NACHT, LRR and PYD domains-containing protein 3 inflammasome is activated and inhibited by berberine via toll-like receptor 4/myeloid differentiation primary response gene 88/nuclear factor-κB pathway, in phorbol 12-myristate 13-acetate-induced macrophages

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Abstract. The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP-3) inflammasome has recently emerged as a pivotal regulator of chronic inflammation. The present study investigated the expression of NLRP3 inflammasome in phorbol 12-myristate 13-acetate (PMA)-induced macrophages, and aimed to identify the effects of berberine on the inflammasome. Human monocytic THP-1 cells were pretreated with berberine for 1 h and then induced with PMA for 48 h. Total RNA and protein were collected for reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Supernatants were collected to determine IL-1β levels by using ELISA. The present study demonstrated that NLRP3 inflammasome and IL-1β were activated in PMA-induced macrophages in a time-dependent manner, whereas berberine significantly inhibited their expression in a dose-dependent manner in PMA-induced macrophages. Furthermore, berberine also suppressed the toll-like receptor 4 (TLR4)/myeloid differentiation primary response gene 88 (Myd88)/nuclear factor (NF)-κB signaling pathway which was activated during the conversion of THP-1 cells to macrophages by PMA. In conclusion, berberine reduced NLRP3 inflammasone expression by suppressing the activation of the TLR4/Myd88/NF-κB signaling pathway in PMA-induced macrophages. This inhibitory effect may imply an important role of berberine on chronic inflammation and atherogenic progression in coronary artery disease.

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Introduction

Chronic inflammation is one of the well-established causative conditions for the development and progression of atherosclerosis, whereas inciting inflammation in the artery wall remains largely unknown. Monocyte-derived macrophages in late atherogenesis are regarded as primary inflammatory stimuli through either synthesis or secretion of numerous substances, such as interleukin (IL)-1β and -18 (1,2). A clinical report has demonstrated that increased expression levels of IL-1β are associated with clinical severity in patients with coronary artery disease (CAD) (3). Additionally, targeting IL-1β with a monoclonal antibody can impede the progression of atherosclerosis in $ApoE^{-/-}$ mice (4).

The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP-3) inflammasome is formed around NLRP3, which also contains the adaptor molecules; apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1. The NLRP3 inflammasome has been demonstrated to be associated with chronic inflammation, resulting in IL-1β and IL-18 maturation (5). Previously, aberrantly elevated NLRP3 inflammasome levels have been reported to be associated with vascular inflammation and endothelial dysfunction in atherosclerotic pig aorta (6). Furthermore, gene silencing of NLRP3 has prevented plaque progression increasing atherosclerotic plaque stability by inhibiting proinflammatory cytokines (7). In a previous study, NLRP3 inflammasome levels were higher in the CAD group compared with the non-CAD group, with a positive association between NLRP3 inflammasome and IL-1β and

IL-18 levels (8). Based on these observations, dysregulated NLRP3 inflammasome activation may have been involved in the pathogenesis of atherosclerosis, and the NLRP3 inflammasome might serve as a potential candidate for ameliorating the development of atherosclerosis.

Toll-like receptor 4 (TLR4), an intensively investigated member of the TLR family, serves a critical role in initiating inflammation (9) and participates in mediating inflammatory cell formation (10). As the downstream effectors of TLR4 (11), myeloid differentiation primary response gene 88 (MyD88) and nuclear factor (NF)-κB regulate the expression of many inflammatory genes and participate in the development of several diseases, such as cancer (12), inflammatory bowel disease and atherosclerosis (13-15). Additionally, studies have demonstrated that NF-κB induces NLRP3 expression and NLRP3 inflammasome activity (16,17). In the present study, the role of the TLR4/Myd88/NF-κB signaling pathway was investigated in phorbol 12-myristate 13-acetate (PMA)-induced macrophages. Furthermore, the effects of isoquinoline alkaloid berberine on this signaling pathway were also studied.

Berberine, a botanical alkaloid, is isolated from medicinal herbs, such as *Rhizoma coptidis* (Chinese name, Huanglian) and Cortex phellodendri (Chinese name, Huangbai) (18). Berberine has been reported to inhibit N-acetyltransferase activity in several tumor cells in a dose-dependent manner, including human bladder tumor (carcinoma) cells (T24) (19). Additionally, berberine has been reported to have an anti-obesity effect by inhibiting adipocyte differentiation and lipid accumulation in 3T3L-1-cells (20). Furthermore, increasing evidence has demonstrated that berberine has an anti-atherosclerosis effect in cardiovascular disease (21-23). Therefore, the present study investigated the effect of berberine on NLRP3 inflammasome activation in PMA-induced macrophages.

