Short communication

Berberine inhibits LPS-induced TF procoagulant activity and expression through NF-κB/p65, Akt and MAPK pathway in THP-1 cells

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A B S T R A C T

Background: Considering the key role of TF in coagulation of sepsis or acute lung injury (ALI), we investigated whether berberine (BBR) could inhibit TF expression and procoagulant activity and explored its possible mechanism.

Methods: The effects of berberine on the expression, procoagulant activity of TF and related signal pathways induced by lipopolysaccharide (LPS) were observed in THP-1 cells.

Results: Our results showed that berberine could inhibit LPS-induced TF activity and expression, and down-regulate NF-κB, Akt and MAPK/JNK/p38/ERK pathways.

Conclusion: Berberine inhibits TF expression and related pathway, which provides some new insights on its mechanism for sepsis treatment.

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Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are characterized by pulmonary edema and neutrophilic inflammation and cause acute respiratory failure in patients of all ages. Recent research has shown that abnormalities in coagulation and fibrinolysis are present in ALI and may be the result of ongoing inflammation in the lungs. Thrombin formation and fibrin deposition, which appear as hyaline membranes lining the denuded alveolar surface, have become one of the typical pathological signs in ALI/ARDS patients [1,2]. Tissue factor (TF), a 47-kDa protein, is the only blood coagulation factor that exists outside the plasma in normal cells and tissue. As the initiator of the coagulation cascade reaction, TF participates in ALI/ARDS and locally activates various cytokines in the lungs as well as the systemic inflammatory reaction in patients suffering from ALI/ARDS [1]. TF-related signaling pathways, including the NF-kappa B and MAPK/JNK/p38/ERK pathways, are involved in ALI development after lipopolysaccharide (LPS) stimulation [2–4], which causes the production of intracellular reactive oxygen species through Toll-like receptor 4. Multiple cell types, including endothelial cells, alveolar macrophages and monocytes, have been associated with ALI [5,6]. Furthermore, monocytes synthesize TF in response to various pathophysiological procedures that are important in in vitro models of sepsis [7]. After stimulation with agents such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and phorbol ester (PMA), the procoagulant activity and expression of tissue factor are up-regulated in human monocytes, which results in inflammation and the development of severe acute coronary syndrome (ACS), sepsis or ALI [8]. Additionally, inhibitors against the complex of TF and FVII can balance coagulation and thrombus formation, resulting in fewer adverse effects such as bleeding compared with other anticoagulant drugs [9]. Blockade of the TF pathway may provide a specific therapeutic strategy for ALI or sepsis [1].

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BBR, berberine; DIC, disseminated intravascular coagulation; ERK, extracellular regulated protein kinases; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor (NF)-κB; PCA, procoagulant activity; TF, tissue factor.

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Berberine (BBR), an isoquinoline alkaloid, is the major active component of the Chinese medicinal herb *Rhizoma coptidis* (Huanglian) and has multiple biochemical and pharmacological activities, including lipid-lowering and anti-inflammatory effects [10, 11]. Recent studies have shown that BBR possesses marked activities against sepsis and ALI [12–14]. Furthermore, we previously found that BBR has a significant inhibitory effect on the TF procoagulation activity (PCA) induced by LPS in human monocytes or THP-1 cells [15, 16]. These findings indicate that inhibiting the TF pathway might be a possible action mechanism of BBR in the treatment of ALI; however, it is still unclear which signaling pathways are involved in this inhibition of TF.

Therefore, in the present study, we investigated the mechanism of BBR in down-regulating LPS-induced TF expression and activity in THP-1 cells to provide further support for its clinical use to treat sepsis and other diseases involving TF.

Materials and methods

Materials

The human monoblastic leukemia cell line THP-1 was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cell culture reagents were purchased from Gibco (Carlsbad, CA, USA) and PAA Laboratories GmbH. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a 95% air–5% CO₂ humidified atmosphere at 37 °C.

