Leptin promotes IL-18 secretion by activating the NLRP3 inflammasome in RAW 264.7 cells

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Abstract. Leptin is a cytokine-like hormone secreted by adipocytes, which serves to control energy expenditure and metabolism. In addition, leptin may modulate the innate and adaptive immune responses. The innate immune cell sensor nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is mainly expressed in myeloid immune cells, including macrophages. The NLRP3 inflammasome serves a pivotal role in the development and maintenance of autoimmunity and inflammation. The expression levels of caspase-1, apoptosis-associated speck-like protein containing a CARD, interleukin (IL)-18, IL-1β and leptin are significantly reduced in the white adipose tissue of nonsteroidal anti-inflammatory drug-activated gene-1 transgenic mice. However, the association between leptin and the NLRP3 inflammasome has not yet to be determined. The aim of the present study, was to explore the role of leptin on NLRP3 inflammasome. In order to do this, IL-1β and IL-18 expression levels were investigated in RAW 264.7 cells after incubation with leptin of increasing doses by Elisa or reverse transcription-quantitative polymerase chain reaction, and to assess whether IL-1β and IL-18 were affected after caspase-1 activity being inhibited by an inhibitor or by silencing NLRP3 expression. The results of the present study demonstrated that leptin enhanced the mRNA and protein expression levels of IL-18 in RAW 264.7 cells via activation of the NLRP3 inflammasome. This is achieved partly by enhancing the production of reactive oxygen species and K^+ efflux. Therefore, leptin may be considered a novel activator and modulator of the NLRP3 inflammasome.

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Introduction

The nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome can stimulate the innate or adaptive immune system in response to certain signals, including endogenous metabolites and microorganisms. It is understood that the underlying mechanism employed by the NLRP3 inflammasome involves the recruitment of apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1. As a result of inflammasome activation, the proinflammatory cytokines interleukin (IL)-1β and IL-18 are released via pyroptosis and proteolytic cleavage (1). Activators of the NLRP3 inflammasome are heterogeneous, ranging from self-originating crystals, such as monosodium urate monohydrate, uric acid, glucose and adenosine 5'triphosphate (ATP) to environment-derived aluminum hydroxide, silica and asbestos, as well as pathogenic molecules (2,3). How such structurally diverse molecules activate the NLRP3 inflammasome is yet to be determined; however, it appears that K^+ efflux and reactive oxygen species (ROS) may serve pivotal roles in activation of the NLRP3 inflammasome (3,4). NLRP3 is usually expressed in myeloid cells, including monocytes, macrophages and dendritic cells; recently it has been revealed that NLRP3 is also expressed in T helper (Th) 1 (5), Th2 (6) and Th17 (7) cells. Providing the association between NLRP3 activation and numerous pathological conditions, including Muckle-Wells syndrome (8), Alzheimer's disease (9), type 2 diabetes (10), as well as autoimmune disorders, such as experimental autoimmune encephalitis (7) and systemic lupus erythematosus (11), increasing efforts to clarify the underlying molecular mechanism are being made.

Secreted by adipocytes, leptin is a cytokine-like hormone that has been reported to control energy expenditure and metabolism, and modulate the innate and adaptive immune responses. The activation of natural killer cells, chemotaxis of neutrophils, and secretion of tumor necrosis factor (TNF)-α, IL-6 and IL-12 from macrophages (12) also involves leptin. Additionally, leptin also promotes Th17 cell responses (13) and downregulates the number of T regulatory cells (14). Previously, it was demonstrated that the mRNA and protein expression levels of caspase-1 and ASC were significantly reduced, in addition to reduced expression levels of IL-18, IL-1β, leptin and macrophage infiltration markers, within

white adipose tissue of nonsteroidal anti-inflammatory drug-activated gene-1 transgenic mice (15). These findings have indicated an association between leptin and the NLRP3 inflammasome within macrophage cells. The present study demonstrated that activation of the NLRP3 inflammasome was promoted by an increase in ROS synthesis and K^+ efflux in response to leptin, which resulted in an increase in IL-18 secretion within RAW 264.7 cells. Leptin may therefore be considered a novel activator and a potential modulator of the NLRP3 inflammasome.