Materials and methods

Reagents. RPMI 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin (pen/strep, 10,000 U/ml each) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Dimethyl sulfoxide (DMSO), PMA, and berberine were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). BAY 11-7082 and nuclear and cytoplasmic extraction reagents were obtained from Beyotime Institute of Biotechnology (Shanghai, China). TRIzol reagent for RNA isolation was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Omniscript reverse transcriptase for first-strand cDNA synthesis was obtained from Qiagen GmbH (Hilden, Germany). Anti-IL-1β (cat. no. 12703), anti-NLRP3 (cat. no. 13158), anti-ASC (cat. no. 13833), anti-p65 (cat. no. 8242), anti-p-IκB-α (cat. no. 9246), anti-lamin B (cat. no. 13435) and anti-GAPDH (cat. no. 5174) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-TLR4 (cat. no. ab22048) and anti-Myd88 (cat. no. ab2064) were produced by Abcam (Cambridge, UK). Anti-caspase-1 (cat. no. sc-56036) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit anti-mouse conjugated-horseradish peroxidase (HRP) (cat. no. A16166) and goat anti-rabbit conjugated-HRP (cat. no. A10547) secondary antibodies were from Invitrogen; Thermo Fisher Scientific, Inc. A Bicinchoninic Acid protein assay kit was produced by Pierce; Thermo Fisher Scientific, Inc. Polyvinylidene difluoride membranes were obtained from EMD Millipore (Billerica, MA, USA).

Cell culture. The THP-1 human monocyte cell line was purchased from the American Type Culture Collection (Manassas, MD, USA) and maintained at a density of 10^6/ml in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin solution at 37˚C in a 5% CO_2 incubator. Cells at a density of $5x10^5$/ml per well were cultured in 6-well plates for 48 h in the presence of 100 nM PMA except the control group, which allowed them to differentiate into adherent macrophages (24). Berberine was dissolved in DMSO at a final concentration of 5, 10, 25 or 50 μM in Berberine-treated groups 1 h prior to adding PMA. THP-1 cells that were treated with DMSO (1:1,000 in culture medium) only in PMA-treated group served as the control.

RNA isolation, cDNA synthesis, and Taqman reverse transcription-quantitative polymerase chain reaction (RT-qPCR). THP-1 cells were homogenized in TRIzol for extraction of RNA according to the manufacturer's protocol. RT-qPCR was carried out using a Superscript III cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) and SYBR-Green reagent kits (Invitrogen; Thermo Fisher Scientific, Inc.). Relative expression data was analyzed using the 2-ΔΔCq method (25). The specific primers of genes were synthesized from Invitrogen; Thermo Fisher Scientific, Inc. The following primers were used: i) *IL-1*β forward, 5'-ACTGCCTGCCTTAGGGTAG-3' and reverse, 5'-GTGGGAGCGAATGACAGAG-3'; ii) *NLRP3* forward, 5'-CTGGAGGATGTGGACTTG-3' and reverse, 5'-GTCTGCCTTCTCTGTCTG-3'; and iii) *GAPDH* forward, 5'-CACCCACTCCTCCACCTTTG-3' and reverse, 5'-CCACCACCCTGTTGCTGTAG-3'. GAPDH was used as an internal housekeeping control.

Protein isolation and western blot analysis. Protein extracts from all treatment groups were isolated from the cytoplasm and the nucleus using nuclear protein and cytoplasmic protein extraction kit (cat. no. P0027; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Protein isolation and Western blot analysis of the cell lysates were performed as previously described (26). Lysates (50 μg) micrograms were separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane. Each membrane was pre-incubated for 1.5 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween-20 and 5% non-fat milk (Sangon Biotech Co., Ltd., Shanghai, China). Each membrane was incubated with primary IL-1β, NLRP3, ASC, p65, p-IκB-α, lamin B, TLR4, Myd88, GAPDH antibodies diluted 1:1,000 and caspase-1 diluted 1:200 at 4˚C overnight. Bands were then detected by incubating with a secondary antibody (diluted 1:1,000) conjugated with HRP at room temperature for 2 h and visualizing using enhanced chemiluminescence reagents (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were then analyzed using Image J analysis software (version no. 2.1.4.7; National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH and lamin B.

ELISA assays of proinflammatory cytokines. For the determination of IL-1β levels in cell medium, a cytokine-specific ELISA kit (cat. no. DLB50; R&D Systems Europe Ltd., Abingdon, UK) was used according to the manufacturer's protocol.