Chemicals and reagents

Berberine chloride was obtained from the Nanjing Qingze Medical Technology Company (Nanjing, China). Curcumin chloride was kindly provided by Dr. Haixia Ge. LPS (from *Escherichia coli* O55:B5), the chromogenic substrate Xa and Triton X-100 were obtained from Sigma (St. Louis, MO, USA). The human prothrombin complex (300 IU, containing factor II, VII, IX and X) was obtained from Hualan Bioengineering Company (Xinxian, China). The p65 inhibitor JSH-23, the Akt inhibitor LY294002, the JNK inhibitor SP600125 and the p38 inhibitor SB203580 were obtained from Alexis-Biochemicals (San Diego, CA, USA). Anti-TF antibody was purchased from R&D Systems (Minneapolis, MN, USA). Anti-p65 and anti-phospho-NF-κB/p65 antibodies, anti-JNK and anti-phospho-JNK antibodies, anti-p38MAPK and anti-phospho-p38MAPK antibodies, and anti-Akt and anti-phospho-Akt antibodies were purchased from Bioworld Technology (Nanjing, China). Anti-ERK antibodies and anti-phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Shanghai Kang Chen Bio-tech Inc. (Shanghai, China). The goat anti-rabbit IgG antibody was purchased from Wuhan Boster Biological Technology, LTD (Wuhan, China). All other reagents were of analytical grade.

Drug treatment

THP-1 cells were grown in medium for 2 h before pretreatment with berberine at 0.01–1.0 μM or pathway inhibitors for 1 h and were stimulated with 500 ng/ml LPS for 5 h.

Simplified chromogenic assays for TF procoagulation activity

Cells lysates were obtained by repetitive freezing and thawing and were incubated with 10 g/l human prothrombin complex in Tris–CaCl₂ buffer (pH 7.3) at 37 °C for 15 min. Thereafter, 0.5 mM chromogenic substrate Xa in Tris–EDTA buffer (pH 8.4) was added. The absorbance was read at 405 nm [16].

Western blotting analysis

Protein expression was determined by western blotting analysis. The antibody against TF was used at a 1:800 dilution. Anti-p65 and anti-phospho-NF-κB/p65 antibodies, anti-JNK and anti-phospho-JNK antibodies, anti-p38MAPK and anti-phospho-p38MAPK antibodies, anti-Akt and anti-phospho-Akt antibodies, and anti-ERK antibodies and anti-phospho-ERK were used at 1:1000 dilutions. The blots were normalized to GAPDH expression (1:5000 dilution). The antigen–antibody complexes were then detected using a chemiluminescence system.

Immunofluorescence

Treated cells were washed with cold PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking, the cells were incubated with the primary antibodies and FITC/TRITC-conjugated secondary antibodies. Fluorescence was detected by using an Axiovert 40 fluorescence microscope (Zeiss, Germany).

Statistical analysis

The data were expressed as the means ± SEM and analyzed by one-way ANOVA or two-tailed unpaired Student’s t-test. A probability value of less than 0.05 was considered statistically significant. All results are representative of at least three independent experiments.

Results

Berberine attenuated LPS-induced TF-PCA and expression in THP-1 cells

THP-1 cells were stimulated with LPS (500 ng/ml) for 5 h. As shown in Fig. 1A, berberine had an extremely significant suppressing effect on LPS-induced TF procoagulant activity at three concentrations between 0.01 and 1 μM. The inhibition rate of berberine on TF-PCA at the highest concentration of 1 μM was approximately 71%. Additionally, pretreatment with berberine (0.01–1 μM) inhibited LPS-induced TF protein expression (Fig. 1B). Similarly, curcumin inhibited LPS-induced TF expression and activity.

Involvement of signaling pathways in the inhibitory effects of berberine on TF procoagulant activity in THP-1 cells

To investigate the mechanism of the inhibitory effect of berberine on TF-PCA in THP-1 cells, we investigated the effect of inhibitors of various signaling pathways on the inhibitory activity of berberine. Pretreatment with the p65 inhibitor JSH-23 (8 μM), the Akt inhibitor LY294002 (10 μM), the JNK inhibitor SP60125 (40 nM) and the p38 inhibitor SB203580 (70 nM) for 1 h significantly decreased the TF procoagulant activity of the cells, as the relative inhibition rates were approximately 31.8%, 40.7%, 16.9% and 53.7%, respectively (Fig. 2). When a combination of the four pathway inhibitors was used with berberine, the inhibiting effect of berberine on TF-PCA was decreased by varying degrees, indicating that the NF-κappa B, Akt and MAPK signaling pathways all participate in the inhibitory effect of berberine on the TF-PCA.