Materials and methods

Cell culture. RAW 264.7 murine macrophage cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, low glucose) supplemented with 10% fetal bovine serum, 100 ng/ml streptomycin and 100 U/ml penicillin (Gibco; Thermo Scientific, Inc., Waltham, MA, USA) at 37˚C, 5% CO_2 and humidity. Various doses of leptin (10, 100 and 500 ng/ml; PeproTech China, Suzhou, China) were added and the cells were cocultured for 24 h in the presence or absence of Ac-YVAD-cmk (18.4 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), KCl (100 μM) or diphenyleneiodonium chloride (DPI; 50 μM, Sigma-Aldrich; Merck KGaA). Lipopolysaccharide (LPS, 100 ng/ml, Sigma-Aldrich; Merck KGaA) and/or adenosine 5'triphosphate (ATP, 5 mM, Sigma-Aldrich; Merck KGaA) were cultured for 3 h respectively and used as positive controls. Cells were centrifuged at 400 x g, for 5 min, 4˚C, and supernatants and sediments were collected and analyzed by ELISA or reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Flow cytometric analysis. Caspase-1 activity was detected using a Fluorochrome-Labeled Inhibitor of caspase-1 kit: FAM-FLICA®Caspase assay kit (cat. no. 655) (ImmunoChemistry Technologies, LLC, Bloomington, MN, USA). ROS synthesis was investigated using a ROS detection assay kit, CFDA Cellular ROS Detection assay kit (cat. no. ab113851, Abcam, Shanghai, China). THBP was used as a positive control. All kits were performed according to the manufacturer's protocol. Results were analyzed using a FACSCanto FlowJo 7.6 (BD Biosciences, Franklin Lakes, CA, USA).

Cytokine measurement. IL-1β and IL-18 expression levels in the supernatant of RAW 264.7 cells were measured using ELISA kits (Mouse IL-18 cat. no. BMS618, Mouse IL-1β cat. no. BMS6002) and were purchased from eBioscience; Thermo Fisher Scientific, Inc., according to the manufacturer's protocols.

RNA isolation, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from 500 ng total RNA in 10 ul volume using a Superscript kit (Invitrogen; Thermo Fisher Scientific, Inc.). The conditions used were 37˚C for 15 min and 85˚C for 5 sec. RT-qPCR reactions were performed using 1ul cDNA, 10 ul SYBR Green mater mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 2 ul of primer mix in a total volume of 20 ul. Thermocycling conditions were set up as follows: 5 min at 95˚C, 40 cycles of denaturation (5 sec at 95˚C), and combined annealing/extension (34 sec at 64˚C). The housekeeping gene GAPDH was used as the internal standard. Analysis of relative gene expression data using the $2^{-\Delta\Delta Cq}$ method (16). RT-qPCR was performed on an ABI Prism 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). Primer sequences were as follows: NLRP3-forward, 5'-ATT ACCCGCCCGAGAAAGG-3', and reverse, 5'-CATGAGTGT GGCTAGATCCAAG-3'; IL-1β forward, 5'-GTACAAGGA GAACCAAGCAA-3' and reverse, 5'-CCGTCTTTCATTACA CAGGA-3'; IL-18 forward, 5'-AGGACACTTTCTTGCTTG CC-3', and reverse, 5'-CACAAACCCTCCCCACCTAA-3'; GAPDH forward 5'-TTCACCACCATGGAGAAGGC-3' and reverse 5'-GGCATGGACTGTGGTCATGA-3'.

