the present work, together with the same effect previously shown in LS14 preadipocytes (D'Espessailles et al., 2018) represent a new model whereby CaSR activation participates in the development of a positive feedback loop and paracrine crosstalk that perpetuates AT inflammation and dysfunction, consistent with our proposed model of CaSR involvement in metabolic events associated with obesity (Bravo-Sagua et al., 2016). Modulating CaSR activation in AT inflammation may be a promising new approach in

preventing obesity-related metabolic disorders associated with AT dysfunction.

Declaration of competing interest

There are no conflicts of interest for any of the authors.

Acknowledgements

This work was supported by FONDECYT grant 1150651 (to MC) and CONICYT grants 21140284 (to AD) and 22170739 (to NS).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.110654.

References

Avila-George, K., Ramos-Olivares, K., Vasquez-Munoz, K., Villanueva-Morales, V., Reyes-Farias, M., Quintero, P., et al., 2017. Chemically induced hypoxia promotes differential outcomes over preadipocyte– or adipocyte–macrophage communication. Arch. Physiol. Biochem. 123 (3), 175–181. https://doi.org/10.1080/13813455.2017.1285318.

Bravo-Sagua, R., Mattar, P., Díaz, X., Lavandero, S., Cifuentes, M., 2016. Calcium sensing receptor as a novel mediator of adipose tissue dysfunction: mechanisms and potential clinical implications. Front. Physiol. https://doi.org/10.3389/fphys.2016.00395.

Brown, E.M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., et al., 1993. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. Nature 366 (6455), 575–580. https://doi.org/10.1038/366575a0.

Caputo, T., Gilardi, F., Desvergne, B., 2017. From chronic overnutrition to metaflammation and insulin resistance: adipose tissue and liver contributions. FEBS (Fed. Eur. Biochem. Soc.) Lett. https://doi.org/10.1002/1873-3468.12742.

Cifuentes, M., Albala, C., Rojas, C., 2005. Calcium-sensing receptor expression in human adipocytes. Endocrinology 146 (5), 2176–2179. https://doi.org/10.1210/en.2004-1281.

Cifuentes, M., Fuentes, C., Tobar, N., Acevedo, I., Villalobos, E., Hugo, E., et al., 2012. Calcium sensing receptor activation elevates proinflammatory factor expression in human adipose cells and adipose tissue. Mol. Cell. Endocrinol. 361 (1–2), 24–30. https://doi.org/10.1016/j.mce.2012.03.006.

Codoñer-Franch, P., Tavaŕez-Alonso, S., Murria-Estal, R., Herrera-Martiń, G., Alonso-Iglesias, E., 2011. Polyamines are increased in obese children and are related to markers of oxidative/nitrosative stress and angiogenesis. J. Clin. Endocrinol. Metab. 96 (9), 2821–2825. https://doi.org/10.1210/jc.2011-0531.

Colella, M., Gerbino, A., Hofer, A.M., Curci, S., 2016. Recent advances in understanding the extracellular calcium-sensing receptor. F1000Res. 5 (2535). https://doi.org/10.12688/f1000research.8963.1.

D'Espessailles, A., Mora, Y.A., Fuentes, C., Cifuentes, M., 2018. Calcium-sensing receptor activates the NLRP3 inflammasome in LS14 preadipocytes mediated by ERK1/2 signaling. J. Cell. Physiol. 233 (8), 6232–6240. https://doi.org/10.1002/jcp.26490.

Díaz-Soto, G., Rocher, A., García-Rodríguez, C., Núñez, L., Villalobos, C., 2016. The calcium-sensing receptor in health and disease. Int. Rev. Cell Mol. Biol. 327, 321–369. https://doi.org/10.1016/bs.ircmb.2016.05.004.

Gerner, R.R., Wieser, V., Moschen, A.R., Tilg, H., 2013. Metabolic inflammation: role of cytokines in the crosstalk between adipose tissue and liver. Can. J. Physiol. Pharmacol. 91 (11), 867–872. https://doi.org/10.1139/cjpp-2013-0050.

Guzik, T.J., Skiba, D.S., Touyz, R.M., Harrison, D.G., 2017. The role of infiltrating immune cells in dysfunctional adipose tissue. Cardiovasc. Res. https://doi.org/10.1093/cvr/cvx108.

