Role of ASM/Cer/TXNIP Signaling Module in the NLRP3 Inflammasome Activation

Jianjun Jiang
First Affiliated Hospital of Anhui Medical University
Jin Yang
First Affiliated Hospital of Anhui Medical University
Yining Shi
First Affiliated Hospital of Anhui Medical University
Jiyu Cao
First Affiliated Hospital of Anhui Medical University
Youjin Lu
First Affiliated Hospital of Anhui Medical University
Gengyun Sun (sungengy@126.com)
First Affiliated Hospital of Anhui Medical University

Research

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Abstract

Background: The NLRP3 inflammasome serves as a crucial component in an array of inflammatory conditions by boosting the secretion of pro-inflammatory cytokines: IL-1β and IL-18. Hence, a thorough investigation of the underlying mechanism of NLRP3 activation could ascertain the requisite directionality to the ongoing studies, along with the identification of the novel drug targets for the management of inflammatory diseases. 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. ASM mediates the ceramide production by sphingomyelin hydrolysis. Furthermore, the participation of the ASM/Cer in NLRP3 activation remains ambiguous.

Methods: We employed lipopoysaccharide (LPS)/Adenosine Triphosphate (ATP)-induced activation of NLRP3 inflammasome in J774A.1 cells as an *in vitro* inflammatory model.

Results: We observed that imipramine, a well-known inhibitor of ASM, significantly inhibited ASM activity & increased ceramide accumulation, which indicates 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 activity and ceramide production. Further examination showed that the exogenous C2-ceramide-treated J774A.1 cells induce the overexpression of TXNIP, NLRP3, Caspase-1, IL-1β, and IL-18. Furthermore, verapamil inhibited C2-Ceramide mediated TXNIP overexpression and NLRP3 inflammasome activation. These findings infer that TXNIP overexpression leads to Cer mediated NLRP3 inflammasome activation.

Conclusion: Our study validated the crucial role of the ASM/Cer/TXNIP signaling pathway in NLRP3 inflammasome activation.

1. Introduction

Inflammation serves as a local protective mechanism evoked by the exposure of harmful stimuli such as irritants, pathogens, or damaged cells. An excessive or chronic presence of inflammation manifests various pathological complications such as tissue damage and dysfunction along with the onset of multiple diseases such as diabetes, sepsis, arteriosclerosis, Alzheimer's disease, liver disorders, and cancer [1]. Routinely prescribed anti-inflammatory drugs fall under two categories: non-steroidal and steroidal. However, the consumption of these drugs is coupled with the considerable adverse impact that challenges their clinical utility [2, 3]. These factors have raised a pressing need to explore the effective alternatives of current anti-inflammatory drugs.

NOD-Like Receptor Protein 3 (NLRP3) inflammasome is the crucial intracellular inflammatory pathway of the innate immune system. An unrestrained activation of NLRP3 inflammasome leads to the accumulation of proinflammatory cytokines: IL-1β and IL-18, which contributes to the unwarranted inflammation and associated inflammatory disorders [4]. The precise regulatory mechanism behind the

NLRP3 inflammasome activation will help in the identification of adept molecular drug targets to treat the inflammatory diseases.

NLRP3 can be activated by exogenous factors such as pathogens or endogenous factors such as ceramide, a product of intracellular lipid metabolism [5, 6]. In line with these findings, recent reports have confirmed that ceramide can activate the NLRP3 inflammasome in various disorders such as obesity, glomerular injury, acute lung injury, and Alzheimer's disease [7, 8]. Sphingolipid metabolism is the primary mode of ceramide generation. Sphingomyelin hydrolysis is the main pathway in the body's stress response, and sphingomyelinase serves as a critical component in the regulation of this pathway [9]. Acid sphingomyelinase (ASM) is found in the lysosomes, and various stress stimuli, such as oxidative stress, LPS, and TNF-α increases its expression. Ceramide is generated through the ASM mediated hydrolysis of sphingomyelin at the cell membrane [10, 11]. Numerous studies have reported that the over-activation of the ASM/Cer pathway is the underlying mechanism behind the stimulus-induced inflammation [12, 13]. As per our previous findings, imipramine remarkably controlled the LPS-induced pulmonary inflammation and increased the survival rate of mice by suppressing the ceramide overbuilt inside the cells [14]. Besides, NLRP3 shRNA curbed the ceramide-induced NLRP3 inflammasome activation & proinflammatory cytokines secretion, which improved the type Ⅱ alveolar epithelial cells permeability [8]. The foremost investigation should focus on the participation of ASM hydrolyzed sphingomyelin in the ceramide-induced NLRP3 inflammasome activation in response to stress stimuli. TXNIP is an essential antioxidant, which directly interacts and activates the NLRP3 inflammasome [15]. Previous studies have reported the crucial role played by TXNIP/NLRP3 inflammasome signaling pathway in NLRP3 inflammasome activation. Nonetheless, the ASM/Cer/TXNIP signaling pathway mediated activation of NLRP3 inflammasome remains unexplored.

