Received: 13 November 2017 | Accepted: 17 January 2018
DOI: 10.1002/jcp.26490

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Calcium-sensing receptor activates the NLRP3 inflammasome in LS14 preadipocytes mediated by ERK1/2 signaling

Amanda D'Espessailles | Yuly A. Mora | Cecilia Fuentes | Mariana Cifuentes

Institute of Nutrition and Food Technology (INTA), Universidad de Chile, Macul, Santiago, Chile

Correspondence
Mariana Cifuentes, Institute of Nutrition and Food Technology (INTA), Universidad de Chile, El Líbano 5524, Macul, Casilla, 138–11, Santiago-Chile.
E-mail address: mcifuentes@inta.uchile.cl

Funding information
Fondo Nacional de Desarrollo Científico y Tecnológico (Chile), Grant number: 1150651; Comisión Nacional de Investigación Científica y Tecnológica (Chile), Grant number: 2114028

The study of the mechanisms that trigger inflammation in adipose tissue is key to understanding and preventing the cardiometabolic consequences of obesity. We have proposed a model where activation of the G protein-coupled calcium sensing receptor (CaSR) leads to inflammation and dysfunction in adipose cells. Upon activation, CaSR can mediate the expression and secretion of proinflammatory factors in human preadipocytes, adipocytes, and adipose tissue explants. One possible pathway involved in CaSR-induced inflammation is the activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome, that promotes maturation and secretion of interleukin (IL)-1β. The present work aimed to study whether CaSR mediates the activation of NLRP3 inflammasome in the human adipose cell model LS14. We assessed NLRP3 inflammasome priming and assembly after cinacalcet-induced CaSR activation and evaluated if this activation is mediated by downstream ERK1/2 signaling in LS14 preadipocytes. Exposure to 2 μM cinacalcet elevated mRNA expression of NLRP3, CASP-1, and IL-1β, as well as an increase in pro-IL-1β protein. In addition, CaSR activation triggered NLRP3 inflammasome assembly, as evidenced by a 25% increase in caspase-1 activity and 63% IL-1β secretion. CaSR silencing (siRNA) abolished the effect. Upstream ERK pathway inhibition decreased cinacalcet-dependent activation of NLRP3 inflammasome. We propose CaSR-dependent NLRP3 inflammasome activation in preadipocytes through ERK signaling as a novel mechanism for the development of adipose dysfunction, that may favor the cardiovascular and metabolic consequences of obesity. To the best of our knowledge, this is the first report linking the inflammatory effect of CaSR to NLRP3 inflammasome induction in adipose cells.

KEYWORDS
calcium sensing receptor, inflammation, NLRP3 inflammasome, obesity, preadipocyte

1 | INTRODUCTION

Obesity is a major worldwide health problem due to its association with insulin resistance, type 2 diabetes mellitus and cardiovascular disease. Adipose tissue expansion can be accompanied by macrophage infiltration and an elevation in proinflammatory factors, resulting in local metabolic dysfunction with consequences at a whole-body level (Kusminski, Bickel, & Scherer, 2016). It is now accepted that while excess body fat defines obesity, it is adipose tissue quality and functionality that defines the development of obesity-related metabolic disorders (Vegiopoulos, Rohm, & Herzig, 2017). Adipose tissue dysfunction leads to an altered secretory profile of adipose cells favoring proinflammatory adipokine production, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL6,

and chemokines (such as chemokine ligand 2, CCL2) (Kusminski et al., 2016; Saltiel & Olefsky, 2017).

The study of the mechanisms that trigger inflammation and dysfunction in adipose tissue is key to understanding and preventing the cardiometabolic consequences of obesity. Our laboratory has proposed a model where activation of the G protein-coupled calcium sensing receptor (CaSR) leads to dysfunction in adipose cells (Bravo-Sagua, Mattar, Díaz, Lavandero, & Cifuentes, 2016). The CaSR was first described in 1993 as a regulator of parathyroid hormone secretion and calcium homeostasis (Brown et al., 1993), and its presence has been described in several organs and associated with diverse functions (Ward, Magno, Walsh, & Ratajczak, 2012).

Our studies have shown that upon activation, CaSR can mediate the expression and secretion of several proinflammatory factors in both human primary adipose cells and in the human cell line LS14 (Cifuentes et al., 2012; Rocha et al., 2015). The molecular signaling triggered after CaSR activation is complex and vast. It includes, among other pathways, inhibition of adenylate cyclase, activation of P38 mitogen-activated protein kinase, c-Jun N-terminal kinase, phosphatidylinositol three kinase, extracellular-signal-regulated kinases (ERK) 1/2, and intracellular Ca^{2+} mobilization (Chakravarti, Chattopadhyay, & Brown, 2012). One of the mechanisms proposed to mediate the CaSR actions in adipose cells is through ERK 1/2 signaling (Rocha et al., 2015).

An additional possible pathway involved in CaSR-induced inflammation in adipose tissue is the activation of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome. The inflammasomes are high-molecular weight signaling platforms assembled in response to numerous exogenous and endogenous signals. Upon assembly, they activate the cysteine protease caspase-1 and control maturation and secretion of interleukins such as IL-1β and IL-18, whose potent proinflammatory activities direct host responses to infection and injury (Schroder & Tschopp, 2010). The best characterized inflammasome at present is NLRP3, and its activation has been associated with obesity-related chronic inflammation, insulin resistance and diabetes (Rheinheimer, de Souza, Cardoso, Bauer, & Crispim, 2017; Robbins, Wen, & Ting, 2014). The formation of this large (>700 kDa) multiprotein cytoplasmic complex involves several cellular proteins. The Nod-like receptor protein 3 (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 triggers the activation of caspase-1 and subsequent conversion of pro-IL-1β and pro-IL-18 into their mature secreted forms. The canonical activation of the NLRP3 requires a priming process, typically induced by the binding of lipopolysaccharide (LPS) to TLR4, that increases mRNA expression of *NLRP3* and *IL-1β* though NF-κB signaling (Bauernfeind et al., 2009; Stienstra et al., 2011). Once primed, the NLRP3 can respond to a second stimuli and assemble to initiate the proteolytic activation of procaspase-1 (Guo, Callaway, & Ting, 2015). Recent work also suggests that ERK phosphorylation is required for NLRP3 inflammasome activation in different cell models (Ghonime et al., 2014; Mezzasoma, Antognelli, & Talesa, 2017). The NLRP3 inflammasome responds to several metabolic stress signals observed in obesity such high glucose (Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010), saturated fatty acid levels (Reynolds et al., 2012) and oxidative stress (Zhou, Yazdi, Menu, & Tschopp, 2010). Accordingly, the NLRP3 pathway has been strongly associated with the pathophysiology of obesity-related inflammation and diseases such as cardiovascular disease and type 2 diabetes (Patel et al., 2017; Stienstra, Tack, Kanneganti, Joosten, & Netea, 2012).