NLRP3 gene knockdown. RAW 264.7 cells were nucleofected with 20 μM NLRP3 specific small interfering (si)RNA (Shanghai Biotend, Shanghai, China) or negative control (NC) siRNA (Shanghai Biotend) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Nucleofected cells were incubated for 24 h at 37˚C, 5% CO_2 and humidity in the presence or absence of leptin, 6 h post-transfection. Knockdown of the NLRP3 gene was determined via RT-qPCR with the following primers: NLRP3 siRNA forward, 5'-GCAGGUUCUACU CUAUCAAdTdT-3' and reverse, 5'-UUGAUAGAGUAGAAC CUGCdTdT-3'. The NC siRNA sequences were as follows: NC siRNA forward, 5'-UUCUCCGAACGUGUCACGUdTdT-3' and reverse, 5'-ACGUGACACGUUCGGAGAAdTdT-3'.

Statistical analysis. A paired t-test was employed for two group analyses and Kruskal-Wallis one-way analysis of variance was used for analyses of >3 groups using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference and the experiments were repeated three times.

Results

Leptin promotes IL-18 secretion in RAW 264.7 cells. To study the effects of leptin on macrophages, leptin was applied at increasing doses to RAW 264.7 cells for 24 h, using LPS and/or ATP as positive controls. Supernatants or cell sediments were collected and analyzed by ELISA or RT-qPCR. The present study reported a leptin-induced increase in IL-1β mRNA expression levels only, whereas a dose-dependent increase was observed in IL-18 mRNA and protein expression levels (Fig. 1)

Leptin activates caspase-1 and NLRP3 to promote IL-18 secretion. Caspase-1 contributes to the NLRP3 inflammasome complex and regulates the synthesis and secretion of IL-18 by proteolytically digesting pro-IL-18 (17,18). Since leptin was observed to promote IL-18 secretion in RAW 264.7 cells, the effects of leptin on caspase-1 were investigated. The findings of the present study indicated that activation of

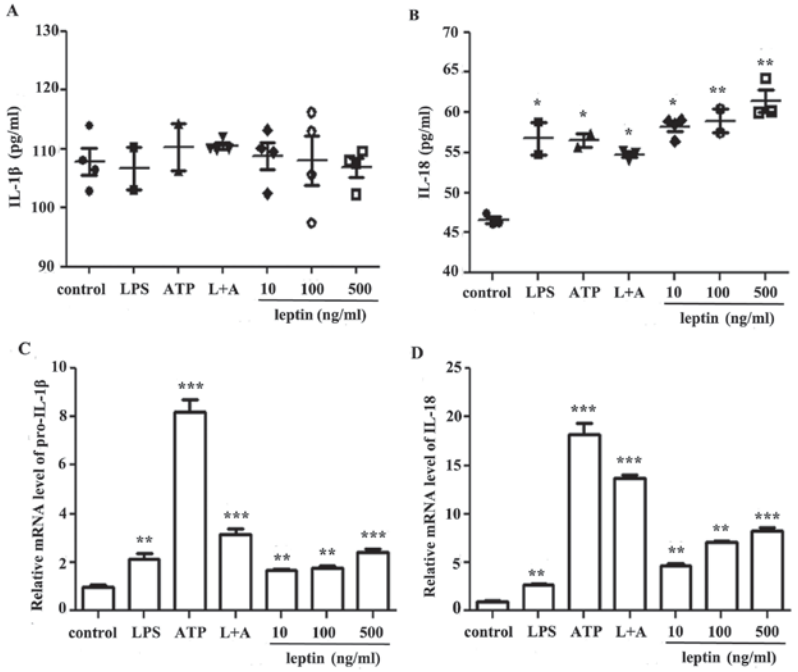

Figure 1. Leptin promotes IL-18, but not IL-1β secretion, in RAW 264.7 cells. (A) IL-1β and (B) IL-18 ELISA of culture supernatants from RAW 264.7 cells incubated with LPS, ATP, L+A or leptin at increasing doses (10, 100 and 500 ng/ml) for 24 h. Results are presented as the mean ± standard error of the mean from four independent experiments. Relative mRNA expression levels of (C) IL-1β and (D) IL-18 in RAW 264.7 cells treated as aforementioned. Results are from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. the control. ATP, adenosine 5'triphosphate; IL, interleukin; LPS, lipopolysaccharide; L+A, LPS and ATP.