Statistical analysis. Data are expressed as the mean ± standard deviation. Results were analyzed by one-way analysis of variance (ANOVA) with Student-Newman-Kewls and Dunnettis methods as post hoc tests using SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA). All experiments were performed at least three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IL-1β level and NLRP3 inflammasomes are activated in PMA-induced macrophages in a time-dependent manner. IL-1β protein expression levels in PMA-induced macrophages were upregulated in a time-dependent manner from 6 to 48 h, reaching a peak at 48 h (Fig. 1A and B). Additionally, NLPR3 inflammasome expression levels were upregulated when macrophages were treated with PMA in a time-dependent manner (Fig. 1A). Furthermore, as a critical component of inflammasomes, NLPR3 activates caspase-1, which is a (p20/p10) tetramer necessary and sufficient for the cleavage of IL-1β precursor, through its adaptor ASC. In the present study, both ASC and pro-caspase-1 were activated in PMA-induced macrophages in a time-dependent manner (Fig. 1A and C). These experiments indicated that NLRP3 inflammasomes and its downstream signaling cascade may be involved in the differentiation process of macrophages when treated by PMA.

Reduced IL-1β and NLRP3 inflammasome levels by berberine, in a dose-dependent manner in PMA-induced macrophages. Our previous study has demonstrated that berberine doses ranging from 5 to 75 μM caused no significant reduction (~5-10%) in cell viability, and doses ranging from 5 to 50 μM were used in subsequent experiments (27). In the present study, ELISA was performed to investigate the effects of berberine on the secretion of IL-1β in the supernatant. THP-1 cells were pretreated with the indicated concentration of berberine for 1 h, followed by treatment with PMA (100 nM) for 48 h. As illustrated in Fig. 2A, IL-1β was sharply increased in the supernatant of PMA-treated group, whereas berberine treatment significantly reduced its secretion in a dose-dependent manner. Similar results were observed in THP-1 cells at the protein (Fig. 2B and C) and mRNA level (Fig. 2C), as detected by western blotting and RT-qPCR, respectively. Thus, IL-1β expression is reduced by berberine, at both transcription and translation level in PMA-induced macrophages.

Given that IL-1β expression is positively regulated by NLRP3 inflammasomes (28) and berberine was demonstrated to reduce IL-1β expression in PMA-induced macrophages, the present study then investigated whether the inhibitory effect of berberine on IL-1β expression might be a consequence of the inhibition of NLRP3 inflammasome expression in PMA-induced macrophages. Results consistently demonstrated that berberine had a similar effect on NLRP3 at both the mRNA and protein level in PMA-induced macrophages

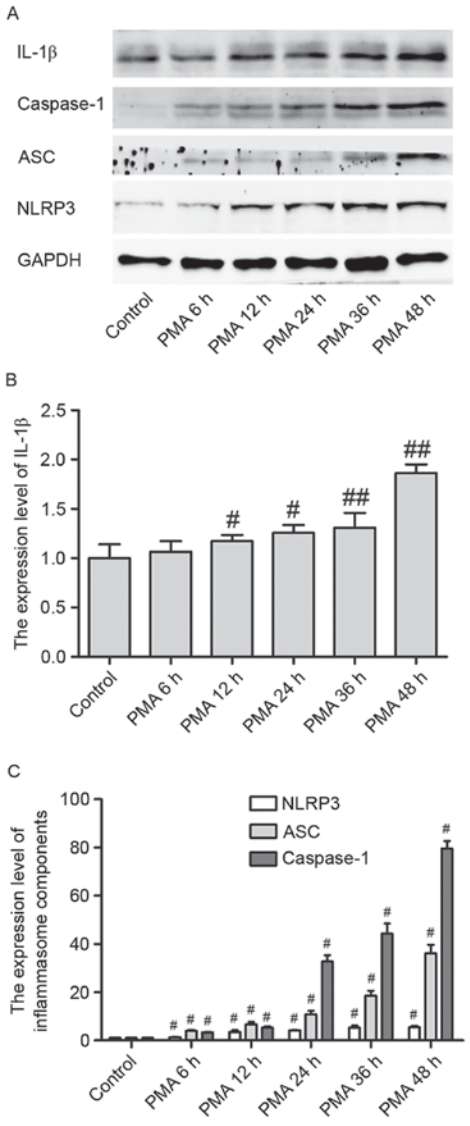

Figure 1. NLRP3 inflammasome activation following the upregulation of IL-1β in PMA-induced macrophages in a time-dependent manner. (A) Representative western blot images and (B and C) quantification of protein expression levels of IL-1β and NLRP3 inflammasome components. Band density of native THP-1 cells were defined as control and set to 1. Data are presented as the mean ± standard deviation. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. control. IL-1β, interleukin-1β; NLRP3, NACHT, LRR and PYD domains-containing protein 3; PMA, phorbol 12-myristate 13-acetate; Ber, berberine; ASC, apoptosis-associated speck-like protein containing a CARD.