In 2012, two independent groups reported the activation of NLRP3 inflammasome after CaSR stimulation in murine macrophages (Lee et al., 2012), primary human monocytes and the human monocyte cell line THP-1 (Rossol et al., 2012). These studies described for the first time the association between the CaSR activation and the inflammasome pathway. More recently, 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 cells.

The present work aimed to study whether CaSR stimulation mediates the activation of NLRP3 inflammasome in a human adipose cell model. We assessed in vitro if CaSR activation mediates the priming and assembly of the NLRP3 inflammasome and if this activation is mediated by ERK1/2 signaling in LS14 preadipocytes.

2 | METHODS

2.1 | LS14 cell line culture and differentiation

Our studies used the preadipose cell line LS14 (RRID:CVCL_DH87), derived from a human metastasic liposarcoma, able to differentiate into lipid-laden adipocytes that express mature adipocyte genes (Hugo et al., 2006). Preadipose LS14 cells were seeded on plastic culture dishes (Nunc, Rochester, NY) and grown in DMEM/Ham's F-12 (1:1) medium (Sigma–Aldrich, 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). The medium was replaced every 2–3 days. Cells were stimulated with 2 μM of the CaSR allosteric activator cinacalcet for 16 hr. The calcilytic NPS2143 (1 μM) and the MAPK kinase (MEK)/ERK inhibitor U0126 (10 μM) were used 0,5 hr prior to cinacalcet stimulation.

2.2 | CaSR silencing

LS14 preadipocytes were harvested using 0.25% trypsin, washed and resuspended in PBS. Transfection with small interfering RNAs (siRNAs) was performed by electroporation using the Gene Pulser Xcell System (BioRad, Hercules, CA) with a pulse of 90 V and 950 μF capacitance. Cells were then resuspended in warm culture medium, seeded, and allowed to grow for 2 days before stimulation with 2 μM of cinacalcet for 16 hr. CaSR-specific and non-targeting control siRNAs were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). The siRNAs were transfected at a final concentration of 80 nM. The success of this protocol for CaSR silencing in our lab was previously reported (Rocha et al., 2015).

2.3 | 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 using the high Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). mRNA expression was evaluated using the Step-one Real-time PCR System (Applied Biosystems) 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 housekeeping gene GAPDH and the expression was calculated using the Pfaffl model (Pfaffl, 2001). Sequences for PCR primers are depicted on Table 1.

2.4 | Western blot

LS14 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–Aldrich), 1.5 μM pepstatin A (Sigma–Aldrich) and Complete® protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration of the lysate was determined by a method based on bicinchoninic acid (Pierce, Rockford, IL). Fifty micrograms of protein were heat denatured in SDS-PAGE loading buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, and 40% glycerol, 20% 2-mercaptoethanol). Proteins were electrophoresed on 12% (ERK) and 15% (IL-1β) 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–Aldrich), with an anti p-ERK (E:4) and ERK (K:23) (Santa Cruz Biotechnology) and anti IL-1β (Santa Cruz Biotechnology) antibodies. β-Actin (AC:15) (Santa Cruz Biotechnology) 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 LI-COR C-DiGit Blot Scanner (Lincoln, NE). The images were digitalized, and the band densities were quantified using Image UN-SCAN-IT software (Silk Scientific Inc.).

2.5 | Caspase-1 activity

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

2.6 | IL-1β secretion

IL-1β secreted into the medium was analyzed using the human IL-1 beta/IL-1F2 QuantiGlo ELISA Kit (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions in the conditioned medium of LS14 cultured cells. The medium was conditioned for 16 hr, collected, centrifuged (800*g* × 10 min) and stored at −80 °C.

2.7 | Statistical analysis

To compare parameters between controls and treated cells, variables were evaluated using Wilcoxon signed rank test or Wilcoxon matched-pairs signed rank test as appropriate. Data are shown in the figures as means ± SEM or as individual values, and a *p*-value less than 0.05 is considered significant.

3 | RESULTS

3.1 | CaSR stimulation upregulates the expression and activation of NLRP3 inflammasome in LS14 preadipocytes

The expression (mRNA) of NLRP3 inflammasome components *Nlrp3*, *Asc*, caspase-1 as well as *IL-1β* was evaluated in LS14 cells treated with the CaSR allosteric activator cinacalcet (2 μM) for 16 hr. Exposure to cinacalcet elevated mRNA expression of *NLRP3* (76%, $p < 0.01$), *CASP1* (118%, $p < 0.05$) and *IL-1β* (62%, $p < 0.01$) in preadipocytes (Figure 1a). NLRP3 component *ASC* mRNA expression was not affected by CaSR stimulation. In addition to the increment in mRNA expression, cinacalcet treatment elevated the protein levels of pro-IL-1β by 44% ($p < 0.005$) (Figure 1b). There was a positive correlation between the expression of *IL-1β* and *NLRP3* (Figure 1c). In addition to enhancing the expression of NLRP3 components, CaSR activation was effective to trigger NLRP3 inflammasome assembly, as evidenced by a 25%