Effects of berberine on the LPS-induced activation of p65, Akt, JNK, p38 and ERK in THP-1 cells by Western blotting assay

To further determine the mechanism by which berberine inhibits TF expression and TF activity, we investigated the expression and phosphorylation of p65, Akt, JNK, ERK and p38...
in LPS-induced THP-1 cells. Compared with the control group, the phosphorylation of NF-κB/p65, Akt and MAPK/JNK/p38/ERK were significantly increased after stimulation with LPS (Fig. 3). The expression levels of total p65, Akt, JNK p38 and ERK were not changed after stimulation with LPS and pretreatment with berberine. Pretreatment with berberine at three concentrations (0.01, 0.1, 1 μM) suppressed the activation of the NF-κB, Akt and MAPK/JNK/p38/ERK pathways. The phosphorylation of Akt and p38 were significantly blocked by berberine, indicating that these two signaling pathways are closely involved in the inflammatory pathway.

**Immunofluorescence analysis of the inhibitory effects of berberine on the expression of TF and the activation of NF-κB/p65 and Akt in LPS-induced THP-1 cells**

Immunofluorescence was performed to further investigate the NF-κB and Akt pathways in the effect of berberine on TF expression. As shown in Fig. 4, berberine, at the indicated concentrations, significantly reduced TF expression in LPS-induced THP-1 cells. Similarly, berberine significantly suppressed the levels of phospho-p65 and phospho-Akt. These results suggest that berberine suppresses the expression of TF through the above-mentioned pathways.

**Fig. 1.** Berberine attenuated LPS-induced TF-PCA and expression in THP-1 cells. (A) THP-1 monocytic cells were exposed to 500 ng/mL LPS for 5 h and pre-incubated with 0.01, 0.1, 1 μM berberine for 1 h before exposure to LPS. The procoagulant activities were measured by simplified chromogenic assays. (B) THP-1 cells were pretreated with berberine (0.01, 0.1, 1 μM) for 1 h and then exposed to 500 ng/mL LPS for 5 h. Cellular lysates were prepared and TF expression was detected by Western blotting. All data presented are the mean ± SEM (n = 4) of one representative experiment from 3 independent experiments. **p < 0.01, ***p < 0.001 significantly different from the control group.

**Fig. 2.** Involvement of the NF-kappaB, Akt and MAPK/JNK/p38 pathways in the inhibitory effect of berberine on TF procoagulant activity in THP-1 cells. THP-1 cells were pretreated with (A) p65 inhibitor JSH-23 (8 μM), (B) Akt inhibitor LY294002 (10 μM), (C) JNK inhibitor SP600125 (40 nM), (D) p38 inhibitor SB203580 (70 nM) for 1 h, then berberine (1 μM) for 1 h and then exposed to 500 ng/mL LPS for 5 h. TF activity was measured by the simplified chromogenic assay described in method and then stimulated with LPS (500 ng/mL) was taken as 100%. All data presented are the mean ± SEM (n = 4) of one representative experiment from 3 independent experiments. **p < 0.01, ***p < 0.001 significantly different from the control group.
representative. 

anti-phospho-p65, phospho-JNK, in Fig. 4.

THP-1 cells. Effects of berberine on TF activity and expression in THP-1 cells. THP-1 cells were pretreated berberine (0.01, 0.1, 1 μM) for 1 h before exposure to LPS (500 ng/mL) for 30 min, and then cells were lysed and proteins were isolated. Activation of p65 and phospho-p65, Akt and phospho-Akt, JNK and phospho-JNK, p38 and phospho-p38 and ERK and phospho-ERK were detected by Western blotting. All data presented are the mean ± SEM (n = 4) of one representative experiment from 3 independent experiments.

Discussion

This study investigated the mechanism by which berberine affects tissue factor in LPS-stimulated THP-1 cells. Our results suggest that berberine inhibits TF activity and expression. Specifically, we demonstrated that berberine exerts a suppressive effect through the concomitant inactivation of the NF-κB, Akt and MAPK/JNK/p38/ERK pathways, leading to down-regulation of TF levels.