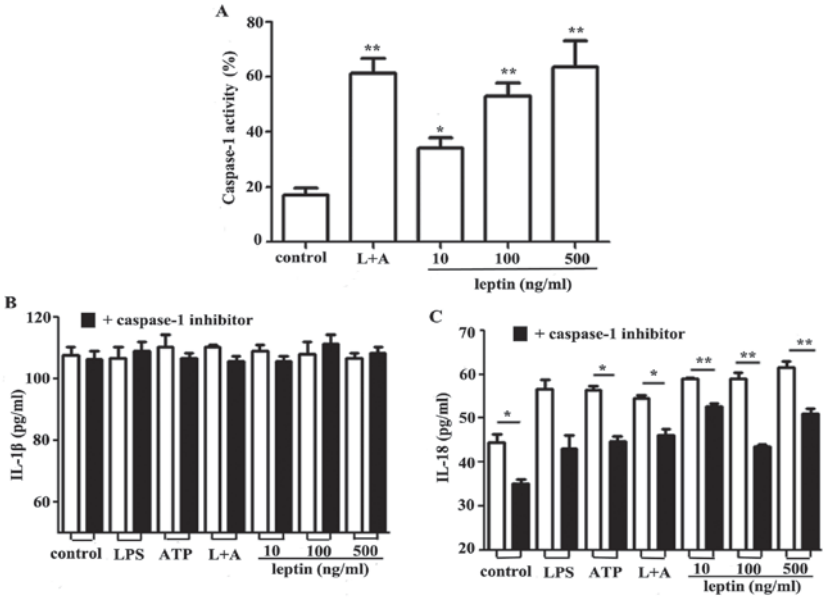

Figure 2. Caspase-1 activation facilitates IL-18 secretion in response to leptin. (A) Caspase-1 activity was measured by Fluorochrome-Labeled Inhibitor of Caspases staining of RAW 264.7 cells incubated with L+A or leptin at increasing doses (10, 100 and 500 ng/ml) for 24 h. Cumulative data are from three experiments. (B) IL-1β and (C) IL-18 expression in the culture supernatants from RAW 264.7 cells following treatment with or without the caspase-1 inhibitor Ac-YVAD-cmk (20 μM) for 24 h, as determined by ELISA. Results are expressed as pg/ml and are presented as the mean ± standard error of the mean from four independent experiments. *P<0.05, **P<0.01 vs. the control group. ATP, adenosine 5'triphosphate; IL, interleukin; LPS, lipopolysaccharide; L+A, LPS and ATP.

caspase-1 was induced by leptin in a dose-dependent manner (Fig. 2A). Inhibition of caspase-1 via Ac-YVAD-cmk had no effect on IL-1β secretion (Fig. 2B); however, IL-18 secretion was markedly decreased in response to the caspase-1 inhibitor (Fig. 2C).

The NLRP3 inflammasome comprises NLRP3, and the adapter and effector proteins, ASC and caspase-1, respectively. The effects of leptin on capsase-1 activation prompted an investigation into the association between leptin and the NLRP3 inflammasome. The results demonstrated that leptin