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
NLRP3	NM_001079821	TCTGTGAGGGACTCTTGCAC	CAGCAGTGTGACGTGAGGTT
ASC	NM_145182	AGCTCACCGCTAACGTGCT	CGGTGCTGGTCTATAAAGTGC
CASP1	NM_001257118.2	CTGGGACTCTCAGCAGATCA	GCTTGACATTCCCTTCTGAGC
IL-1β	NM_000576	GGACAAGCTGAGGAAGATGC	TCGTTATCCCATGTGTCGAA
IL6	NM_000600	CAATCTGGATTCAATGAGGAGAC	CTCTGGCTTGTTCCTCACTACTC
GAPDH	NM_002046	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT

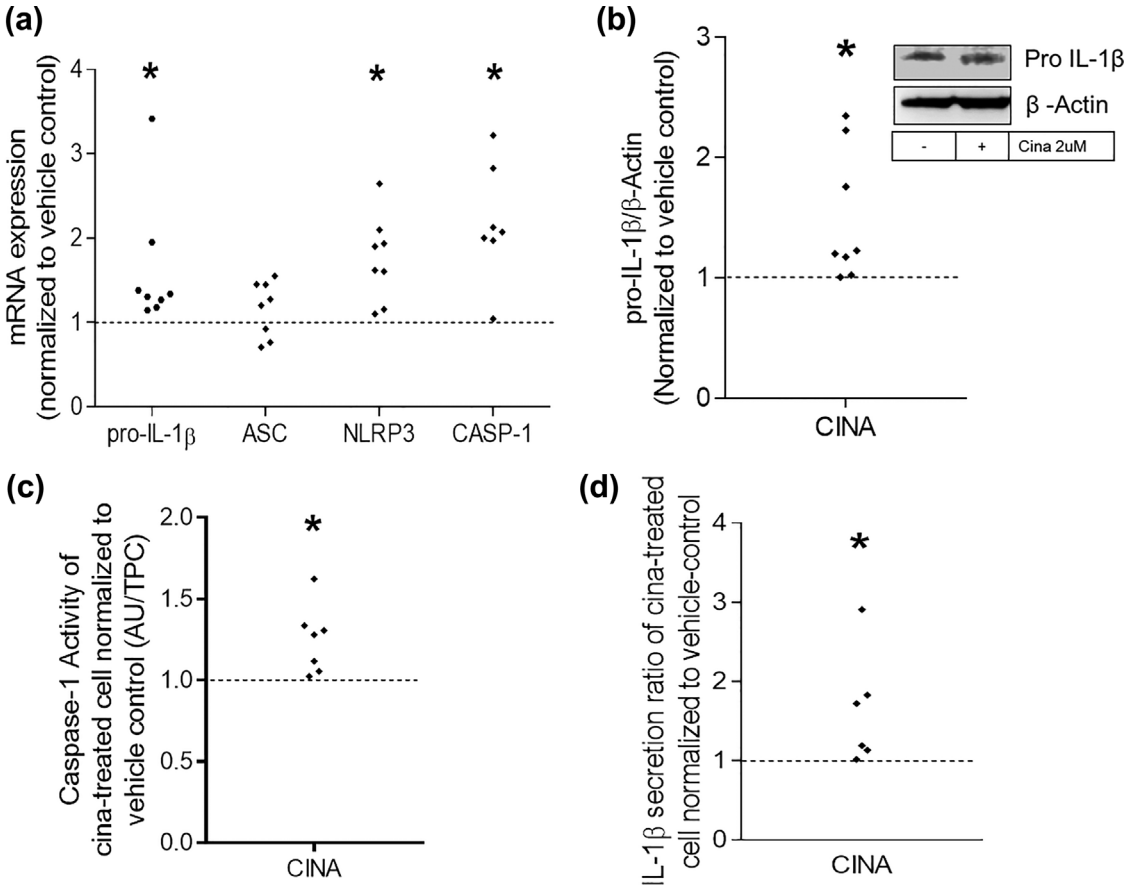

FIGURE 1 Cinacalcet (Cina)-mediated CaSR stimulation increases NLRP3 inflammasome activation. (a) mRNA expression of NLRP3 components in LS14 preadipocytes was evaluated by qPCR, using each sample's GAPDH expression as the internal control gene ($n = 7–8$). (b) pro-IL-1β protein levels in cell lysates of preadipocytes (WB, $n = 9$). (c) Caspase-1 activity in LS14 preadipocytes exposed to cinacalcet for 16 hr ($n = 7$) and (d) IL-1β secretion evaluated in conditioned medium of preadipocytes (ELISA, $n = 6$) exposed for 16 hr to 2 μM cinacalcet. Data are reported as fold of untreated cells (value of 1, represented by the dotted lines) and each dot represents one independent experiment. *$p < 0.05$ for the difference from 1, Wilcoxon Signed Rank Test

increase in caspase-1 activity ($p < 0.05$, Figure 1d) and IL-1β secretion (63%, $p < 0.05$) (Figure 1e).

3.2 | Inhibition and silencing of CaSR abolish cinacalcet-induced NLRP3 inflammasome upregulation

To support the role of CaSR in NLRP3 activation, the calcilytic NPS2143 was used to decrease CaSR signaling upon cinacalcet treatment. NPS2143 pretreatment in our model prevented the increase in mRNA expression of *IL-1β* ($p < 0.05$), NLRP3 ($p < 0.05$) and CASP-1 ($p < 0.05$) after 2 μM cinacalcet treatment for 16 hr. (Figure 2). Consistent with the lack of effect shown in Figure 1a, there were no changes in *ASC* expression.

