Role of ASM/Cer/TXNIP signaling module in the NLRP3 inflammasome activation

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Research

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Abstract

Background: The NOD-Like Receptor Protein 3 (NLRP3) inflammasome is a crucial component of an array of inflammatory conditions. It functions by boosting the secretion of pro-inflammatory cytokines: interleukin-1β (IL-1β) and interleukin-18 (IL-18). Previous studies have established the vital role of the acid sphingomyelinase (ASM)/ceramide (Cer) pathway in the functional outcome of cells, with a particular emphasis on the inflammatory processes. This study aimed to explore the effects and associated underlying mechanism of Cer-induced NLRP3 inflammasome activation.

Methods: Lipopolysaccharide (LPS)/adenosine triphosphate (ATP)-induced NLRP3 inflammasome activation in J774A.1 cells was used as an in vitro inflammatory model. Western blotting and Real-time PCR (RT-PCR) were used to detect the protein and mRNA levels, respectively. IL-1β and IL-18 levels were evaluated using ELISA kits. ASM assay kit and immunofluorescence were used to detect ASM activity and Cer content.

Results: Imipramine, a well-known inhibitor of ASM, significantly inhibited ASM activity and inhibited Cer accumulation, which indicated ASM activation. Besides, it also suppressed the LPS/ATP-induced expression of proteins and mRNA: thioredoxin interacting protein (TXNIP), NLRP3, caspase-1, IL-1β and IL-18. Interestingly verapamil, a TXNIP inhibitor, suppressed LPS/ATP-induced TXNIP/NLRP3 inflammasome activation; however, it did not affect LPS/ATP-induced ASM activation and ceramide production. Further analysis showed that the exogenous C2-Cer treated J774A.1 cells induced the overexpression of TXNIP, NLRP3, caspase-1, IL-1β and IL-18. Besides, TXNIP siRNA or verapamil inhibited C2-Cer-induced TXNIP overexpression and NLRP3 inflammasome activation.

Conclusion: This study demonstrated the involvement of the ASM/Cer/TXNIP signaling pathway in NLRP3 inflammasome activation.

1. Introduction

Inflammation is a local protective mechanism evoked by cells' exposure to harmful stimuli, such as irritants, pathogens, or damaged cells. Excessive or chronic inflammation gives rise to multiple pathological complications, such as tissue damage and dysfunction, diabetes, sepsis, arteriosclerosis, Alzheimer's disease, liver disorders, and various malignancies [1]. Routinely prescribed anti-inflammatory drugs fall under two categories: non-steroidal and steroidal. However, the consumption of these drugs is associated with various side-effects, which restricts their clinical utility [2, 3]. It has necessitated a pressing need for effective alternatives for current anti-inflammatory medications.

NOD-Like Receptor Protein 3 (NLRP3) inflammasome is the crucial intracellular inflammatory pathway of the innate immune system. Incessant activation of NLRP3 inflammasome culminates in the accumulation of pro-inflammatory cytokines, i.e., interleukin-1β (IL-1β) and interleukin-18 (IL-18), which contributes to excessive inflammation and inflammatory disorders [4]. Unraveling the precise regulatory
mechanism leading to the NLRP3 inflammasome activation could help identify adept molecular drug targets to treat inflammatory diseases.

NLRP3 is activated by exogenous factors, such as pathogens or endogenous factors such as Ceramide (Cer), an intracellular lipid metabolism [5, 6]. In line with these findings, recent reports demonstrated Cer-mediated activation of NLRP3 inflammasome in multiple disorders, such as obesity, glomerular injury, acute lung injury, and Alzheimer's disease [7, 8]. Cer is primarily synthesized through sphingolipid metabolism. Sphingomyelin hydrolysis one of the first pathways to get activated as a part of host's stress response, and sphingomyelinase serves as a crucial component in the regulation of this pathway [9]. Acid sphingomyelinase (ASM) is present in the lysosomes. Various stress stimuli, such as oxidative stress lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α), promote ASM's overexpression. Cer is generated through the ASM mediated hydrolysis of sphingomyelin at the cell membrane [10, 11]. Numerous studies have reported that the ASM/Cer pathway over-activation is the underlying mechanism for the stimulus-induced inflammation [12, 13]. As per our previous findings, imipramine remarkably reduced the LPS-induced pulmonary inflammation and thus increased mice's survival rate by suppressing the Cer overbuilt inside the cells [14]. Besides, NLRP3 shRNA curtailed the Cer-induced NLRP3 inflammasome activation & pro-inflammatory cytokines secretion, which improved the type alveolar epithelial cells permeability [8]. The foremost investigation should focus on the participation of ASM hydrolyzed sphingomyelin in the Cer-induced NLRP3 inflammasome activation in response to stress stimuli. Thioredoxin interacting protein (TXNIP) can directly interacts and activates the NLRP3 inflammasome [15]. Previous studies have elucidated the crucial role played by TXNIP/NLRP3 inflammasome signaling pathway in NLRP3 inflammasome activation. However, the ASM/Cer/TXNIP signaling pathway mediated activation of NLRP3 inflammasome remains unexplored.