The main objective of this study was to validate the involvement of ASM derived ceramide in the NLRP3 inflammasome activation. We also made an attempt to unravel the underlying mechanism behind the NLRP3 inflammasome activation with a particular emphasis on ASM/Cer/TXNIP signaling pathway.

2. Materials And Methods

2.1. Cell culture and treatment

In this study, we used the J774A.1 cell line obtained from the University of Science and Technology of China. Cells were cultured in DMEM medium with 10% FBS and antibiotics (100 U/mL penicillinand 100 μg/mL streptomycin) and incubated at 37 °C with 5% CO2. After attaining ~ 70% confluencey, cells were used for further experimentation. The J774A.1 cells were randomly grouped into 1) normal control group; LPS/ATP group ; imipramine intervention + LPS/ATP group (The cells were treated with 1 μg/mL LPS for 4 h followed by pretreatment with imipramine (10 μmol/L) for 3 h, and then 30 min of incubation with 5 mM ATP) and imipramine control group (imipramine group); 2) normal control group; LPS/ATP group ; verapamil intervention + LPS/ATP group (The cells were treated with 1 μg/mL LPS for 4 h followed by pretreatment with verapamil (10 μmol/L) for 3 h, and then 30 min of incubation with 5 mM

ATP) and verapamil control group (verapamil group) and 3) normal control group; ceramide group (30 μmol/L ceramide); verapamil intervention + ceramide group (J774A.1 cells pretreated with 10 μmol/L verapamil for 3 h and incubated with 30 μmol/L ceramide for 5 h) and the verapamil control group (verapamil group).

2.2. Cell viability assays

The J774A.1 cells (5×10^4/well) were seeded in 96-well plates and treated with imipramine (0, 25, 50, 75, 100 μmol/L), C2-ceramide (0, 15, 30, 45, 60 μmol/L) and verapamil (0, 25, 50, 75, 100 μmol/L) for 24 h. Then, 200 μL MTT solution was added to each well, followed by a 4 h incubation at 37 °C. Further, 150 μL dimethylsulfoxide (DMSO) was added to each well to dissolve the resulting formazan crystals, and the absorbance was recorded by the ELx800 microplate reader at 490 nm.

2.3. Western blot analysis

We quantified the protein expression of TXNIP, NLRP3, and Caspase-1 by western blot analysis. Total protein was extracted from J774A.1 cells with the help of an ice-cold RIPA lysis buffer. Protein estimation was done by using the BCA protein Assay kit. 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 50V, 1 h, and separated gel voltage 100V, 1.5 h). The cellular proteins were electrophoretically transferred at 200 mA for 3.5 h onto a PVDF membrane by parallel electrophoresis. The PVDF membrane was blocked with 5% skim milk overnight at 4 °C. Further, it was incubated with primary antibodies (β-actin, 1:1000, Cell Signaling Technology; TXNIP, 1:500, Abcam; NLRP3, 1:800, Abcam; Caspase-1, 1:800, Cell Signaling Technology) at 4 °C for 16 h and washed with TBST. It was followed by incubation with corresponding secondary antibody in a shaker for 2 h at room temperature. Protein bands were visualized by the enhanced chemiluminescence system and scanned using the chemiluminescence imaging system.

2.4. Real-time PCR

The J774A.1 cells (1×10^6/well) were seeded in 6-well plates for treatment mentioned above. Total RNA was isolated from cells using Promega reagent (PromegaLS1040, Beijing, China) as per manufacturer's instructions. Total mRNA concentration was determined by Thermo Scientific NanoDrop instrument. Reverse transcription kit (Promega-A3500) for reversing the total RNA to cDNA on a Bio-Rad T100™ Thermal Cycler instrument.Promega-A6001 kit was used to detect cDNA expression on Light Cycler® 96 real-time PCR instrument (Roche, Switzerland). Referring to the expression of GAPDH, the relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Primers used in real time-PCR.

Forward Primer	Reverse Primer	
TXNIP	GATACCCCAGAAGCTCCTCC	ACCTCA GTGTAAGTGGGTGG
NLRP3	AGCCTTCCAGGATCCTCTTC	CTTGGGCAGCAGTTTCTTTC
Caspase-1	TGGCAGGAATTCTGGAGCTT	CTTGAGGGTCCCAGTCAGTC
IL-1β	CTG CTT CCA AAC CTT TGA CC	AGC TTC TCC ACA GCC ACA AT
IL-18	AAGACTCTTGCGTCAACTTCAAGGA	AGTCGGCCAAAGTTGTCTGATTC
GAPDH	TGATGGGTGTGAACCACGAG	AGTGATGGCATGGACTGTGG

2.5. ELISA assay

After the treatments, as mentioned earlier, the supernatant of J774A.1 cells was collected for cytokine analysis. ELISA assay kits measured the levels of IL-1β and IL-18 in the supernatant according to the manufacturer's instruction (MultiSciences(Lianke)Biotech company, Hangzhou, China). The absorbance of each well was measured on a microplate reader (wavelength 450 nm) and repeated 3 times. Absorbance values of each well were averaged and cytokine contents in the supernatants were calculated according to standard curves.