He, Y.H., He, Y., Liao, X.L., Niu, Y.C., Wang, G., Zhao, C., et al., 2012. The calcium-sensing receptor promotes adipocyte differentiation and adipogenesis through PPARγ pathway. Mol. Cell. Biochem. 361 (1–2), 321–328. https://doi.org/10.1007/s11010-011-1118-5.

He, Y., Perry, B., Bi, M., Sun, H., Zhao, T., Li, Y., Sun, C., 2013. Allosteric regulation of the calcium-sensing receptor in obese individuals. Int. J. Mol. Med. 32 (2), 511–518. https://doi.org/10.3892/ijmm.2013.1408.

Hendy, G.N., Canaff, L., 2016. Calcium-sensing receptor, proinflammatory cytokines and calcium homeostasis. Semin. Cell Dev. Biol. https://doi.org/10.1016/j.semcdb.2015.11.006.

Hugo, E.R., Brandebourg, T.D., Comstock, C.E.S., Gersin, K.S., Sussman, J.J., Ben-Jonathan, N., 2006. LS14: a novel human adipocyte cell line that produces prolactin. Endocrinology 147 (1), 306–313. https://doi.org/10.1210/en.2005-0989.

Kaslick, R.S., Mandel, I.D., Chasens, A.I., Lazzara, R., Egitto, J., Weinberg, S., 1973. Quantitative analysis of inorganic phosphorus and magnesium in gingival fluid. J. Dent. Res. 52 (1), 180. https://doi.org/10.1177/00220345730520011801.

Keophiphath, M., Achard, V., Henegar, C., Rouault, C., Clément, K., Lacasa, D., 2009. Macrophage-secreted factors promote a profibrotic phenotype in human preadipocytes. Mol. Endocrinol. 23 (1), 11–24. https://doi.org/10.1210/me.2008-0183.

Kusminski, C.M., Bickel, P.E., Scherer, P.E., 2016. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat. Rev. Drug Discov. 15 (9), 639–660. https://doi.org/10.1038/nrd.2016.75.

Lacasa, D., Taleb, S., Keophiphath, M., Miranville, A., Clement, K., 2007. Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. Endocrinology 148 (2), 868–877. https://doi.org/10.1210/en.2006-0687.

Lee, G.-S., Subramanian, N., Kim, A.I., Aksentijevich, I., Goldbach-Mansky, R., Sacks, D.B., et al., 2012. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP. Nature 492 (7427), 123–127. https://doi.org/10.1038/nature11588.

Lee, J.W., Park, H.A., Kwon, O.K., Park, J.W., Lee, G., Lee, H.J., et al., 2017. NPS 2143, a selective calcium-sensing receptor antagonist inhibits lipopolysaccharide-induced pulmonary inflammation. Mol. Immunol. 90, 150–157. https://doi.org/10.1016/j.molimm.2017.07.012.

Liu, W., Zhang, X., Zhao, M., Zhang, X., Chi, J., Liu, Y., et al., 2015. Activation in M1 but not M2 macrophages contributes to cardiac remodeling after myocardial infarction in rats: a critical role of the calcium sensing receptor/NRLP3 inflammasome. Cell. Physiol. Biochem. 35 (6), 2483–2500. https://doi.org/10.1159/000374048.

Nemeth, E.F., Van Wagenen, B.C., Balandrin, M.F., 2018. Discovery and development of calcimimetic and calcilytic compounds. Prog. Med. Chem. 57, 1–86. https://doi.org/10.1016/bs.pmch.2017.12.001.

Olszak, I.T., Poznansky, M.C., Evans, R.H., Olson, D., Kos, C., Pollak, M.R., et al., 2000. Extracellular calcium elicits a chemokinetic response from monocytes in vitro and in vivo. J. Clin. Investig. 105 (9), 1299–1305. https://doi.org/10.1172/JCI9799.

Penn, R.B., 2015. Calcilytics for asthma relief. Science. https://doi.org/10.1126/science.aab2173.

Permana, P.A., Menge, C., Reaven, P.D., 2006. Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. Biochem. Biophys. Res. Commun. 341 (2), 507–514. https://doi.org/10.1016/j.bbrc.2006.01.012.

Peshdary, V., Gagnon, A.M., Sorisky, A., 2016. Effect of high glucose concentration on human preadipocytes and their response to macrophage-conditioned medium. Can. J. Diabetes 40 (5), 411–418. https://doi.org/10.1016/j.jcjd.2016.02.012.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45. https://doi.org/10.1093/nar/29.9.e45.