This study's main objective was to validate the involvement of ASM derived Cer in the NLRP3 inflammasome activation. We also attempted identification of the underlying mechanism for the NLRP3 inflammasome activation with a particular emphasis on the ASM/Cer/TXNIP signaling pathway.

2. Materials And Methods

2.1. Materials

Murine macrophage cell line J774A.1 cells were obtained from the University of Science and Technology of China. C2-Cer, LPS, Adenosine 5′-triphosphate (ATP) disodium salthydrate, verapamil, and imipramine were acquired from Sigma-Aldrich (St Louis, MO, USA). Anti-NLRP3, anti-TXNIP antibodies were acquired from Abcam (San Francisco, USA). The secondary antibody was obtained from ZSGB-BIO (Beijing, China). Anti-caspase-1, anti-β-actin antibodies, and Lysis buffer were obtained from CST (Beverly, MA, USA).

2.2. Cell culture and treatment
The J774A.1 cells were randomly grouped into 1) normal control group; LPS/ATP group; imipramine intervention + LPS/ATP group (J774A.1 cells were incubated with LPS (1 μg/mL) for 4 h, treated with imipramine (10 μmol/L) for 3 h, and finally added ATP (5 mM) for 30 min) and imipramine control group (imipramine group); 2) normal control group; LPS/ATP group; verapamil intervention + LPS/ATP group (J774A.1 cells were incubated with LPS (1 μg/mL) for 4 h, treated with verapamil (10 μmol/L) for 3 h, and finally added ATP (5 mM) for 30 min) and verapamil control group (verapamil group) and 3) normal control group; Cer group (30 μmol/L C2-Cer); verapamil intervention + Cer group (J774A.1 cell were pretreated with verapamil (10 μmol/L for 3 h) and co-incubated with C2-Cer (30 μmol/L for 5 h)) and the TXNIP siRNA + Cer group (cells were transfected with TXNIP siRNA and incubated with C2-Cer (30 μmol/L for 5 h)).

2.3. Transfection

Control siRNA and TXNIP siRNA were synthesized from GenePharma (Shanghai, China). Cell transfection was using Lipofectamine® 3000 RNAi Max reagent, as per manufacturer's instructions (Invitrogen, Karlsruhe, Germany). The cells were collected for western blotting and RT-PCR analysis at 48 h post-transfection.

2.4. Cell viability assays

The J774A.1 cells were treated with imipramine (0, 25, 50, 75, 100 μmol/L), C2-Cer (0, 15, 30, 45, 60 μmol/L) and verapamil (0, 25, 50, 75, 100 μmol/L) for 24 h. It was followed by incubation of J774A.1 cells with MTT (Beyotime, Jiangsu, China) for 4 h. Furthermore, dimethylsulfoxide (DMSO) (Beyotime, Jiangsu, China) was added per well, and the absorbance was recorded by the microplate reader (BioTek, Winooski, VT, USA) at 490 nm.

2.5. Western blotting

An equal amount of protein (10–20 μg) was taken from each sample, loaded into the individual lane, and subjected to vertical SDS-PAGE electrophoresis (concentrated gel voltage 50 V, 1 h, and separated gel voltage 100 V, 1.5 h). The cellular proteins were electrophoretically transferred at 200 mA for 3.5 h onto a PVDF membrane (Millipore Corporation, Billerica, USA) by parallel electrophoresis. It was incubated with primary antibodies and corresponding secondary antibody. Protein bands were scanned using the chemiluminescence imaging system (GE Healthcare, Bucks, UK).