2.6. Ceramide content detection by immunofluorescence

J774A.1 cells suspension were seeded into 12-well plates with a 50 μL density of 5×10^7/L and incubated for 12 h. They were fixed with 4% paraformaldehyde for 40 min, permeabilized with 0.4% Triton X-100 for 20 min, and blocked with 0.1% BSA for 1 h. Cells were incubated at 4 °C for 12 h with anti-Cer antibodies (1:500, ENZO), 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 equipped with an Olympus DP80 and an Olympus IX73 digital camera (Olympus Instruments, Inc.) was used to visualize the cells and record the images.

2.7. Acid sphingomyelinases measurements

The activity of acid sphingomyelinases was measured as described previously. Briefly, cells were lysed in 1 × mammalian lysis buffer. Then, according to the manufacturer's recommended protocol, these samples are reacted with acid sphingomyelinases assay reagents (ASMase assay kit, Abcam). After incubating for one hour at room temperature, the fluorescence of each sample was measured by using a microplate reader (GeminiEM; Molecular Devices) at Ex/Em = 540/590 nm. The fluorescence in the blank wells was only used as a negative control.

2.8. Statistical analysis

All experiments in this study have been performed for three times. All experimental data are presented as the mean ± standard deviation and statistical analysis was performed using SPSS 23.0. Statistical

differences between the groups were determined using a one-way analysis of variance (ANOVA). Statistical significance was considered when $p < 0.05$.

3. Results

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

The MTT assay was used to determine the effects of different concentrations of imipramine (0, 25, 50, 75, 100 μmol/L), C2-ceramide (0, 15, 30, 45, 60 μmol/L) and verapamil (0, 25, 50, 75, 100 μmol/L) on the J774A.1 cells viability. A 24 h treatment resulted in a significant shift in cell viability. As shown in Fig. 1A, no significant toxicity was seen in imipramine (0–50 μmol/L) treated J774A.1 cells, and the cell viability did not vary significantly as compared to the control group. As shown in Fig. 1B, at increasing C2-ceramide concentrations, the activity of the cells decreased, but the concentration of C2-ceramide showed no significant toxicity to J774A.1 cells within the range of 0–30 μmol/L. As shown in Fig. 1C, no toxicity was seen in verapamil (0–50 μmol/L) treated J774A.1 cells as well, and its cell viability was not significantly different from that of the control group. According to MTT assays, we used imipramine at a concentration of 10 μmol/L, C2-ceramide at 30 μmol/L and verapamil at 10 μmol/L for subsequent experiments.

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

We first examined the effect of imipramine as an ASM activity inhibitor on LPS/ATP-induced ASM activity and ceramide production as the primary measure to unravel the role of the ASM/Cer pathway in TXNIP/NLRP3 inflammasome activation in J774A.1 cells. The cells were treated with 1 μg/mL LPS for 4 h followed by pretreatment with or not imipramine (10 μmol/L) for 3 h, and then 30 min of incubation with 5 mM ATP. Later, we estimated the ASM activity and Cer content in J774A.1 cells by using ASM assay kit and immunofluorescence, respectively. As depicted in Fig. 1D, LPS/ATP significantly increased ASM activity as against the control cells, which were later restored to a normal level by imipramine treatment. Similarly, LPS/ATP significantly elevated the Cer content as compared to the J774A.1 control cells; however, the Cer level was reverted with imipramine treatment (Fig. 1F).

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

We also estimated the outcome of imipramine treatment on LPS/ATP-induced TXNIP protein expression and the NLRP3 inflammasome activation. As evident in Fig. 2A-C, LPS/ATP remarkably elevated TXNIP, NLRP3, and Caspase-1 protein levels that were later completely suppressed by imipramine treatment. We further examined the NLRP3 inflammasome induced expression of IL-1β and IL-18 cytokines to examine the inhibitory effect of imipramine on NLRP3 inflammasome activation. The outcomes indicated that

LPS/ATP significantly increased IL-1β and IL-18 secretion in the supernatant, and pretreatment with imipramine significantly inhibited the secretion of IL-1β and IL-18 (Fig. 2D, E).