(Fig. 2B and D). Furthermore, the expression of ASC and activated caspase-1 were both significantly inhibited in the berberine-treated group (Fig. 2B and D). This downregulation of NLRP3 inflammasomes by berberine may be partly responsible for the reduction of IL-1β expression in PMA-induced macrophages.

TLR4/Myd88/NF-κB pathway is involved in the activation of NLRP3 inflammation in PMA-induced macrophages. In the present study, in order to assess whether the TLR4/Myd88/NF-κB signaling pathway is stimulated by PMA, its expression pattern at different time points (6, 12, 24, 24, 36 and 48 h) was investigated, in THP-1 cells. The expression of TLR4, Myd88, p-IκBα and p65 in cell nuclei were all upregulated by PMA in a time dependent manner (Fig. 3A-D).

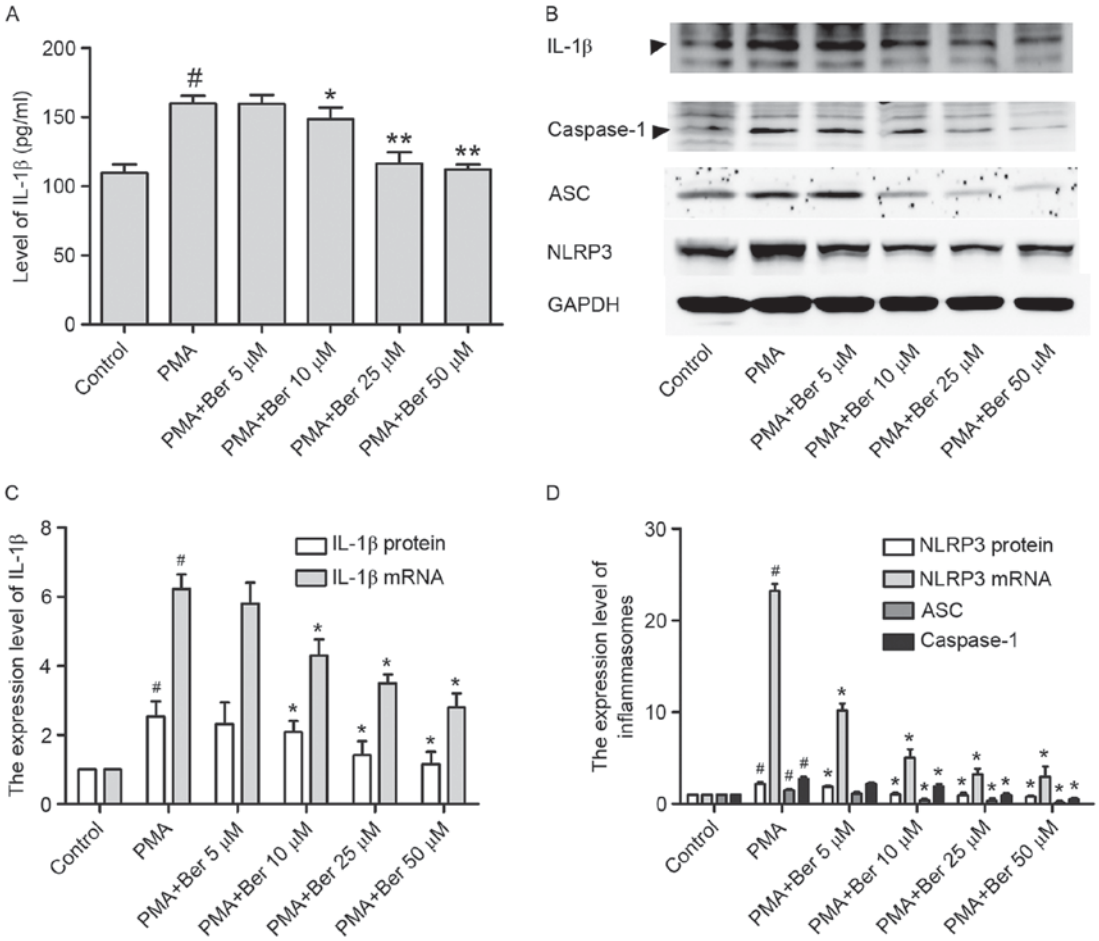

Figure 2. Berberine inhibits the NLRP3 inflammasome activation and IL-1β synthesis in PMA-induced macrophages. (A) Berberine effects on the secretion of IL-1β in cell supernatant of THP-1 cells using ELISA. (B) Berberine reduces protein expression levels of IL-1β (black arrow), Caspase-1 (black arrow), ASC and NLRP3 in PMA-induced macrophages. (C) Berberine suppresses IL-1β both at the protein and mRNA levels in PMA-induced macrophages. (D) Berberine inhibits NLRP3 both at the protein and mRNA levels in PMA-induced macrophages. Band density of native THP-1 cells were defined as control and set to 1. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. PMA group; $^{\#}$P<0.05 vs. control. IL-1β, interleukin-1β; NLRP3, NACHT, LRR and PYD domains-containing protein 3; Ber, berberine; PMA, phorbol 12-myristate 13-acetate; ASC, apoptosis-associated speck-like protein containing a CARD.

Whether this pathway is also involved in the upregulation of NLRP3 inflammasome expression in PMA-induced macrophages was investigated. Fig. 3E and F illustrate that the NLRP3 increased levels were partly abrogated in PMA-induced macrophages by treating cells with BAY 11-7082 (NF-κB inhibitor), suggesting that the activation of NF-κB signaling pathway may be involved in the activation of NLRP3 inflammasomes.