To further confirm that the observed effects were due to CaSR activation we performed CaSR silencing using siRNA. Expression of our target genes was evaluated after 16 hr exposure to cinacalcet in adipose cells previously transfected with siRNA targeted for the human CaSR (siRNA CaSR) or a scrambled sequence siRNA used as

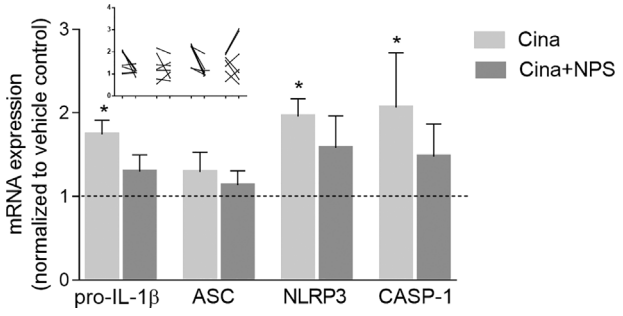

FIGURE 2 Effect of the CaSR antagonist NPS2341 on cinacalcet-induced elevation of NLRP3 components mRNA expression in LS14 preadipocytes. 16-hr Cinacalcet (Cina, 2 μM)-exposed LS14 preadipocytes were pre-exposed (1 hr) to 1 μM NPS2341 or vehicle. The expression of NLRP3, ASC, CASP-1, and IL-1β was evaluated by qPCR 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). Bars represent mean ± SEM for independent experiments ($n = 8$). *$p < 0.05$ for the difference from 1, Wilcoxon Signed Rank Test

control (siRNA CTL). Consistent with our previous results, cells exposed to siRNA CTL and treated with cinacalcet presented an increase in the expression of *IL-1β* ($p < 0.01$), *NLRP3* ($p < 0.01$), and *CASP-1* ($p < 0.05$). As shown in Figure 3a, CaSR silencing prevented this effect. Consistently, the increment observed in caspase-1 activity (23%, $p < 0.05$, Figure 3b) and IL-1β secretion (23%, $p < 0.05$, Figure 3c) was abolished by silencing the CaSR.

3.3 | ERK signaling mediates the CaSR-dependent NLRP3 inflammasome activation in preadipocytes

The MAPK kinase (MEK)/ERK inhibitor, U0126, was used to evaluate whether ERK1/2 phosphorylation mediates the upregulation of NLRP3 observed after CaSR activation. We observed that exposure to cinacalcet induced the phosphorylation of ERK after 16 hr ($p < 0.05$), effect that was reversed by incubation with U0126 0,5 hr before and during cinacalcet treatment (Figure 4a). Under those conditions, U0126 also prevented the elevation of *IL-1β* and *CASP-1* mRNA expression ($p < 0.05$, Figure 4b), pro-IL-1β cell protein levels ($p < 0.05$, Figure 4c) and Caspase-1 activity ($n = 7$, $p < 0.05$) observed with cinacalcet (Figure 4d).

4 | DISCUSSION

Our previous report established that CaSR activation with the calcimimetic cinacalcet enhances the expression and secretion of the cytokine IL-1β in human adipose cells and adipose tissue (Cifuentes et al., 2012; Rocha et al., 2015). Considering that extracellular calcium activates the NLRP3 inflammasome via stimulation of CaSR in monocytes and macrophages (Lee et al., 2012; Liu et al., 2015; Rossol et al., 2012), we evaluated whether the elevation of IL-1β through CaSR stimulation could be mediated by inflammasome activation in the LS14 preadipocyte cell model. We provide evidence showing that cinacalcet-induced CaSR activation in LS14 preadipocytes upregulates the expression of NLRP3 inflammasome components, triggers inflammasome assembly and elevates CASP-1 activity, leading to NLRP3 inflammasome-induced IL-1β secretion.