3.4 Inhibition of ASM activity attenuates LPS/ATP-induced NLRP3 inflammasome and TXNIP mRNA expression

The expressions of NLRP3 inflammasome and TXNIP mRNA were detected by RT-PCR. As shown in Fig. 2F-J, the expression of TXNIP, NLRP3, Caspase-1, IL-1β and IL-18 increased significantly after treated with LPS/ATP, compared with the control group. While imipramine pretreatment significantly reduced the mRNA levels 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.

We next tested the effect of verapamil as an inhibitor of Txnip on LPS/ATP-induced TXNIP protein expression and NLRP3 inflammasome activation. As evident in Fig. 3A-E, LPS/ATP remarkably elevated TXNIP, NLRP3, Caspase-1, IL-1β and IL-18 expression that were later completely suppressed by verapamil treatment. Besides, we also examined the effects of Txnip inhibitor verapamil on LPS/ATP-induced ASM activity and Cer content. As depicted in Fig. 1D, F, the Txnip inhibitor verapamil did not affect LPS/ATP-induced ASM activity and Cer content.

3.6 Inhibition of Txnip attenuates C2-Ceramide induced Txnip expression and NLRP3 inflammasome activation.

We further examined the effect of verapamil, a TXNIP inhibitor, on C2-Ceramide induced TXNIP protein expression and NLRP3 inflammasome activation to investigate if ASM/Cer pathway has TXNIP/NLRP3 inflammasome as a downstream module. In short, cells were pretreated with verapamil (10 μmol/L for 3 h) and co-incubated with C2-Ceramide (30 μmol/L for 5 h). The expression level of TXNIP, NLRP3, and Caspase-1 in J774A.1 cells was measured by western blot analysis. As shown in Fig. 4A-C, C2-Ceramide induced the TXNIP, NLRP3, and Caspase-1 protein expression, which was later suppressed by verapamil. We also investigated the effect of verapamil on C2-Ceramide induced IL-1β and IL-18 secretion. The supernatant quantification of IL-1β and IL-18 levels with the help of ELISA has shown that C2-Ceramide significantly elevated IL-1β and IL-18 secretion. However, pretreatment with verapamil significantly inhibited this effect and IL-1β and IL-18 levels in the supernatant (Fig. 4D, E).

4. Discussion

Macrophage, a vital part of the immune system, plays a crucial role in the innate immune response [16]. LPS is an essential molecule of the extracellular membrane of gram-negative bacteria, which besides maintaining the structural integrity of the cells, elicits the 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, we used the LPS/ATP-stimulated J774A.1 macrophage cells 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 can enable the development of more efficient drug targets for effective management of the resulting inflammatory disorders. Our study reveals the involvement of ASM in LPS/ATP-induced ceramide production in J774A.1 cells, which in turn elicits the NLRP3 inflammasome activation by mediating the TXNIP overexpression. We herein reported for the first time that the ASM/Cer/TXNIP signaling module braces the NLRP3 inflammasome activation.

Sphingolipid metabolism constitutes a significant part of lipid metabolism, which generates an array of active cellular lipids, imparts structural integrity to the cell and regulates a large number of crucial cellular functions [19, 20]. A plethora of stress stimuli generate 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 the ceramide accumulation triggers the assembly and activation of NLRP3 inflammasome in various pathological conditions [7, 8]. Two major 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 have been reported for the accumulation of intracellular ceramide [21, 23]. However, recent studies suggest that the elevated level of ceramide 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 ceramide levels [14]. Imipramine is a well known ASM inhibitor, which interferes with the ASM and lysosomal membrane interaction and elicits lysosomal destruction of ASM [24].

In this study, we found that ASM activity and the content of Cer significantly increased after LPS/ATP treatment, whilst the ASM inhibitor imipramine significantly attenuated the increase in ASM activity and Cer content in response to LPS/ATP. 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 if the ASM/Cer signaling pathway involves TXNIP/NLRP3 inflammasome activation as a downstream component.

TXNIP contributes significantly to biological processe such as the regulation of 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 the researchers to suppress TXNIP expression and the associated NLRP3 inflammasome activation with an aim to manage the inflammatory conditions [28]. In this study, verapamil suppressed LPS/ATP-induced TXNIP/NLRP3 inflammasome activation. However, it did not affect LPS/ATP-induced ASM activity and ceramide production. We further stimulated J774A.1 cells with C2-Ceramide 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-Ceramide could remarkably enhance the TXNIP, NLRP3, and Caspase-1 protein expression and IL-1β, IL-18 secretion. Furthermore, verapamil inhibited C2-Ceramide mediated TXNIP overexpression and NLRP3 inflammasome activation. Therefore, our findings suggest that ceramide leads to TXNIP overexpression and subsequent NLRP3 inflammasome activation.