2.6. Real-time PCR

After the treatments mentioned above, the total RNA was extracted from cells using Promega reagent (Promega, Beijing, China), as per manufacturer's instructions. Promega-A3500 kit was used for reverse transcription of RNA. The Promega-A6001 kit was used to detect cDNA expression. Based on the GAPDH expression, the 2\(^{-ΔΔCt}\) method used for the calculation of relative expression level of target genes.

Primers used in real time-PCR.
### 2.7. ELISA assay

After distinct treatments of J774A.1 cells, as mentioned earlier, the supernatant of J774A.1 cells was collected for cytokine analysis. ELISA kits were used to measure IL-1β and IL-18 concentrations, as per the manufacturer's instruction (MultiSciences Biotechnology, Hangzhou, China). Each well's absorbance was measured on a microplate reader (wavelength 450 nm) (BioTek, Winooski, VT, USA) and repeated 3 times. Each well’s absorption values were averaged, and cytokine contents in the supernatants were calculated using standard curves.

### 2.8. Cer content detection by immunofluorescence

After distinct treatments of J774A.1 cells, as mentioned earlier, the cells were subsequently fixed and permeabilized. Cells were incubated at 4°C for 12 h with anti-Cer antibodies (1:500, ENZO, Switzerland), subsequently with secondary antibodies in the dark at room temperature for 1 h. After washing, cells were DAPI stained in the dark for 10 min. Nikon Eclipse 90i Fluorescence Microscope system (Nikon, Japan) was used to visualize the cells and record the images.

### 2.9. ASM activity measurement

Briefly, after the treatments mentioned above, the cells were lysed using a 1X mammalian lysis buffer. Then, as per the manufacturer's instruction, these samples were treated with ASM assay reagents (ASM assay kit, Abcam, San Francisco, USA). After incubating for one hour at room temperature, each sample's
fluorescence was detected using a microplate reader (BioTek, Winooski, VT, USA) at Ex/Em = 540/590nm. The fluorescence in the blank wells was only used as a negative control.

2.10. Statistical analysis

All experimental data are presented as the mean ± SD, and statistical analysis was conducted using SPSS version 23.0. Statistical differences between the groups were determined using a one-way analysis of variance (ANOVA). \( P< 0.05 \) indicates statistical significance.

3. Results

3.1 Effects of imipramine, C2-Cer, and verapamil on the cell viability

The MTT method was used to measure the effects of imipramine (0, 25, 50, 75, 100 \( \mu \text{mol/L} \)), C2-Cer (0, 15, 30, 45, 60 \( \mu \text{mol/L} \)), and verapamil (0, 25, 50, 75, 100 \( \mu \text{mol/L} \)) on the J774A.1 cells viability. As shown in Fig. 1A, no significant toxicity was observed in imipramine (0–50 \( \mu \text{mol/L} \)) treated J774A.1 cells. As shown in Fig. 1B, as C2-Cer concentration increased, the cells' activity decreased. However, increased C2-Cer concentration, within the range of 0–30 \( \mu \text{mol/L} \), did not exert any toxic effect on J774A.1 cells. As shown in Fig. 1C, no toxicity was seen in verapamil (0–50 \( \mu \text{mol/L} \)) treated J774A.1 cells. According to MTT assays, 10 \( \mu \text{mol/L} \) of imipramine, 30 \( \mu \text{mol/L} \) of C2-Cer, and 10 \( \mu \text{mol/L} \) of verapamil were used in this study.

3.2 Imipramine pretreatment inhibits LPS/ATP-induced ASM activity and Cer production

Firstly, as an ASM activity inhibitor, the imipramine effect on LPS/ATP-induced ASM activation and Cer generation was examined to unravel the ASM/Cer pathway’s role in TXNIP/NLRP3 inflammasome activation in J774A.1 cells. ASM activity and Cer level in cells were evaluated using ASM assay kit and immunofluorescence, respectively. As depicted in Fig. 1D, LPS/ATP significantly increased ASM activity as against the control cells, later restored to baseline level by imipramine treatment. Similarly, LPS/ATP significantly elevated the Cer content compared to the J774A.1 control cells; however, the Cer level was reverted with imipramine treatment (Fig. 1E, F).