As previously being demonstrated, TF is a critical initiator of the physiological and pathological coagulation cascade and plays a key role in inflammation. Blockade of TF activity and expression may decrease inflammation and coagulation [9,17]. The cellular source of TF in sepsis or ALI, however, still remains to be determined. In vitro, endothelial cells (ECs) and monocytes are the major cellular origins of TF under pathological conditions. Previous studies have suggested that activated monocytes-macrophages are the main triggers of blood coagulation during sepsis. LPS activates the plasma membrane of monocytes, which promotes TF expression and activation in these cells and increases the risk of thrombosis [6,7]. Previous studies have shown that berberine retards LPS-induced inflammation as well as TF-PCA in monocytes [11,15,16]. In this study, we confirmed that berberine, at concentrations of 0.01–1 μM, markedly inhibits TF-PCA and protein expression in LPS-induced THP-1 monocytic cells. It has been recently found that the up-regulation of monocyte tissue factor activity is significantly

Fig. 3. Effects of berberine on LPS-induced activation of p65, Akt, JNK, p38 and ERK in THP-1 cells. THP-1 cells were pretreated berberine (0.01, 0.1, 1 μM) for 1 h before exposure to LPS (500 ng/mL) for 30 min, and then cells were lysed and proteins were isolated. Activation of p65 and phospho-p65, Akt and phospho-Akt, JNK and phospho-JNK, p38 and phospho-p38 and ERK and phospho-ERK were detected by Western blotting. All data presented are the mean ± SEM (n = 4) of one representative experiment from 3 independent experiments.

Fig. 4. Effects of berberine on LPS-induced TF expression and activation of NF-κB/p65 and Akt in THP-1 cells. Immunofluorescences visualizing FITC-conjugated goat anti-TF, anti-phospho-p65 and anti-phospho-Akt in each group of THP-1 cells treated with berberine (1 μM) 1 h before stimulation with LPS (500 ng/ml) were observed by microscopy. TF-induction was stimulated for 5 h, and NF-κB/p65 and Akt were stimulated by LPS for 30 min. Scale bar 10 μm.
associated with low-grade chronic inflammation and insulin resistance in patients with metabolic syndrome, which is a risk factor for cardiovascular disease [17]. Our studies of the inhibitory activity of berberine at relatively low concentrations provide a possible explanation for its application in cardiovascular disease or other TF-related diseases. However, our findings are different from previous reports that showed that berberine could enhance TNF-α-induced endothelial TF expression, which may promote the development of thrombosis [18]. It is possible that under different conditions and in different types of cells, berberine may have bidirectional modulation on TF activity and expression.

It is well known that various signaling pathways, including the NF-κB, Akt and MAPK/JNK/p38/ERK pathways, are involved in TF-PCA and expression [19–21]. The phosphorylation of MAPK/p38 and NF-κB is increased in LPS-induced monocytes [13]. TF pathways mediate inflammatory signaling pathway activation through protease-activated receptors (PARs), which respond to coagulation and inflammation [2,20]. To investigate the possible mechanism of berberine in modulating TF, we utilized several pathway inhibitors. As illustrated in Fig. 2, pretreatment with the inhibitors of the NF-κB, Akt and MAPK/JNK/p38 signaling pathways down-regulated TF-PCA and partially suppressed the inhibitory activity of berberine, indicating the involvement of these signaling pathways in the inhibitory effects of berberine on TF procoagulant activity in THP-1 cells. Similarly, berberine, at a concentration of 0.1 μM in THP-1 cells, significantly inhibited the phosphorylation of NF-κB/p65 and Akt induced by LPS. Furthermore, the phosphorylation of MAPK/p38 was markedly decreased by berberine at three different concentrations (Fig. 3). We also confirmed the inhibitory effect of berberine on TF expression and the phosphorylation of Akt and p65 by an immunofluorescence assay (Fig. 4). The activation of MAPKs was found to be highly associated with an increase in the expression levels of TF in THP-1 cells [22] and was also shown to be involved in LPS-mediated NF-kappa B activation in the development of lung injury [23]. Furthermore, phosphorylation and activation of MAPK has been shown to stimulate NF-κB; whereas activation of the NF-κB pathway by LPS releases large amounts of IL-1, which causes priming of MAPK/p38 [24,25]. These findings suggest that the anti-TF protein expression profile of berberine is related to the inhibition of the NF-κB, Akt and MAPK/JNK/p38/ERK pathways, which is partly consistent with its regulation of such signaling pathways in LPS-induced mice [13].

In conclusion, the present study demonstrated that pretreatment with berberine significantly attenuated LPS-induced TF expression and activity and down-regulated the NF-κB, Akt and MAPK/JNK/p38/ERK pathways in THP-1 cells. These results suggest that the modulation of the TF pathway by berberine could be a promising therapeutic approach for sepsis and acute lung injury treatment.

Conflict of interest

No conflict of interest.

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References