Conclusion

In conclusion, this study revealed that ASM activation leads to LPS/ATP-induced Cer accumulation in J774A.1 cells. Besides, ceramide mediates the NLRP3 inflammasome activation by overexpression of TXNIP. Hence, in the current study, we successfully confirmed the crucial role of the ASM/Cer/TXNIP signaling pathway in NLRP3 inflammasome activation.

Abbreviations

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

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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Figures

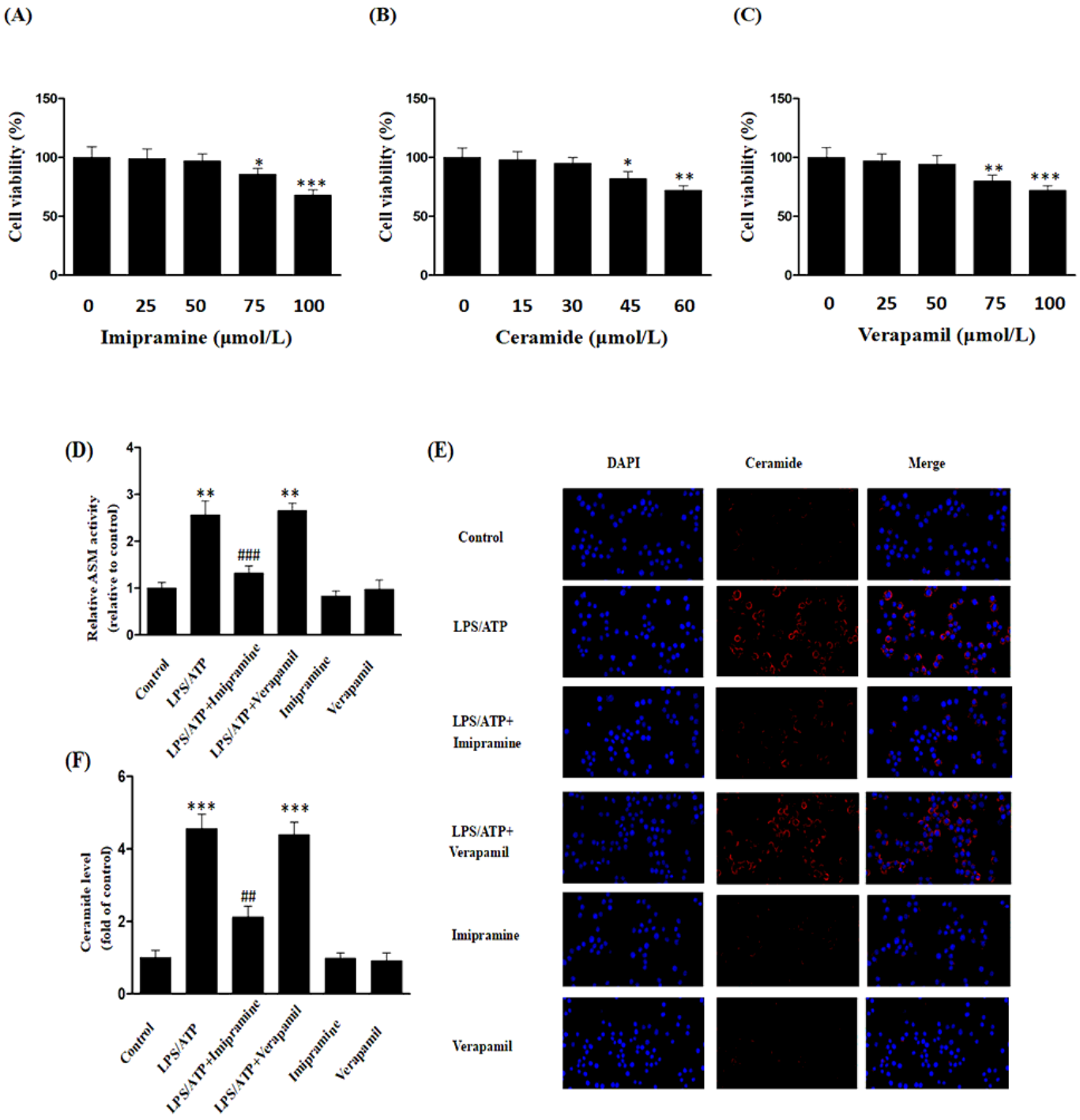

Figure 1

Effects of imipramine or verapamil on LPS/ATP-induced ASM activity and Cer production. Cells treated with a range of concentrations of imipramine (0, 25, 50, 75, 100 μmol/L), C2-ceramide (0, 15, 30, 45, 60 μmol/L) and verapamil (0, 25, 50, 75, 100 μmol/L) for 24 h. A) Effects of imipramine on cell viability; B) Effects of C2-ceramide on cell viability; C) Effects of verapamil on cell viability. J774A.1 cells were treated with 1 μg/mL LPS for 4 h followed by pretreatment with or not imipramine or verapamil (10 μmol/L) for 3

h, and then 30 min of incubation with 5mM ATP. D) Effects of imipramine or verapamil on ASM activity; E, F) Effects of imipramine or verapamil on Cer production. Compared with the control group, *P< 0.05, **P< 0.01, ***P< 0.001; Compared with the corresponding LPS/ATP treatment group. #P < 0.05, ##P< 0.01, ###P< 0.001.