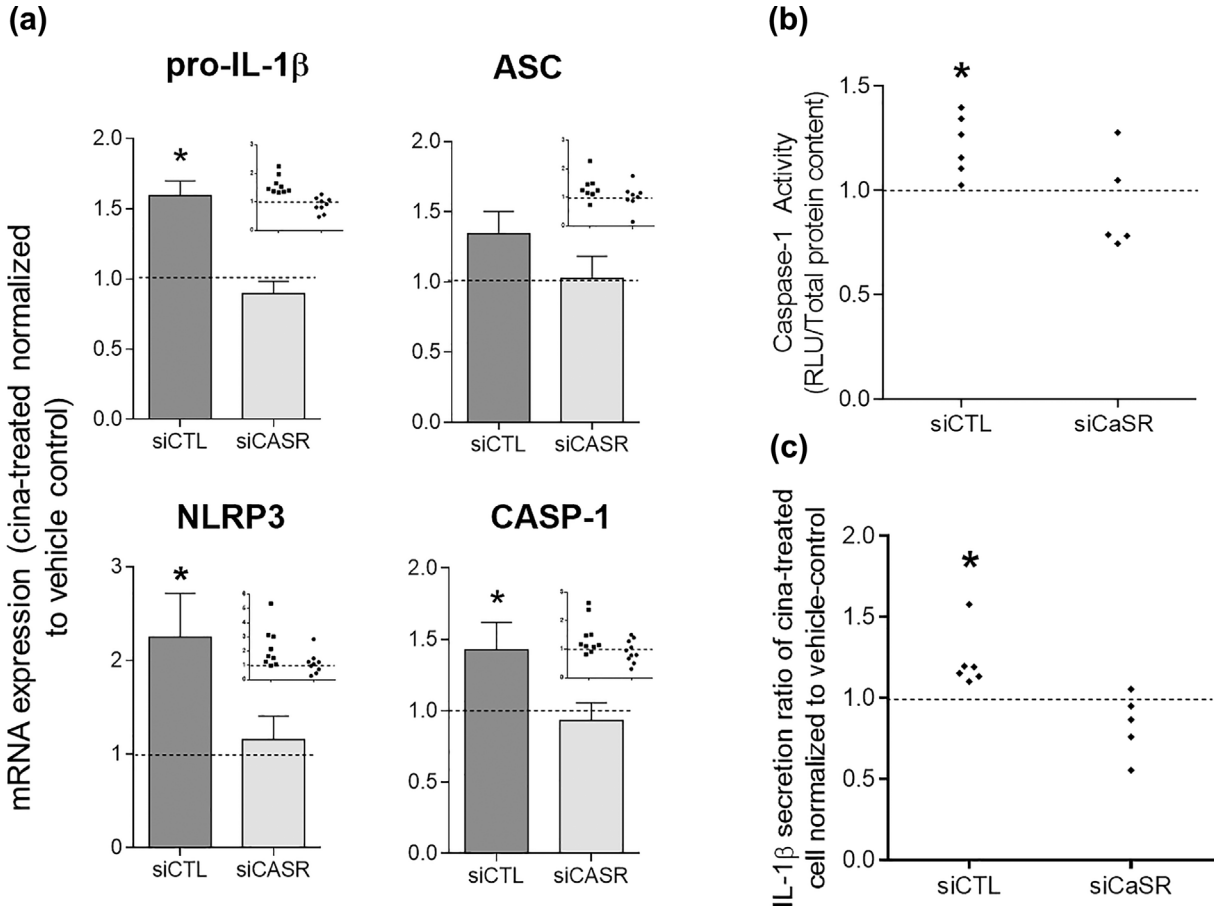

FIGURE 3 CaSR gene silencing abolishes cinacalcet-induced NLRP3 upregulation and activation in LS14 preadipocytes. (a) mRNA expression of NLRP3 components (qPCR) in LS14 preadipocytes subjected to CaSR or control silencing (siRNA) and exposed for 16 hr to 2 μM cinacalcet. Bars represent mean ± SEM for independent experiments ($n = 8–10$). (b) Caspase-1 activity in cultured cells exposed to cinacalcet 2 μM for 16 hr ($n = 6$). (c) IL-1β secretion was evaluated (ELISA) in the medium. Data ($n = 6$) are reported as fold of vehicle treated cells (value of 1, represented by the dotted line). *$p < 0.05$ for the difference from 1, Wilcoxon Signed Rank Test

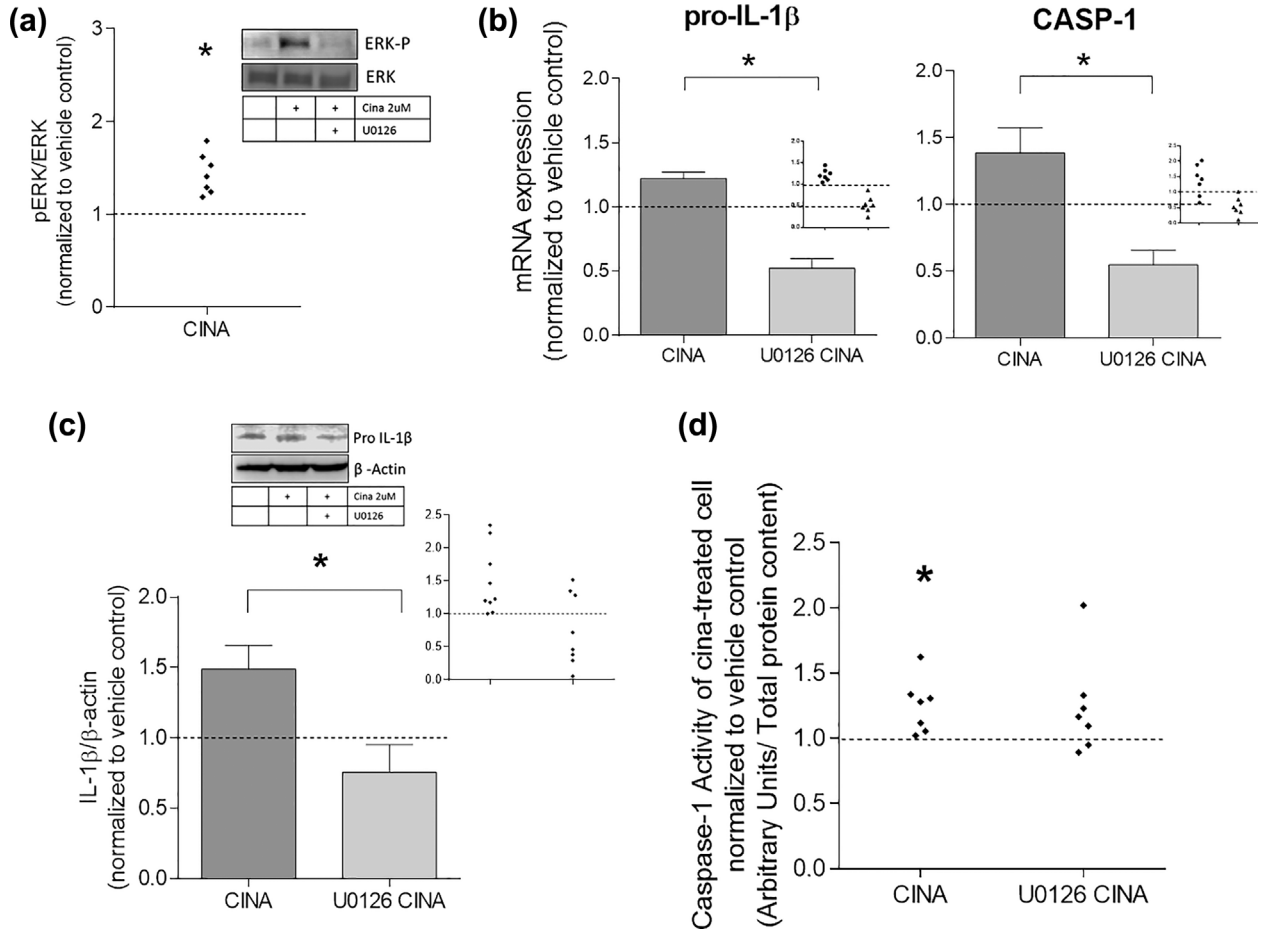

FIGURE 4 The upstream ERK inhibitor U0126 inhibits CaSR-induced NLRP3 activation. LS14 preadipocytes were exposed for 16 hr to 2 μM cinacalcet alone or together with 10 μM U0126 (30 min additional U0126 pre-incubation). (a) pERK/ERK protein in cinacalcet-treated cells, normalized by vehicle-treated controls (value of 1, represented by the dotted line) (n = 4–8, WB). The right panels show a representative image of the blot (top) and a subset of experiments showing the effect of U0126 reverting cinacalcet-induced ERK phosphorylation (bottom). (b) IL-1β and CASP-1 mRNA expression, normalized for GAPDH expression and expressed as fold from vehicle control (value 1, dotted line, n = 7, qPCR). (c) Pro-IL-1β protein levels in cell lysates from cinacalcet and U0126-treated preadipocytes (n = 8, WB) and D. Caspase-1 activity in cultured cells exposed for 16 hr to 2 μM cinacalcet alone or after 1 hr pre-exposure to 10 μM U0126. Bars represent mean ± SEM for independent experiments. *p < 0.05 for the difference between the groups, Wilcoxon matched-pairs signed rank test. β-actin was used as loading control for WB