TLR4/Myd88/NF-κB signaling pathway is required for the inhibitory effect of berberine on NLRP3 inflammasome expression in PMA-induced macrophages. Given that berberine may lead to downregulation of NLRP3 expression and that activation of the TLR4/Myd88/NF-κB signaling pathway is involved in activating NLRP3 in PMA-induced macrophages, whether the inhibitory effect of berberine on NLRP3 expression was associated with the TLR4/Myd88/NF-κB signaling pathway was investigated. THP-1 cells in berbeirne-treated groups were pretreated with berberine at the indicated concentration (5 to 50 μM) for 1 h and were subsequently cultured with PMA for 48 h. Berberine significantly inhibited the protein expression of TLR4, Myd88, p-IκBα and p65 induced by PMA in a dose-dependent manner (Fig. 4). By inhibiting the activation of the TLR4/Myd88/NF-κB signaling pathway, berberine conclusively downregulates NLRP3 expression in PMA-induced THP-1 macrophages.

Discussion

The main findings of the present study were the following: i) Expression levels of NLRP3 (both at mRNA and protein level), ASC and caspase-1 were all upregulated in a time-dependent manner, leading to IL-1β maturation during the conversion of THP-1 cells to macrophages by PMA; ii) berberine effectively suppressed mRNA and protein expression levels of NLRP3 inflammasomes in a dose-dependent manner in PMA-induced macrophages; and iii) the TLR4/Myd88/NF-κB signaling pathway was partly involved in the inhibition of NLRP3 inflammasome by berberine.

Atherosclerosis is considered as a chronic inflammation process and is influenced by inflammatory mediators. A recent study has suggested the causal role of IL-1β in the development and progression of atherosclerotic vascular disease (29). Studies in mouse models have demonstrated that mice with IL-1β mutation/repression have low atherosclerosis risk (30), whereas mice over-expressing IL-1β can develop atherogenesis (31). Furthermore, genetic deletion of IL-1 receptor type I