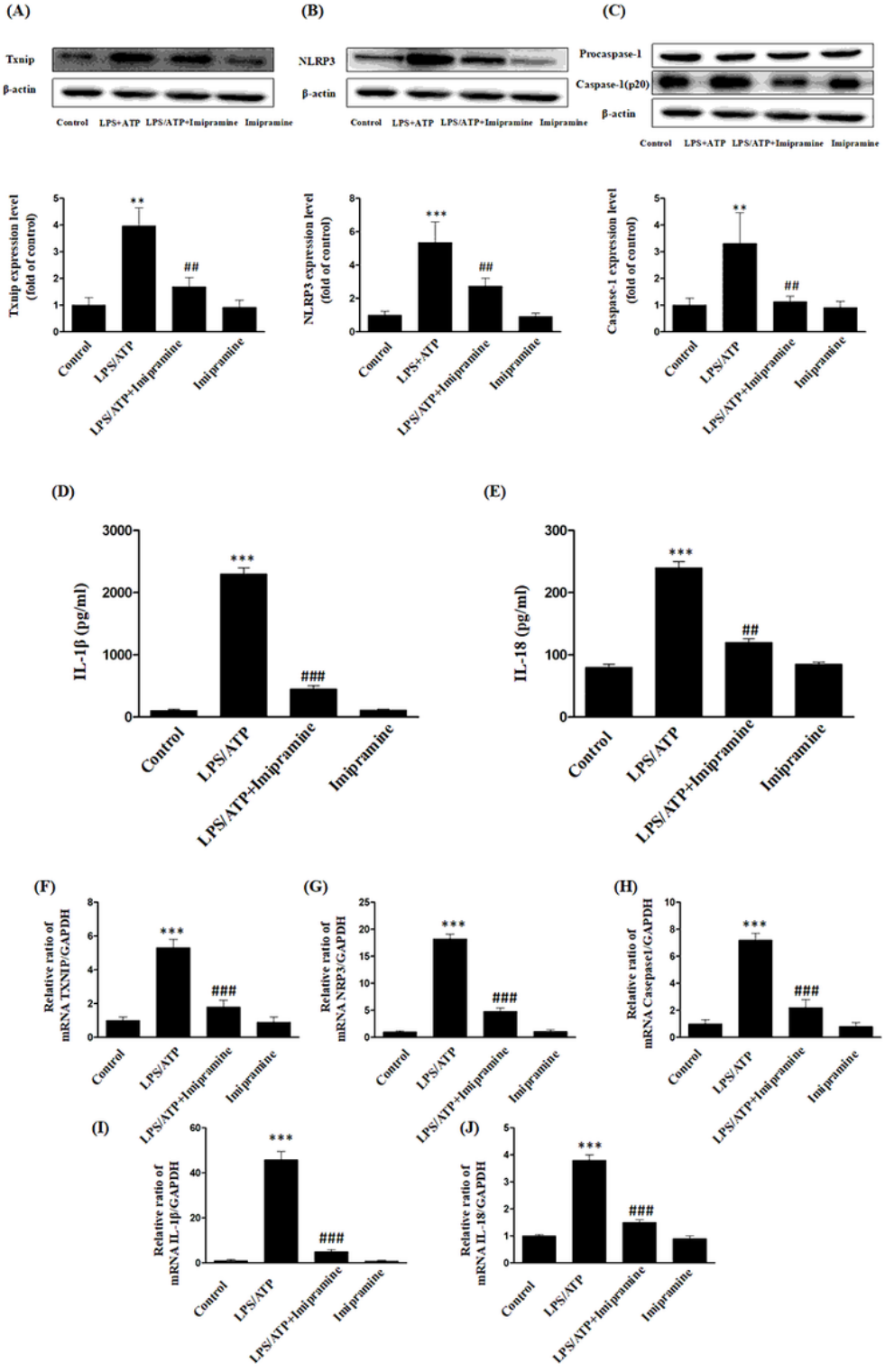

Figure 2

Inhibition of ASM activity attenuates LPS/ATP-induced Txnip expression and NLRP3 inflammasome activation. J774A.1 cells were treated with 1 μg/mL LPS for 4 h followed by pretreatment with or not imipramine (10 μmol/L) for 3 h, and then 30 min of incubation with 5mM ATP. Effects of imipramine on the protein expression: A) Txnip; B) NLRP3; C) Caspase-1; D) IL-1β; E) IL-18. Effects of imipramine on the mRNA expression: F) Txnip; G) NLRP3; H) Caspase-1; I) IL-1β; J) IL-18. Compared with the control group, *P< 0.05, **P< 0.01, ***P< 0.001; Compared with the corresponding LPS/ATP treatment group. #P< 0.05, ##P < 0.01, ###P< 0.001.