In our model, 16-hr exposure to cinacalcet increased *IL-1β*, *NLRP3*, and *CASP-1* mRNA expression. In order to confirm the association between NLRP3 priming and IL-1β expression triggered by cinacalcet (as opposed to a general inflammatory response), we evaluated the correlation between the cinacalcet-induced expression of the *NLRP3* gene, with that of *IL-1β* and *IL6*, whose expression has not been linked to NLRP3 inflammasome priming. There was a positive correlation between the up regulation of NLRP3 and the expression of IL-1β, but none with the NLRP3-independent cytokine IL6 (Supplementary Figure S1), suggesting a coordinated and NLRP3-IL1β specific regulation despite other pro-inflammatory pathways triggered by cinacalcet (Cifuentes et al., 2012).

Activation of NLRP3 inflammasome requires two events, the first of which upregulates NLRP3 and pro-IL-1β mRNA expression (priming) (Bauernfeind et al., 2009). This is followed by a second signal that triggers inflammasome assembly and caspase activation, leading to IL-1β maturation and secretion. A previous report of CaSR-dependent NLRP3 activation using $CaCl_2$ in human monocytes, showed that 16 hr exposure to extracellular Ca^{2+} triggered the proteolytic cleavage of pro-IL-1β protein, but had no influence on its mRNA expression (Rossol et al., 2012), which differs from our observations. However, it is widely accepted that the intracellular pathways triggered by CaSR stimulation depend on the cellular type, ligand, and physiological condition, so differences between studies may explain the inconsistent results. Data from a study addressing CaSR biased signaling (Davey et al., 2012) suggest that the effect of cinacalcet at doses similar to those used in our work elicit a higher p-ERK/ERK response as compared to that triggered by Ca^{2+} alone at the concentration used by Rossol et al. (2012). Therefore, it is possible that our CaSR-induced elevation in *IL-1β* expression was due to ERK activation in our model using cinacalcet. Accordingly, upstream inhibition of this pathway with U0126 reverted *IL-1β*

mRNA elevation, which is consistent with other reports (Scherle et al., 1998; Wang, Wu, Huang, & Yang, 2004). An additional possible mechanistic support for the role of CaSR elevating NRLP3 inflammasome activation is provided by evidence that cinacalcet increases the expression and secretion of TNF-α in adipose cells (Cifuentes et al., 2012). It has been demonstrated that activation of the TNF-α receptor by TNF-α can initiate the NLRP3 inflammasome priming process, leading to the upregulation of its components (Bauernfeind et al., 2009).

In the second event of NLRP3 inflammasome activation, the NLRP3 components are assembled and caspase-1 becomes catalytically active, able to cleave pro-IL1β to yield active IL-1β. We here report an increment in both caspase-1 activity and IL-1β secretion, showing in LS14 preadipocytes that cinacalcet not only induces the earliest phase of NLRP3 inflammasome activation, but also its IL-1β endpoint. In agreement with this, we previously showed that 48 hr of CaSR activation increased the secretion of IL-1β in human adipose tissue explants (Cifuentes et al., 2012).

To verify the specific role of the CaSR in our findings, we used two approaches: the pharmacological inhibition with the selective CaSR antagonist NPS 2143 and RNA silencing of the *CaSR* gene. Preincubation with NPS 2143 decreased the effect of cinacalcet on the expression of the inflammasome components. The inhibition was in this case partial, which is consistent with our proposed pathway involving ERK phosphorylation to an important extent. It has been reported that NPS 2143 shows greater negative CaSR modulation of cytosolic Ca^{2+} mobilization than ERK1/2 phosphorylation (Leach et al., 2013, Leach, Sexton, Christopoulos, & Conigrave, 2014). Accordingly, silencing the *CaSR* gene exerted a more extensive inhibition of the cinacalcet-induced up regulation of NRLP3 components, *pro-IL-1β* gene expression, caspase-1 activity and IL-1β secretion. Our observations thus indicate that cinacalcet participates in the NLRP3 inflammasome activation via CaSR stimulation.

As mentioned above, CaSR activation may trigger different intracellular pathways, and the use of a specific ligand in a certain cellular model determines the particular outcome observed (Thomsen, Hvidtfeldt, & Bräuner-Osborne, 2012). Rossol et al. (2012) proposed that the CaSR-mediated NLRP3 activation in monocytes was via phosphatidyl inositol/Ca^{2+} pathway. Lee et al. (2012) also observed that CaSR activates the NLRP3 inflammasome in a murine model by increases in intracellular Ca^{2+}, but they additionally observed a decrease in cellular cyclic AMP, which differed from Rossol et al. (2012) observations. In the LS14 preadipocyte model, we observed that ERK1/2 activation was involved in the cell proliferation effect induced by CaSR stimulation (Rocha et al., 2015), and the present work on NLRP3 activation corroborates ERK1/2 involvement. We observed a 44% increase in ERK phosphorylation after 16 hr of exposure to cinacalcet, an effect that was lost with the pre-incubation with the upstream ERK1/2 phosphorylation inhibitor U0126. Furthermore, inhibition of phospho-ERK pathway abolished the CaSR-dependent upregulation of the expression of NLRP3 components and activation of the inflammasome. These data suggest a key role for cinacalcet-induced ERK1/2 pathway in the activation of NLRP3 in human preadipocytes. In support of the present findings, ERK1 has been described as a key player in the early events associated with NLRP3 inflammasome priming in human macrophages; and U0126 significantly blocked inflammasome priming and activation (Ghonime et al., 2014).