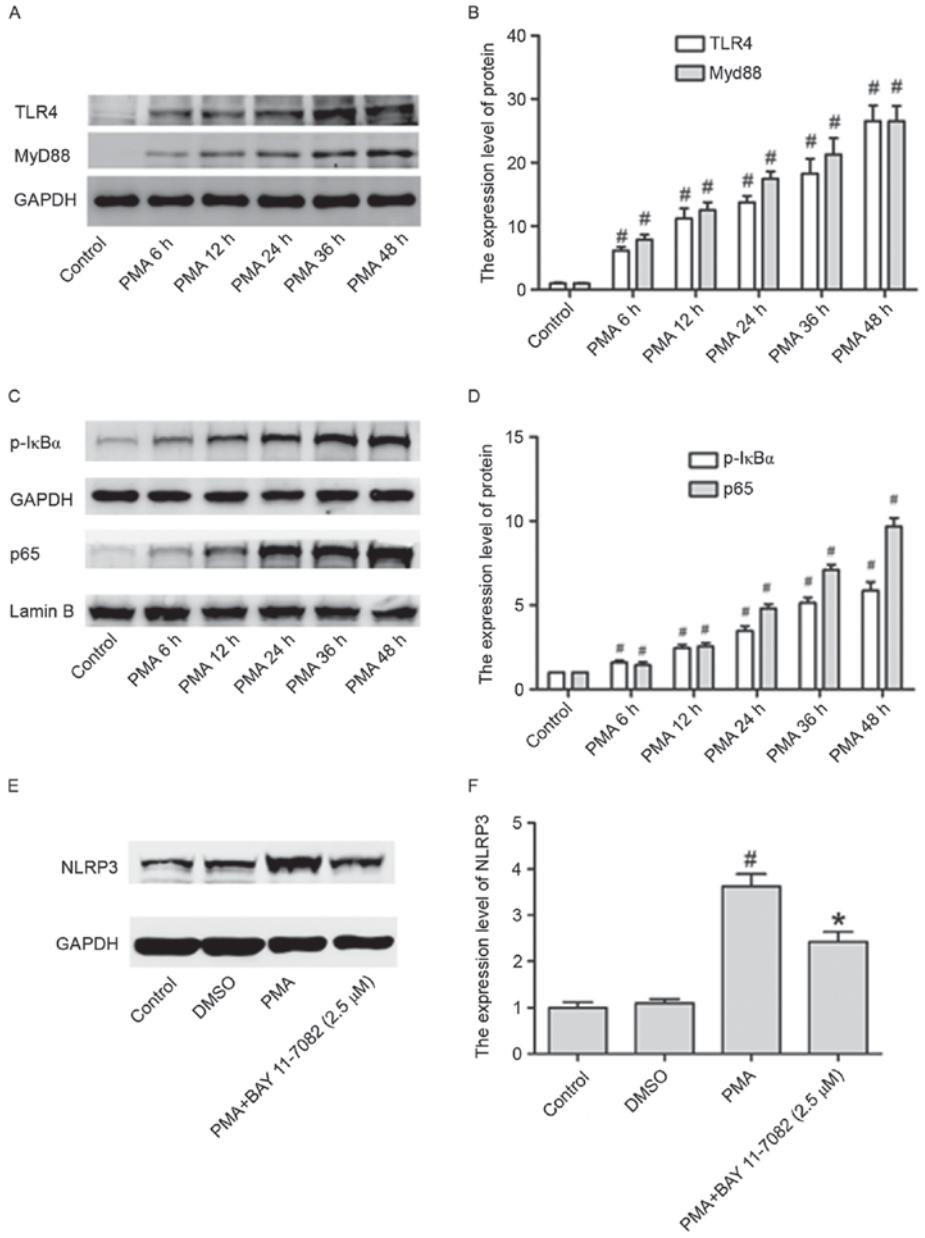

Figure 3. TLR4/Myd88/NF-κB signaling pathway is involved in the activation of NLRP3 inflammation in PMA-induced macrophages. (A) Representative western blot images of TLR4 and Myd88 stimulated by PMA in macrophages and (B) quantification. (C) Protein expression levels of p-IκB-α in the cytoplasm and p65 in the nuclei were upregulated during conversion of THP-1 cells to macrophages by PMA and (D) quantification. (E) NLRP3 activation was abrogated in PMA-induced macrophages by treating cells with BAY 11-7082 (NF-κB inhibitor) and (F) quantification. Band density of native THP-1 cells were defined as control and set to 1. Data are presented as the mean ± standard deviation. $^{\#}$P<0.01 vs. control group, *P<0.01 vs. PMA group. TLR4, toll-like receptor 4; PMA, phorbol 12-myristate 13-acetate; MyD88, myeloid differentiation primary response gene 88; PMA, phorbol 12-myristate 13-acetate; p-IκB-α, phosphorylated inhibitor of κB-α; DMSO, dimethyl sulfoxide; NF-κB, nuclear factor-κB.

in atherosclerosis-prone mice leads to a reduction of advanced atherosclerotic plaques size in the aortic root (32). In the present study, both mRNA and protein expression levels of IL-1β were increased in PMA-induced macrophages, in a time-dependent manner. Furthermore, an increase of IL-1β expression in the supernatant of the PMA-induced group was demonstrated by ELISA, indicating IL-1β as a key factor in the course of atherosclerosis. Additionally, berberine significantly inhibited IL-1β levels in PMA-induced macrophages, reducing its secretion.