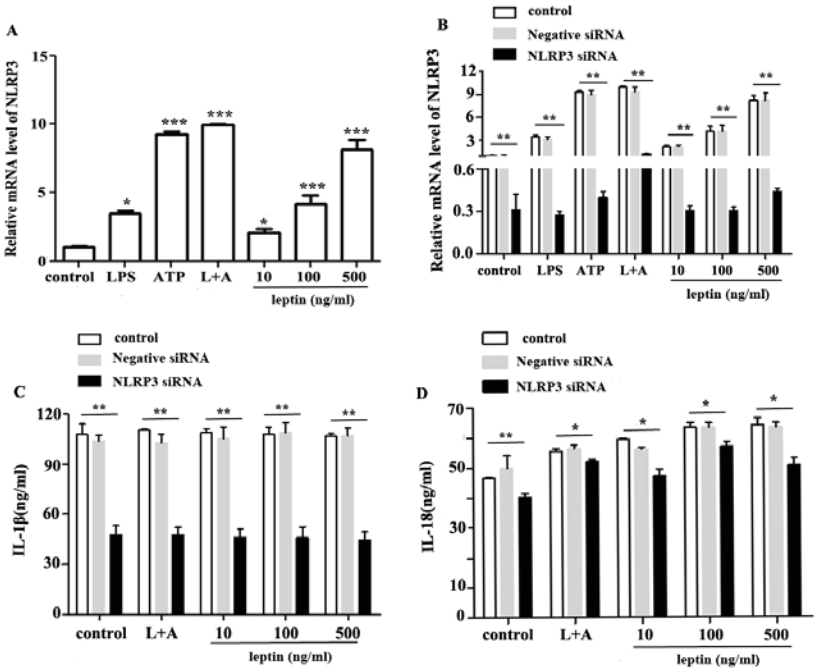

Figure 3. NLRP3 upregulation promotes IL-18 expression in response to leptin. (A) Relative mRNA expression levels of NLRP3 in RAW 264.7 cells incubated with LPS, ATP, L+A or leptin at increasing doses (10,100 and 500 ng/ml) for 24 h. (B) Relative mRNA expression levels of NLRP3 in RAW 264.7 cells following nucleofection with NLRP3 specific siRNA or negative control siRNA. (C) IL-1β or (D) IL-18 expression in the culture supernatants from RAW 264.7 cells treated as aforementioned, as determined by ELISA. *P<0.05, **P<0.01, ***P<0. 001 vs. the control. ATP, adenosine 5'triphosphate; IL, interleukin; LPS, lipopolysaccharide; L+A, LPS and ATP; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3; siRNA, small interfering RNA.

upregulated the mRNA expression levels of NLRP3 (Fig. 3A). Conversely, NLRP3, IL-1β and IL-18 expression levels were reduced following nucleofection with NLRP3-specific siRNA (Fig. 3B, C and D respectively).

ROS synthesis and K^+ efflux is involved in leptin-induced IL-18. ROS have been reported to be key mediators in the activation of the NLRP3 inflammasome (19-21). Intracellular ROS generation was measured in response to leptin within RAW 264.7 cells. Leptin-induced ROS generation was observed compared with in the control group (Fig. 4A and B). In addition, a significant decrease in IL-18 production was observed in response to the NADPH oxidase inhibitor DPI (Fig. 4C).

Previous studies have suggested that K^+ efflux appears to be an important mediator in the activation of the NLRP3 inflammasome (22-24). RAW 264.7 cells were incubated with leptin at increasing doses for 24 h in the presence or absence of 100 mM KCl. The results of the present study indicated that although IL-1β secretion was unaffected (Fig. 4D), an impairment in IL-18 secretion following elimination of K^+ efflux was observed (Fig. 4E).

Discussion

A previous study revealed that leptin induced the upregulation of phagocytic function-associated markers, and stimulated the secretion of proinflammatory cytokines, including TNF-α, IL-6 and IL-12 (12). The results of the present study suggested an association between leptin-induced IL-18 secretion and activation of the NLRP3 inflammasome, via increases in ROS generation and K^+ efflux within RAW 264.7 cells. As a member of the IL-1 cytokine family, IL-18 possesses similarities to IL-1 with regards to structure and receptor utilization. IL-18 serves a key proinflammatory role by inducing interferon-γ expression (25). The expression, synthesis and processing of IL-18 however, is distinct compared with other members of the IL-1 cytokine family (26-28) and is regulated by caspase-1. Following processing, IL-18 is released into the extracellular milieu and can also be expressed as a membrane-bound form. Active IL-18 attaches to the IL-18 receptor, which is expressed on various cells, including macrophages. Myeloid differentiation primary response gene 88-IRAF6-nuclear factor-κB and signal transduction and activator of transcription 3-mitogen activated protein kinase signaling pathways are two major pathways for IL-18 (25). Recently, it has been reported that breast cancer cell metastasis may be associated with leptin-induced secretion of IL-18 (29), which requires future investigation.