To the best of our knowledge, this is the first report linking the inflammatory effect of CaSR in adipose cells to NLRP3 inflammasome induction. The NLRP3 inflammasome has received considerable attention in the obesity field over the last years. Adipose tissue inflammation is a major culprit for its dysfunction and the development of obesity-related lipotoxicity, insulin resistance, and type 2 diabetes mellitus. A recent systematic review (Rheinheimer et al., 2017), concluded that evidence from most human and mice studies support an association between obesity and NLRP3 expression in adipose tissue, with a relevant role of the ensuing IL-1β production on the development of obesity-related insulin resistance and diabetes. High fat diet-fed mice deficient in NLRP3 inflammasome show improved insulin sensitivity, glucose tolerance, and adipose tissue insulin sensitivity as compared with wild type animals (Wen et al., 2011). It has been observed that a diet rich in saturated fatty acids (the most associated to obesity-related insulin resistance), activates NLRP3 inflammasome in adipose tissue (Finucane et al., 2015). Moreover, a diet rich in monounsaturated fatty acids in these mice did not stimulate inflammasome activation (as evidenced by lower caspase activity and IL-1β production), which was accompanied with an improvement of insulin sensitivity.

The NLRP3 inflammasome has been mostly studied in macrophages, and pathogenic inflammation in adipose tissue is greatly influenced by infiltration and activation of M1 (proinflammatory) macrophages. However, preadipocytes also have an important role in adipose inflammation and express the NLRP3 inflammasome machinery. Preadipocytes from caspase-1 deficient mice show improved ability to differentiate into mature adipocytes (Stienstra et al., 2010), a trait that is considered favorable, as it promotes adipose tissue expansibility through new adipocyte formation. The same effect was observed in preadipocytes isolated from NLRP3-deficient mice, confirming this observation (Stienstra et al., 2010). Moreover, caspase-1 and NLRP3 deficiency in white adipose tissue was associated with improved local insulin sensitivity, accompanied by the expected decrease in IL-1β secretion (Stienstra et al., 2010). The stromal vascular fraction of adipose tissue (particularly visceral), which contains macrophages and up to 50% preadipocytes (Tchkonia et al., 2010) expresses and produces more caspase-1-dependent IL-1β as compared to the fraction containing mature adipocytes (Koenen et al., 2011). Notwithstanding the important expected influence of macrophage NLRP3 activation in adipose stroma vascular fraction, few studies have addressed the relevance of preadipocytes. These cells, and particularly those from visceral origin, have an important inflammatory capacity (Zhu et al., 2015), and we previously reported that CaSR enhances their proliferation (Rocha et al., 2015). The present study suggests that activation of NLRP3 inflammasome by CaSR in preadipocytes may worsen the adipose proinflammatory profile and obesity-related consequences.

Altogether, the inflammasome has been proposed as an important mediator of inflammation and metabolic disease, with a relevant role in dysfunctional fat and the development of the diseases associated with obesity. The CaSR-dependent NLRP3 inflammasome activation in preadipocytes strongly suggests a novel mechanism for CaSR in the development of metabolic events linked with adipose dysfunction and the cardiovascular and metabolic consequences of obesity. Identifying the mechanisms involved in NLRP3 inflammasome activation in obesity is relevant to understand and target the link between obesity and disease. The ability to dissociate obesity from inflamed and dysfunctional adipose tissue may help in preventing the severe consequences of the current obesity pandemic.

ACKNOWLEDGMENT

Funded by FONDECYT grant 1150651 (to MC) and CONICYT grant 21140284 (to AD)

ORCID

Mariana Cifuentes http://orcid.org/0000-0001-9485-3490

REFERENCES

Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., … Latz, E. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *Journal of Immunology*, *183*, 787–791. https://doi.org/10.4049/jimmunol.0901363

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. *Frontiers in Physiology*, *7*, 395. https://doi.org/10.3389/fphys.2016.00395

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

Chakravarti, B., Chattopadhyay, N., & Brown, E. M. (2012). Signaling through the extracellular calcium-sensing receptor (CaSR). *Advances in Experimental Medicine and Biology*, *740*, 103–142. https://doi.org/10.1007/978-94-007-2888-2_5

Cifuentes, M., Fuentes, C., Tobar, N., Acevedo, I., Villalobos, E., Hugo, E., … Reyes, M. (2012). Calcium sensing receptor activation elevates proinflammatory factor expression in human adipose cells and adipose tissue. *Molecular and Cellular Endocrinology*, *361*, 24–30. https://doi.org/10.1016/j.mce.2012.03.006

Davey, A. E., Leach, K., Valant, C., Conigrave, A. D., Sexton, P. M., & Christopoulos, A. (2012). Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor. *Endocrinology*, *153*, 1232–1241. https://doi.org/10.1210/en.2011-1426

Finucane, O. M., Lyons, C. L., Murphy, A. M., Reynolds, C. M., Klinger, R., Healy, N. P., … Roche, H. M. (2015). Monounsaturated fatty acid-enriched high-fat diets impede adipose NLRP3 inflammasome-mediated IL-1β secretion and insulin resistance despite obesity. *Diabetes*, *64*, 2116–2128. https://doi.org/10.2337/db14-1098

Ghonime, M. G., Shamaa, O. R., Das, S., Eldomany, R. A., Fernandes-Alnemri, T., Alnemri, E. S., … Wewers, M. D. (2014). Inflammasome priming by lipopolysaccharide is dependent upon ERK signaling and proteasome function. *Journal of Immunology*, *192*, 3881–3888. https://doi.org/10.4049/jimmunol.1301974

Guo, H., Callaway, J. B., & Ting, J. P. Y. (2015). Inflammasomes: Mechanism of action, role in disease, and therapeutics. *Nature Medicine*, *21*, 677–687. https://doi.org/10.1038/nm.3893

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*, 306–313. https://doi.org/10.1210/en.2005-0989

Koenen, T. B., Stienstra, R., Van Tits, L. J., Joosten, L. A. B., Van Velzen, J. F., Hijmans, A., … De Graaf, J. (2011). The inflammasome and caspase-1 activation: A new mechanism underlying increased inflammatory activity in human visceral adipose tissue. *Endocrinology*, *152*, 3769–3778. https://doi.org/10.1210/en.2010-1480

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

Leach, K., Sexton, P. M., Christopoulos, A., & Conigrave, A. D. (2014). Engendering biased signalling from the calcium-sensing receptor for the pharmacotherapy of diverse disorders. *British Journal of Pharmacology*, *171*, 1142–1155. https://doi.org/10.1111/bph.12420