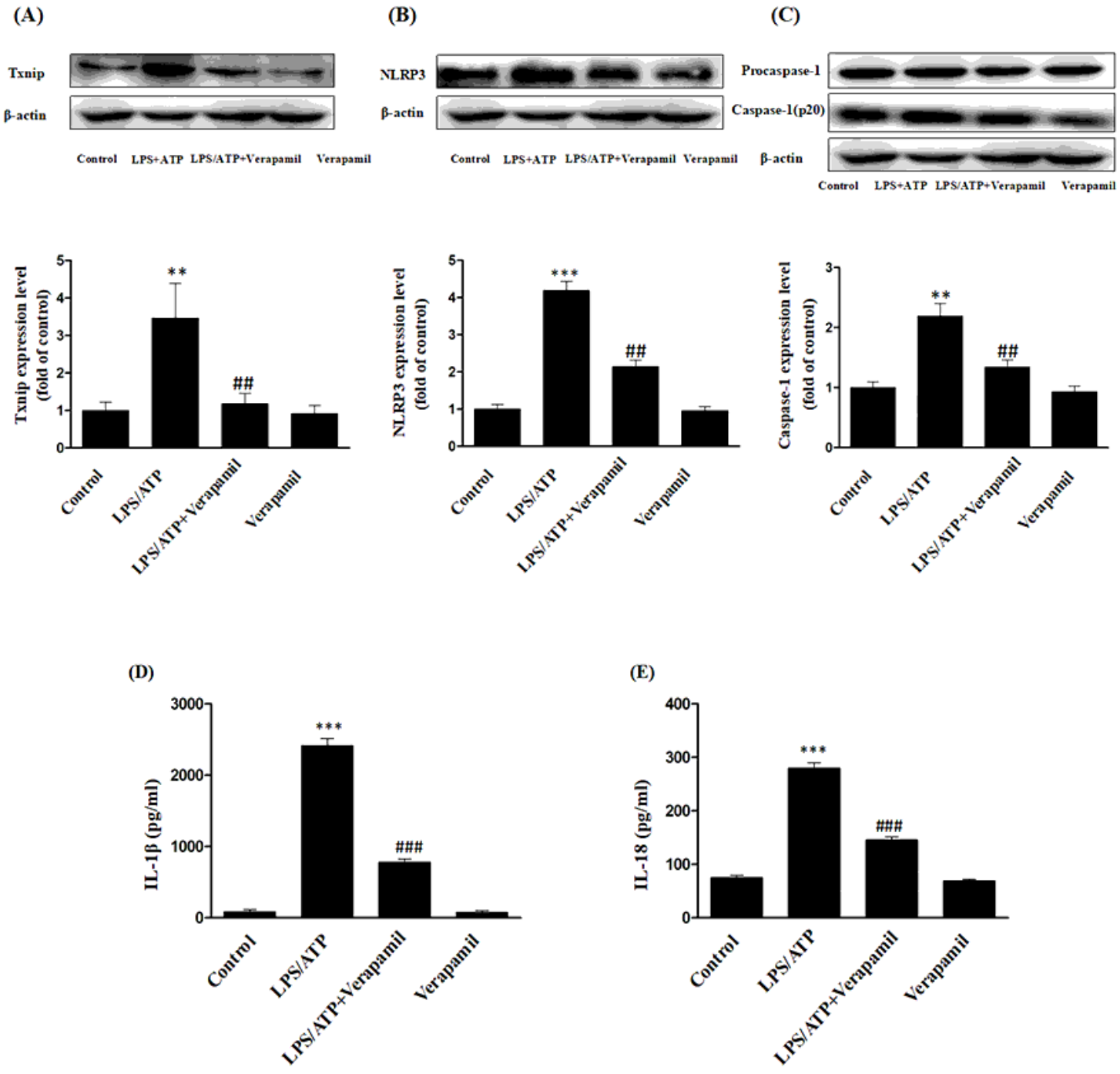

Figure 3

Inhibition of Txnip attenuates LPS/ATP-induced Txnip expression and NLRP3 inflammasome activation. J774A.1 cells were treated with 1 μg/mL LPS for 4 h followed by pretreatment with or not verapamil (10 μmol/L) for 3 h, and then 30 min of incubation with 5mM ATP. Effects of verapamil on the protein expression: A) Txnip; B) NLRP3; C) Caspase-1. Effects of verapamil on the IL-1β and IL-18 secretion: D) IL-1β; E) IL-18. Compared with the control group, *P< 0.05, **P< 0.01, ***P< 0.001; Compared with the corresponding LPS/ATP treatment group. #P< 0.05, ##P < 0.01, ###P< 0.001.