In the present study, leptin was reported to increase IL-1β gene expression; however, protein secretion remained unaffected within RAW 264.7 cells. Additionally, IL-1β was unaffected by caspase-1 inhibition. Martin *et al* (7) detected IL-1β secretion within Th17 cells in a NLRP3-ASC-caspase-8-dependent manner in the absence of caspase-1. These findings suggested an association between caspase-8 and the leptin-induced effects observed in macrophage cells. In addition, the secreted protein p60 from *Listeria monocytogenes* serves as a 'non-canonical' stimulus, which activates the NLRP3 inflammasome. In the present study, leptin mainly promoted IL-18 secretion and caspase-1

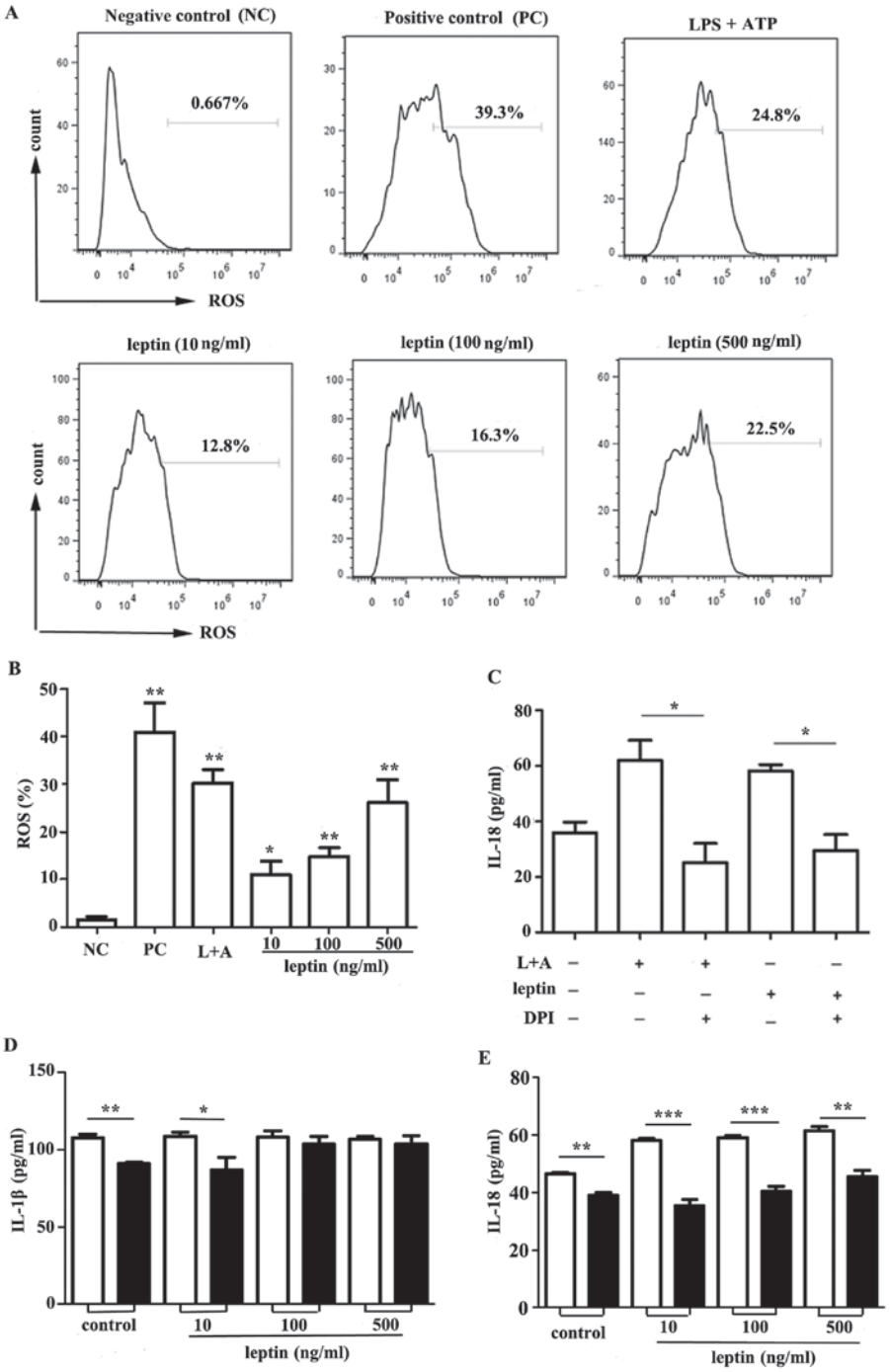

Figure 4. ROS synthesis and K^+ efflux is involved in producing IL-18 in response to leptin. (A) Flow cytometric analysis of ROS in RAW 264.7 cells incubated with leptin at increasing doses (10, 100 and 500 ng/ml) for 24 h. THBP was used as a positive control. Representative data from three independent experiments are presented. (B) Cumulative data of ROS in RAW 264.7 cells from three experiments. (C) IL-18 ELISA of culture supernatants from RAW 264.7 cells incubated with leptin (500 ng/ml) for 24 h in the presence or absence of the ROS inhibitor DPI (50 μM). (D) IL-1β or (E) IL-18 expression in the culture supernatants from RAW 264.7 cells incubated with leptin at increasing doses (10, 100 and 500 ng/ml) for 24 h in the presence or absence of KCl (100 mM), as determined by ELISA. *P<0.05, **P<0.01, ***P<0.001 vs. the control. ATP, adenosine 5'triphosphate; DPI, diphenyleneiodonium chloride; IL, interleukin; LPS, lipopolysaccharide; L+A, LPS and ATP; ROS, reactive oxygen species.