IL-1β is involved in at least two separate signaling cascades (33,34). In the first cascade, pattern recognition and cytokine receptors in host cells control pro-IL-1β transcription (33). In the second cascade, the protein complex known as the inflammasome regulates the proteolytic process of pro-IL-1β into a mature IL-1β (28). In the present study, focus was given on the NLRP3 inflammasome functions and the NLRP3 inflammasome was illustrated to be activated in PMA-induced macrophages, in a time-dependent manner. The NLRP3 inflammasome consists of NLRP3, ASC and pro-caspase-1. Inflammation induces NLRP3 inflammasome oligomerization leading to the recruitment of ASC, which controls the activation of caspase-1. Activated p10 and p20 caspase-1 are able to cleave 117 amino acids of pro-IL-1β N-terminus leading to the bioactive form of 17 kD (35,36). The NLRP3 inflammasome has recently been reported to be activated by several endogenous molecules, such as glucose (37),

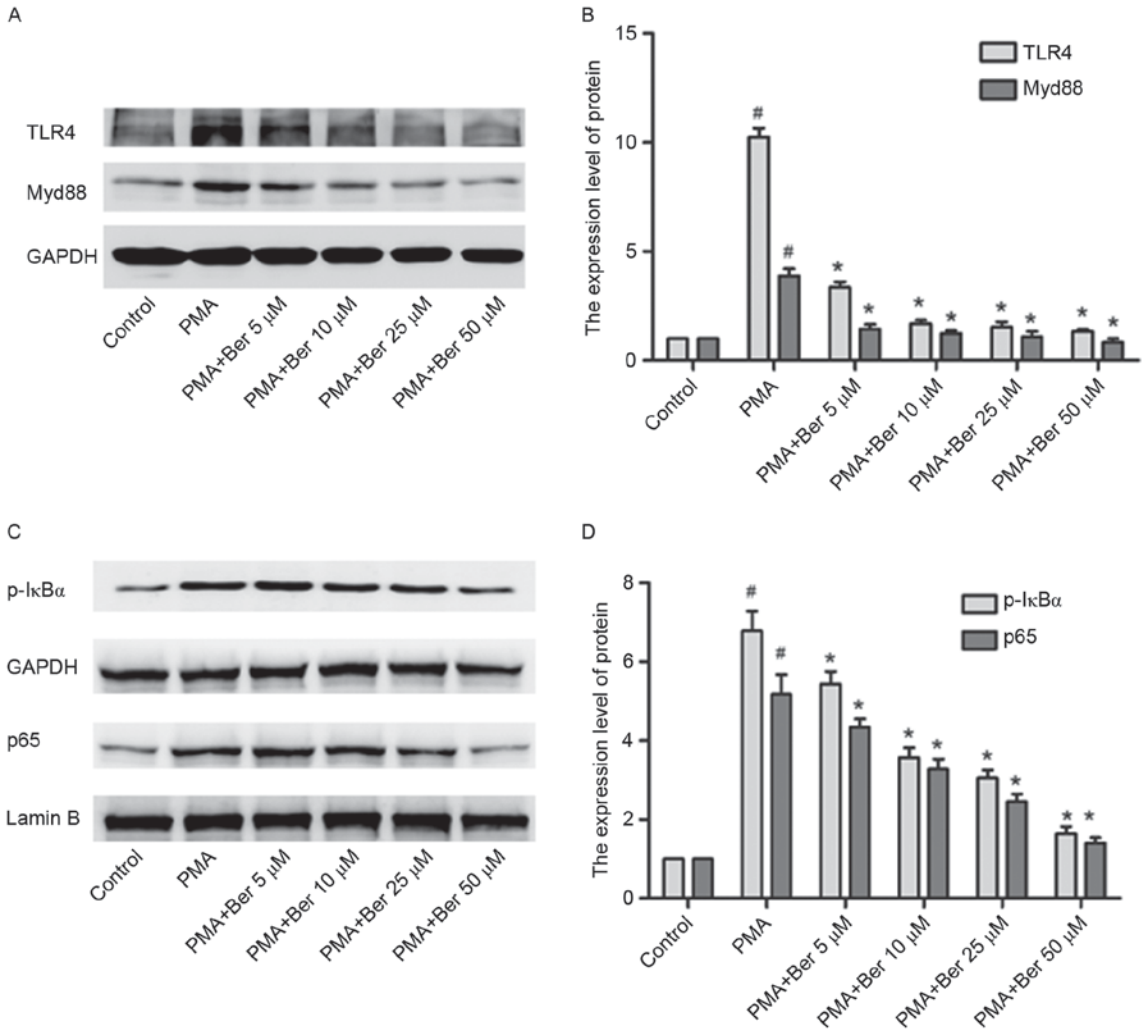

Figure 4. TLR4/Myd88/NF-κB signaling pathway is inhibited by berberine in a dose-dependent manner in PMA-induced THP-1 macrophages. (A) Representative western blot images of TLR4 and Myd88 and (B) quantification. (C) Representative western blot images of p-IκB-α and p65 expression and (D) quantification. Data are presented as the mean ± standard deviation. $^{\#}$P<0.01 vs. control group, *P<0.01 vs. PMA group. NLRP3, NACHT, LRR and PYD domains-containing protein 3; Ber, berberine; PMA, phorbol 12-myristate 13-acetate; MyD88, myeloid differentiation primary response gene 88; TLR4, toll-like receptor 4; p-IκB-α, phosphorylated inhibitor of κB-α.

oxidized low density lipoprotein (LDL) (38) and cholesterol crystals (38), which are recognized as a hallmark in atherosclerotic lesions. NLRP3- or ASC-deficient mice fed with a high-cholesterol diet exhibit markedly decreased atherosclerosis and inflammasome-dependent proinflammatory cytokine levels (38). Furthermore, Vandanmagsar *et al* (39) demonstrated that ablation of NLRP3 in mice fed a high-fat diet prevents inflammasome activation and reduces production of IL-1β. Furthermore, increasing evidence has demonstrated that the activation of the NLRP3 inflammasome is involved in atherosclerosis-associated inflammation (7,8,40). In the current study, the expression of the NLRP3 inflammasome, which regulates the activated forms of ACS and pro-caspase-1, were strongly upregulated in PMA-induced macrophages. Berberine markedly inhibited NLRP3 and ACS expression and suppressed pro-caspase-1 activation in a dose-dependent manner. Given the vital roles of the NLRP3 inflammasome in the development of atherosclerosis, and considering the inhibitory effects of berberine on NLRP3 inflammasomes, berberine may exert beneficial properties by ameliorating chronic inflammation caused by macrophages.