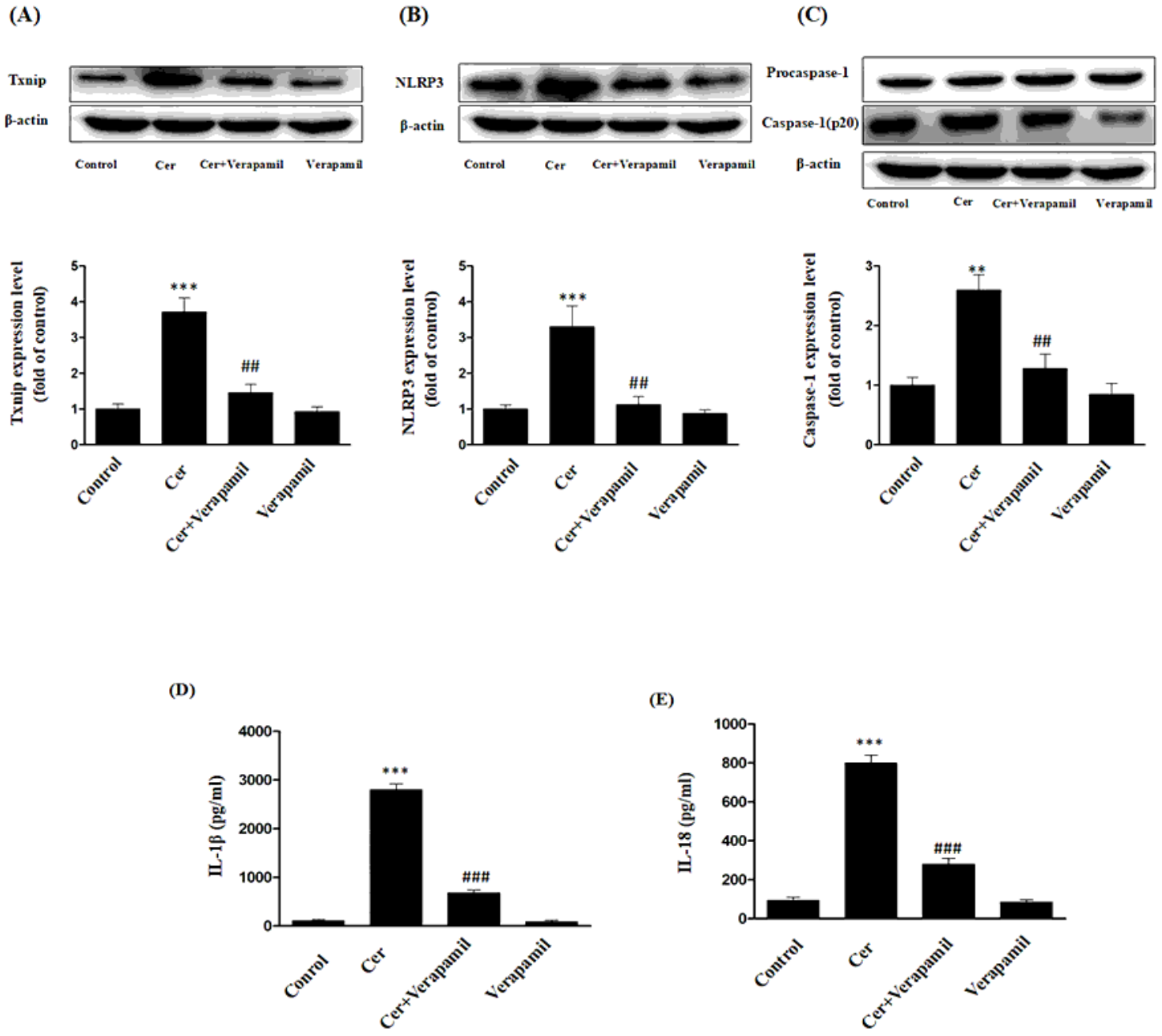

Figure 4

Inhibition of Txnip attenuates C2-Ceramide induced Txnip expression and NLRP3 inflammasome activation. J774A.1 cells were pretreated with verapamil (10 μmol/L for 3 h) and co-incubated with C2-Ceramide (30 μmol/L for 5 h). Effects of verapamil on the protein expression: A) Txnip; B) NLRP3; C) Caspase-1. Effects of verapamil on the IL-1β and IL-18 secretion: D) IL-1β; E) IL-18. Compared with the control group, *P< 0.05, **P< 0.01, ***P< 0.001; Compared with the corresponding C2-Ceramide treatment group. #P< 0.05, ##P < 0.01, ###P< 0.001.