Tenuigenin exhibits protective effects against LPS-induced acute kidney injury via inhibiting TLR4/NF- κ B signaling pathway

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Abstract

Tenuigenin (TNG) has been reported to have various pharmacological activities, such as anti-oxidative and anti-inflammatory activities. However, the protective effects of TNG on lipopolysaccharides (LPS)-induced acute kidney injury (AKI) are still not clear. The aim of this study was to investigate the protective effects and mechanism of TNG on LPS-induced AKI in mice. The kidney histological change, levels of blood urea nitrogen (BUN), and creatinine were measured to assess the protective effects of TNG on LPS-induced AKI. The levels of TNF- α , IL-1 β , and IL-6 in serum and kidney tissues were detected by ELISA. The extent of nuclear factor kappa-B (NF- κ B) p65 and the expression of Toll-like receptor-4 (TLR4) were detected by western blot analysis. The results showed that TNG markedly attenuated the histological alterations, BUN and creatinine levels in kidney. TNG also suppressed LPS-induced TNF- α , IL-1 β , and IL-6 production. Furthermore, the expression of TLR4 and NF- κ B activation induced by LPS were markedly inhibited by TNG. In conclusion, this study demonstrated that TNG protected against LPS-induced AKI by inhibiting TLR4/NF- κ B signaling pathway.

Key words: acute kidney injury; tenuigenin; LPS; NF- κ B

1. Introduction

AKI is defined as the rapid loss of kidney function that often occurs during endotoxemia (Ronco et al., 2008). AKI is a common and important problem in clinical that often results in the dysregulation of volume and electrolyte homeostasis (Murugan and Kellum, 2011). Many factors are accountable for AKI. LPS is one of the most important factors that lead to AKI (Kim et al., 2009; Schrier and Wang, 2004). Studies showed that LPS could induce AKI by activating TLR4/NF- κ B signaling pathway, which subsequently induced the production of inflammatory mediators (Anders, 2010; Cunningham et al., 2004). As we know, LPS induces a “cytokine storm” during the early stage of infection (Wang and Ma, 2008). Thus, reducing of cytokines production may help guide new therapeutic approaches to AKI.

TLR4, the major receptor of LPS, plays an important role in the innate immune system (Takeda and Akira, 2005). When activated by LPS, TLR4 activates the NF- κ B signaling pathway and induces the release of inflammatory cytokines production (Fu et al., 2015). These inflammatory

cytokines lead to kidney injury (Haase, 2015). Previous studies showed that TLR4 plays critical roles in LPS-induced acute kidney injury (Nair et al., 2014). And many compounds isolated from herbal medicine could inhibit LPS-induced acute kidney injury by inhibiting TLR4 signaling pathway (Huang et al., 2015). Therefore, it is urgent to seek compounds that target TLR4 signaling pathway to prevent LPS-induced AKI.

Tenuigenin (TNG), a major active component isolated from the root of the Chinese herb *Polygala tenuifolia*, has been reported to possess various biological and pharmacological activities (Chen et al., 2010; Jia et al., 2004). In vitro, TNG has been reported to inhibit inflammatory cytokine production in macrophages. TNG also inhibited IL-1 β -induced inflammation in human chondrocytes (Wang et al., 2016a). In vivo, TNG has been reported to protect against RANKL-induced osteoclastogenesis (Yang et al., 2015). Furthermore, TNG has been reported to protect against LPS-induced acute lung injury in mice (Lv et al., 2015). Nevertheless, studies on the protective effects of TNG on LPS-induced kidney injury remain few. Thus, we investigated the protective effects of TNG on LPS-induced AKI in this study.

2. Materials and methods

2.1. Chemicals and Reagents

Hyperin (purity >98%), isolated from the root of the Chinese herb *Polygala tenuifolia*, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LPS (*Escherichia coli* 055:B5) was provided from Sigma Chemical Co. (St. Louis, MO, USA). Mouse TNF- α , IL-6 and IL-1 β ELISA kits were purchased from R&D Systems Inc. Mouse TLR4, NF- κ B p-p65, I κ B, p-I κ B, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Animals

Male BALB/c mice (6 to 8 weeks old), were purchased from the Center of Experimental Animals of Jinzhou Medical University. The mice were allowed free access to food and water. The mice were housed in special room with 24 $\pm$ 2 $^{\circ}$ C and 40-80% humidity. All the animal studies were approved by the ethical committee for Laboratory Animals Care and Use of Jinzhou Medical University.

2.3. Experimental design

Sixty mice were randomly divided into five groups as follows (n=12): Control group, LPS group, LPS+TNG (2, 4, 8 mg/kg) groups. The doses of TNG used in this study were based on previous studies (Lv et al., 2015). The mice of LPS group and LPS+TNG (2, 4, 8 mg/kg) groups were injected intraperitoneally (i.p.) with 15 mg/kg body weight of LPS in 50 μ L PBS. 1 h after LPS treatment, the mice of LPS+TNG (2, 4, 8 mg/kg) groups were given i.p. of 2, 4, 8 mg/kg TNG. The mice were euthanized and the blood and kidney tissues were collected 24 h after LPS treatment.