Reynolds, C.M., Mcgillicuddy, F.C., Harford, K.A., Finucane, O.M., Mills, K.H.G., Roche, H.M., 2012. Dietary saturated fatty acids prime the NLRP3 inflammasome via TLR4 in dendritic cells-implications for diet-induced insulin resistance. Mol. Nutr. Food Res. 56 (8), 1212–1222. https://doi.org/10.1002/mnfr.201200058.

Rheinheimer, J., de Souza, B.M., Cardoso, N.S., Bauer, A.C., Crispim, D., 2017. Current role of the NLRP3 inflammasome on obesity and insulin resistance: a systematic review. Metab. Clin. Exp. https://doi.org/10.1016/j.metabol.2017.06.002.

Riccardi, D., Kemp, P.J., 2012. The calcium-sensing receptor beyond extracellular calcium homeostasis: conception, development, adult physiology, and disease. Annu. Rev. Physiol. 74 (1), 271–297. https://doi.org/10.1146/annurev-physiol-020911-153318.

Rocha, G., Villalobos, E., Fuentes, C., Villarroel, P., Reyes, M., Díaz, X., et al., 2015. Preadipocyte proliferation is elevated by calcium sensing receptor activation. Mol. Cell. Endocrinol. 412, 251–256. https://doi.org/10.1016/j.mce.2015.05.011.

Rossol, M., Pierer, M., Raulien, N., Quandt, D., Meusch, U., Rothe, K., et al., 2012. Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. Nat. Commun. 3 (1329). https://doi.org/10.1038/ncomms2339.

Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell. https://doi.org/10.1016/j.cell.2010.01.040.

Sorisky, A., Molgat, A.S.D., Gagnon, A., 2013. Macrophage-induced adipose tissue dysfunction and the preadipocyte: should I stay (and differentiate) or should I go? Adv. Nutr.: Int. Rev. J. 4 (1), 67–75. https://doi.org/10.3945/an.112.003020.

Stienstra, R., van Diepen, J.A., Tack, C.J., Zaki, M.H., van de Veerdonk, F.L., Perera, D., et al., 2011. Inflammasome is a central player in the induction of obesity and insulin resistance. Proc. Natl. Acad. Sci. 108 (37), 15324–15329. https://doi.org/10.1073/pnas.1100255108.

Vegiopoulos, A., Rohm, M., Herzig, S., 2017. Adipose tissue: between the extremes. EMBO J. 36 (14), 1999–2017. https://doi.org/10.15252/embj.201696206.

Ward, B.K., Magno, A.L., Walsh, J.P., Ratajczak, T., 2012. The role of the calcium-sensing receptor in human disease. Clin. Biochem. https://doi.org/10.1016/j.clinbiochem.2012.03.034.

Wellendorph, P., Johansen, L.D., Bräuner-Osborne, H., 2010. The emerging role of promiscuous 7TM receptors as chemosensors for food intake. Vitam. Horm. 84. https://doi.org/10.1016/B978-0-12-381517-0.00005-9.

Xi, Y.H., Li, H.Z., Zhang, W.H., Wang, L.N., Zhang, L., Lin, Y., et al., 2010. The functional expression of calcium-sensing receptor in the differentiated THP-1 cells. Mol. Cell. Biochem. 342 (1–2), 233–240. https://doi.org/10.1007/s11010-010-0489-3.

Yarova, P.L., Stewart, A.L., Sathish, V., Britt, R.D., Thompson, M.A., Lowe, A.P.P., et al., 2015. Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. Sci. Transl. Med. 7 (284). https://doi.org/10.1126/scitranslmed.aaa0282. 284ra58.

Zeng, J.Y., Du, J.J., Pan, Y., Wu, J., Bi, H.L., Cui, B.H., et al., 2016. Calcium-sensing receptor in human peripheral blood t lymphocytes is involved in the ami onset and progression through the nf-κb signaling pathway. Int. J. Mol. Sci. 17 (9). https://doi.org/10.3390/ijms17091397.

Zhou, R., Tardivel, A., Thorens, B., Choi, I., Tschopp, J., 2010a. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat. Immunol. 11 (2), 136–140. https://doi.org/10.1038/ni.1831.

Zhou, R., Yazdi, A.S., Menu, P., Tschopp, J., 2010b. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225. https://doi.org/10.1038/nature09663.