inhibitor mainly inhibited IL-18. It could be hypothesized that other caspases, such as caspase-8 may influence IL-1β response to leptin. The production of IL-1β or IL-18 is independently regulated following activation of the inflammasome. Inhibitors of ROS production inhibited secretion of IL-1β, but did not impair IL-18 secretion. Furthermore, DCs from caspase-11 (casp11)-deficient mice failed to secrete IL-1β in response to p63 but were fully responsive for IL-18 secretion. Therefore, other disparate licensing factors may control IL-18 vs. IL-1β within dendritic cells (30). Consistent with previous studies of human monocytes, the present study demonstrated that leptin augmented the secretion of IL-18, but not IL-1β (31). Conversely, leptin has been reported to induce IL-1β secretion within human peripheral blood mononuclear cells (PBMCs) (32,33); elevated IL-1β mRNA and IL-18 protein expression levels, and increased cytokine responses to

LPS have also been observed in monocyte-derived dendritic cells (34). In addition, it has been reported that leptin may expedite IL-1β secretion within bovine PBMCs; however, it appears that the mRNA expression levels of IL-1β and IL-18 were unaffected (35). Therefore, these findings indicated that leptin-mediated responses within myeloid immune cells may differ between species, lineage and differentiation state.

Reactive oxygen species and K^+ efflux appear to be important mediators for the activation of the NLRP3 inflammasome. The present study revealed that leptin promotes IL-18 secretion by increasing ROS synthesis and K^+ efflux, which may activate the NLRP3 inflammasome in RAW 264.7 cells. Numerous studies have indicated that ROS and K^+ efflux may serve as strong activators for the NLRP3 inflammasome (36,37).

In conclusion, the findings of the present study revealed that leptin promotes IL-18 secretion by enhancing ROS synthesis and K^+ efflux, which activates the NLRP3 inflammasome in RAW 264.7 cells. Leptin may therefore serve as a novel activator of the NLRP3 inflammasome through blocking leptin may be considered as a target for future antimicrobial and anti-inflammatory treatment.

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