Leach, K., Wen, A., Cook, A. E., Sexton, P. M., Conigrave, A. D., & Christopoulos, A. (2013). Impact of clinically relevant mutations on the pharmacoregulation and signaling bias of the calcium-sensing receptor by positive and negative allosteric modulators. *Endocrinology*, *154*, 1105–1116. https://doi.org/10.1210/en.2012-1887

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

Liu, W., Zhang, X., Zhao, M., Zhang, X., Chi, J., Liu, Y., … Yin, X. (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. *Cellular Physiology and Biochemistry*, *35*, 2483–2500. https://doi.org/10.1159/000374048

Mezzasoma, L., Antognelli, C., & Talesa, V. N. (2017). A novel role for brain natriuretic peptide: Inhibition of IL-1 β secretion via downregulation of NF-kB/Erk 1/2 and NALP3/ASC/Caspase-1 activation in human THP-1 monocyte. *Mediators of Inflammation*, *2017*, Article ID 5858315. https://doi.org/10.1155/2017/5858315

Patel, M. N., Carroll, R. G., Galván-Peña, S., Mills, E. L., Olden, R., Triantafilou, M., … Masters, S. L. (2017). Inflammasome priming in sterile inflammatory disease. *Trends in Molecular Medicine*, *23*, 165–180. https://doi.org/10.1016/j.molmed.2016.12.007

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*, 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. *Molecular Nutrition & Food Research*, *56*, 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. *Metabolism: Clinical and Experimental*, *74*, 1–9. https://doi.org/10.1016/j.metabol.2017.06.002

Robbins, G. R., Wen, H., & Ting, J. P. Y. (2014). Inflammasomes and metabolic disorders: Old genes in modern diseases. *Molecular Cell*, *54*, 297–308. https://doi.org/10.1016/j.molcel.2014.03.029

Rocha, G., Villalobos, E., Fuentes, C., Villarroel, P., Reyes, M., Díaz, X., … Cifuentes, M. (2015). Preadipocyte proliferation is elevated by calcium sensing receptor activation. *Molecular and Cellular Endocrinology.*, *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., … Wagner, U. (2012). Extracellular Ca2+ is a danger signal activating the

NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nature Communications*, *3*, 1329. https://doi.org/10.1038/ncomms2339

Saltiel, A. R., & Olefsky, J. M. (2017). Inflammatory mechanisms linking obesity and metabolic disease. *Journal of Clinical Investigation*, *127*, 1–4. https://doi.org/10.1172/JCI92035

Scherle, P. A., Jones, E. A., Favata, M. F., Daulerio, A. J., Covington, M. B., Nurnberg, S. A., . . . Trzaskos, J. M. (1998). Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. *Journal of Immunology*, *161*, 5681–5686.

Schroder, K., & Tschopp, J. (2010). The inflammasomes. *Cell*, *140*, 821–832. https://doi.org/10.1016/j.cell.2010.01.040

Stienstra, R., Joosten, L. A. B., Koenen, T., Van Tits, B., Van Diepen, J. A., Van Den Berg, S. A. A., . . . Netea, M. G. (2010). The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity. *Cell Metabolism*, *12*, 593–605. https://doi.org/10.1016/j.cmet.2010.11.011

Stienstra, R., Tack, C. J., Kanneganti, T. D., Joosten, L. A. B., & Netea, M. G. (2012). The inflammasome puts obesity in the danger zone. *Cell Metabolism*, *15*, 10–18. https://doi.org/10.1016/j.cmet.2011.10.011

Stienstra, R., van Diepen, J. A., Tack, C. J., Zaki, M. H., van de Veerdonk, F. L., Perera, D., . . . Kanneganti, T.-D. (2011). Inflammasome is a central player in the induction of obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 15324–15329. https://doi.org/10.1073/pnas.1100255108

Tchkonia, T., Morbeck, D. E., Von Zglinicki, T., Van Deursen, J., Lustgarten, J., Scrable, H., . . . Kirkland, J. L. (2010). Fat tissue, aging, and cellular senescence. *Aging Cell.*, https://doi.org/10.1111/j.1474-9726. 2010.00608.x

Thomsen, A. R. B., Hvidtfeldt, M., & Bräuner-Osborne, H. (2012). Biased agonism of the calcium-sensing receptor. *Cell Calcium*, *51*, 107–116. https://doi.org/10.1016/j.ceca.2011.11.009

Vegiopoulos, A., Rohm, M., & Herzig, S. (2017). Adipose tissue: Between the extremes. *The EMBO Journals*, *36*, 1999–2017. https://doi.org/10.15252/embj.201696206

Wang, Z. Q., Wu, D. C., Huang, F. P., & Yang, G. Y. (2004). Inhibition of MEK/ERK 1/2 pathway reduces pro-inflammatory cytokine interleukin-1 expression in focal cerebral ischemia. *Brain Research*, *996*, 55–66. https://doi.org/10.1016/j.brainres.2003.09.074

Ward, B. K., Magno, A. L., Walsh, J. P., & Ratajczak, T. (2012). The role of the calcium-sensing receptor in human disease. *Clinical Biochemistry*, *45*, 943–953. https://doi.org/10.1016/j.clinbiochem.2012.03.034

Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M. T. H., . . . Ting, J. P. Y. (2011). Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nature Immunology*, *12*, 408–415. https://doi.org/10.1038/ni.2022

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

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

Zhu, Y., Tchkonia, T., Stout, M. B., Giorgadze, N., Wang, L., Li, P. W., . . . Kirkland, J. L. (2015). Inflammation and the depot-specific secretome of human preadipocytes. *Obesity*, *23*, 989–999. https://doi.org/10.1002/oby.21053

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: D'Espessailles A, Mora YA, Fuentes C, Cifuentes M. Calcium-sensing receptor activates the NLRP3 inflammasome in LS14 preadipocytes mediated by ERK1/2 signaling. *J Cell Physiol*. 2018;233:6232–6240. https://doi.org/10.1002/jcp.26490