3.3 Inhibition of ASM activity attenuates LPS/ATP-induced TXNIP expression and NLRP3 inflammasome activation

Effect of imipramine treatment on LPS/ATP-induced TXNIP protein expression and the NLRP3 inflammasome activation was estimated. Imipramine treatment suppressed the LPS/ATP induced overexpression of TXNIP, NLRP3, and caspase-1 (Fig. 2B-D). NLRP3 inflammasome induced expression of IL-1\( \beta \) and IL-18 cytokines were evaluated to unravel the inhibitory effect of imipramine on NLRP3 inflammasome activation. As shown in Fig. 2E-F, the outcomes indicated that LPS/ATP significantly increased IL-1\( \beta \) and IL-18 secretion in the supernatant, and pretreatment with imipramine significantly inhibited the secretion of IL-1\( \beta \) and IL-18.
3.4 Inhibition of ASM activity attenuates LPS/ATP-induced NLRP3 inflammasome and TXNIP mRNA expression

As shown in Fig. 2G-K, the TXNIP, NLRP3, caspase-1, IL-1β and IL-18 mRNA levels increased significantly after being treated with LPS/ATP as compared with the control group. Imipramine treatment significantly inhibited the mRNA expression of TXNIP, NLRP3, caspase-1, IL-1β and IL-18.

3.5 Inhibition of TXNIP attenuates LPS/ATP-induced TXNIP expression and NLRP3 inflammasome activation

Effects of verapamil (TXNIP inhibitor) on LPS/ATP-induced TXNIP protein expression level and NLRP3 inflammasome activation were evaluated. As shown in Fig. 3B-F, LPS/ATP mediated elevated TXNIP, NLRP3, caspase-1, IL-1β and IL-18 expression, were mitigated by verapamil treatment. Besides, the effects of verapamil on LPS/ATP-induced ASM activity and Cer content were also examined. As depicted in Fig. 1D, F, verapamil did not affect LPS/ATP-induced ASM activity and Cer content.

3.6 Inhibition of TXNIP attenuates C2-Cer induced TXNIP expression and NLRP3 inflammasome activation

Effect of verapamil or TXNIP siRNA on C2-Cer induced TXNIP protein expression and NLRP3 inflammasome activation was examined to investigate if ASM/Cer pathway has TXNIP/NLRP3 inflammasome as a downstream module. As shown in Fig. 4A-C, siRNA can effectively silence TXNIP target genes. As expected, C2-Cer induced the TXNIP, NLRP3, and caspase-1 protein expression, which was later suppressed by verapamil or TXNIP siRNA (Fig. 4E-G). The effect of verapamil or TXNIP siRNA on C2-Cer induced IL-1β and IL-18 secretion levels were also evaluated. The outcomes showed that C2-Cer significantly elevated IL-1β and IL-18 secretion. However, pretreatment with verapamil or TXNIP siRNA significantly inhibited this effect (Fig. 4H, I).

4. Discussion

Macrophage, a vital part of the immune system, plays a vital role in the innate immune response [16]. LPS is a crucial component of gram-negative bacteria's extracellular membrane, which maintains the cells' structural integrity, and elicits pathogen-induced inflammation [17]. The LPS/ATP-stimulated J774A.1 macrophage cells are widely accepted in-vitro model of inflammatory cell NLRP3 inflammasome employed for the investigation of NLRP3 inflammasome activation mechanism [18]. Hence, in the current study, the LPS/ATP-stimulated J774A.1 macrophage cells were used as an inflammatory cell NLRP3 inflammasome activation model. Accumulating pieces of evidence states that the aberrant activation or dysregulation of NLRP3 inflammasome manifests the most prevalent inflammatory conditions [4]. An in-depth investigation of the NLRP3 inflammasome activation signaling pathway could lead to the identification of more relevant drug targets for effective management of the resulting inflammatory disorders. This study demonstrated ASM’s involvement in LPS/ATP-induced Cer production in J774A.1 cells, which elicited the NLRP3 inflammasome activation by mediating the TXNIP overexpression. This
study demonstrated that the ASM/Cer/TXNIP signaling module induced the NLRP3 inflammasome activation.