Berberine is a common drug used for treating diarrhea and gastrointestinal disorders (41). Increasing evidence has demonstrated that berberine has protective effects against atherosclerotic diseases. Berberine can counteract high-fat diet-elicited hyperhomocysteinemia and hyperlipidemia by upregulating LDL receptor and apolipoprotein E mRNA levels and by suppressing 3-hydroxy-3-methylglutaryl-CoA reductase gene expression. Furthermore, no atherosclerotic lesions were developed in berberine-treated rats for 16 weeks (21). Berberine abrogates the formation of foam cells, which serve a critical role in the progression of atherosclerosis, by enhancing LXRalpha-ABCA1-dependent cholesterol efflux (42). Furthermore, berberine reduces oxidative stress and vascular inflammation, and suppresses atherogenesis via stimulation of AMPK-dependent UCP2 expression (42). Our previous study has illustrated that berberine exerts anti-atherogenic effects by inhibiting matrix metalloproteinase-9 and extracellular matrix metalloproteinase inducer (27). This provides some evidence that supports the multiple functions of berberine in the prevention of atherosclerosis. In the present study, berberine suppressed NLRP3 inflammasome expression and decreased

the production and secretion of IL-1β. These results illustrate some of the anti-atherogenic effects of berberine.

Few studies have demonstrated that the TLR4/Myd88/NF-κB signaling pathway is involved in regulatng NLRP3 inflammasome expression (16,43,44). In the present study, the expression levels of TLR4, Myd88 and NF-κB were all increased in PMA-induced macrophages. The expression of the NLRP3 inflammasome was partly abrogated by BAY 11-7082 (a recognized NF-κB inhibitor). Additionally, berberine significantly inhibited activation of the TLR4/Myd88/NF-κB signaling pathway. Berberine was observed to suppress the activation of NLRP3 inflammasome via the inhibition of the TLR4/Myd88/NF-κB signaling pathway.

In conclusion, berberine significantly inhibited the upregulation of the NLRP3 inflammasome, decreasing the production and secretion of IL-1β via inhibition of the TLR4/Myd88/NF-κB signaling pathway in PMA-induced macrophages. Considering the vital role of the IL-1β and NLRP3 inflammasome in chronic inflammatory responses, these results provide novel insights of how berberine may mitigate atherogenic progression, and further confirm its anti-inflammatory effects in atherosclerotic disease. However, further studies are required to confirm if the above effects of berberine also occur *in vivo*.

Acknowledgements

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