Sphingolipid metabolism is a significant part of lipid metabolism, which generates an array of active cellular lipids, imparts structural integrity to the cell, and regulates many crucial cellular functions [19, 20]. A plethora of stress stimuli generates excessive ceramide through sphingolipid metabolism. As a secondary signaling molecule, it activates the signal transduction required for the occurrence of biological processes, such as inflammation, apoptosis, and cellular differentiation [21, 22]. Accumulating evidence has validated that Cer accumulation triggers NLRP3 inflammasome activation in various pathological conditions [7, 8]. Intracellular Cer accumulation occurs through two significant routes of ceramide synthesis, i.e., de novo synthesis of ceramide via ceramide synthase serine palmitoyltransferase (SPT) and sphingomyelinases-neutral sphingomyelinase (NSM)/ASM-mediated hydrolysis of sphingomyelin membrane [21,23]. However, recent studies suggested that the elevated level of Cer in stress response is an outcome of ASM-mediated sphingomyelin hydrolysis [9, 10]. Our previous findings have demonstrated the participation of imipramine in the amelioration of LPS-induced pulmonary inflammation in mice mediated by suppression of Cer levels [14]. Imipramine is a well-known ASM inhibitor, which disrupts ASM and lysosomal membrane interaction, and elicits lysosomal destruction of ASM [24].

In this study, LPS/ATP treatment significantly elevated the ASM activity and the Cer level, which was significantly attenuated by the ASM inhibitor imipramine. This outcome indicates a positive correlation between the LPS/ATP-induced Cer accumulation and ASM activation in J774A.1 cells. Additionally, imipramine suppressed LPS/ATP-induced TXNIP/NLRP3 inflammasome activation. However, its role in ASM/Cer mediated TXNIP/NLRP3 inflammasome activation remains unexplored. This prompted us to investigate the involvement of the ASM/Cer signaling pathway in TXNIP/NLRP3 inflammasome activation as a downstream component.

TXNIP contributes significantly to biological processes, such as inflammation, oxidative stress, cell apoptosis, glucose, and lipid metabolism [25]. An escalated level of TXNIP negatively impacts these biological processes, which majorly contribute to the onset of inflammatory disorders [26, 27]. As per the previous findings, TXNIP serves the crucial task of NLRP3 assembly by directly interacting with NLRP3 [15, 27]. TXNIP inhibitor verapamil has been widely employed by researchers to suppress TXNIP expression and the associated NLRP3 inflammasome activation to manage the inflammatory conditions [28]. In this study, verapamil suppressed LPS/ATP-induced TXNIP/NLRP3 inflammasome activation. However, verapamil did not affect LPS/ATP-induced ASM activation and Cer production. J774A.1 cells were stimulated with C2-Cer to elucidate that the TXNIP/NLRP3 inflammasome activation is a downstream event of the ASM/Cer signaling pathway. The outcome of this study indicated that C2-Cer significantly increased the TXNIP, NLRP3, and caspase-1 protein expression levels and IL-1β, IL-18 secretion. Furthermore, verapamil or TXNIP siRNA inhibited C2-Cer mediated TXNIP overexpression and NLRP3 inflammasome activation. Therefore, it suggested that Cer induced TXNIP overexpression and subsequent NLRP3 inflammasome activation.
Study strengths and limitations

The advantages of this study are as follows. Firstly, this study reported that LPS/ATP induced the Cer accumulation through ASM activation in J774A.1 cells, which further led to the activation of NLRP3 inflammasomes. Secondly, this study elucidated the involvement of the ASM/Cer/TXNIP signaling pathway in NLRP3 inflammasome activation. However, the main limitation of this study was that only one macrophage cell line was used.

Conclusions

In conclusion, this study successfully validated the crucial role of the ASM/Cer/TXNIP signaling pathway in NLRP3 inflammasome activation. Further understanding of the NLRP3 inflammasome activation mechanism might highlight novel treatment strategies for inflammatory diseases, such as acute lung injury and glomerular injury. The targeted regulation of the Cer signaling pathway may provide a potential therapeutic pathway for inflammatory diseases.

Abbreviations

ASM: Acid sphingomyelinase; ATP: Adenosine Triphosphate; Cer: Ceramide; ELISA: Enzyme-linked immunosorbent assay; DMSO: Dimethylsulfoxide; IL-18: Interleukin-18; IL-1β: Interleukin-1β; LPS: Lipopolysaccharide; NLRP3: NOD-Like Receptor Protein 3; NSM: Neutral sphingomyelinase; RT-PCR: Real-time PCR; SPT: Serine palmitoyltransferase; TXNIP: Thioredoxin interacting protein; TNF-α: Tumor necrosis factor-α.

Declarations

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Author Contribution

Jianjun Jiang wrote this paper. Jianjun Jiang and Yining Shi conducted the experiments. Jin Yang and Youjin Lu analyzed the data. Jiyu Cao and Gengyun Sun supervised this study.

Availability of data and materials

Data are available from the authors on request.
Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

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