2.4. Histological Analysis

The kidney tissues were excised 24 h after LPS treatment. The kidney tissues were perfused in 4% paraformaldehyde for more than 24 h. Then the kidney tissues were embedded in paraffin and cut into 5 μ m sections. The sections were stained with H&E staining to observe the damage by light microscopy. The histological changes in the kidneys were scored as previously described (Tilyek et al., 2016). Each histological characteristic was scored 0 to 5.

2.5. ELISA assay

24 h after LPS treatment, kidney tissues and blood were collected. The levels of inflammatory cytokines TNF- α , IL-1 β , and IL-6 in serum and kidney tissues were detected by ELISA kits (R&D

Systems Inc.) according to the manufacturer's instructions. .

2.6. Measurement of serum creatinine and BUN

The levels of creatinine and BUN in serum were measured by using an AutoAnalyzer (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.7. Western blot analysis

Total proteins were extracted with T-PER protein extract kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration was measured by the bicinchoninic acid (BCA) method. 20 µg protein was separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies that recognized TLR4, NF-κB p65, NF-κB p-p65, p-IκBα, IκBα, and β-actin. Then the membranes were incubated with HRP-conjugated secondary antibodies. The band intensity was quantified by Image J software.

2.8. Statistical analysis

Statistical analyses were performed using the SPSS software package (ver. 17 for Windows; SPSS Inc., USA). The data were expressed as the mean ± **S.E.M.** Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post test. Statistical significance was accepted at $P < 0.05$ or $P < 0.01$.

3. Results

3.1 Effect of TNG on histopathological changes

The pathological changes of the kidney tissues were analyzed by staining with H&E. As shown in Fig. 1, normal renal histology was observed in the control group. In contrast, an obvious renal pathological damage was observed in LPS group, such as edema of renal tubular epithelial cells, tubular dilation, and distortion (Fig. 1B). However, these pathological changes induced by LPS were significantly attenuated by TNG (Fig. 1C, D, E).

3.2 Effects of TNG on cytokine production in the kidney tissues of LPS-stimulated mice

To analyses the anti-inflammatory effects of TNG, we measured the levels of TNF-α, IL-1β and IL-6 in serum and kidney tissues by ELISA. The results showed that after LPS stimulating, the levels of TNF-α, IL-1β and IL-6 was significantly increased. However, TNG attenuated the levels of TNF-α, IL-1β and IL-6 significantly (Fig. 2).

3.3 Effect of TNG on renal dysfunction

To analyze kidney damage, BUN and serum creatinine were detected. The results showed that after LPS stimulating, the levels of BUN and serum creatinine were significantly increased. However, compared with LPS group, TNG dose-dependently inhibited LPS-induced BUN and serum creatinine levels (Fig. 3).

3.4 Effect of TNG on LPS-induced activation of NF-κB and TLR4 expression

The effects of TNG on TLR4 expression and NF-κB activation were detected by Western blot analysis. Compared with the control group, the expression of TLR4, the phosphorylation level of NF-κB and IκBα are obviously increased in LPS group. However, compared with LPS group, TNG dose-dependently inhibited LPS-induced TLR4 expression and NF-κB activation (Fig. 4).

Discussion

In this study, we used the mouse model of LPS-induced AKI to investigate the protective effects of

TNG on AKI. The results showed that TNG protected LPS-induced AKI by inhibiting inflammatory response. TNG may be a potential candidate for the treatment of AKI.

BUN and creatinine are important markers of kidney injury (Clarkson et al., 2006; Urbchat et al., 2011). The levels of BUN and creatinine increased significantly in LPS-challenged mice. In this study, we found that TNG significantly attenuated the production of BUN and creatinine, which suggests that TNG could protect LPS-induced kidney injury. Furthermore, the histopathological examination also indicated that TNG significantly attenuate LPS-induced kidney injury.

Inflammatory cytokines have been reported to play critical roles in the development of acute kidney injury (Akca et al., 2010). A large body of studies showed that inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, were contributed to kidney injury induced by LPS (Grigoryev et al., 2008). TNF- α is the key mediator of sepsis. It can induce kidney injury by activating TNF receptor. These inflammatory cytokines have been reported to be involved in the pathogenesis of kidney injury. IL-1 β and IL-6 play a primary role in the inflammatory response by inducing fever and increasing the synthesis of acute-phase proteins. Patients with sepsis or kidney injury showed increased levels of inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, in blood (Chawla et al., 2007). In LPS-challenged mice, the levels of inflammatory cytokines TNF- α , IL-1 β and IL-6 increased significantly (Liu et al., 2015; Xu et al., 2015). Suppression of these cytokines production could attenuate LPS-induced AKI. In the present study, our results showed that TNG dose-dependently inhibited LPS-induced inflammatory cytokines production. Serum BUN and creatinine are important markers of renal function. In this study, TGN significantly attenuated LPS-induced serum BUN and creatinine levels. In addition, histological analysis showed that TGN could attenuate LPS-induced kidney injury. Taken together, these results indicated that TNG protected against LPS-induced AKI by inhibiting inflammatory cytokines production.

NF- κ B is responsible for the regulation of inflammatory cytokines production (DiDonato et al., 2012; Fu et al., 2015). Normally, NF- κ B is located in the cytoplasm with I κ B α . Once stimulating by LPS, NF- κ B detaches from I κ B and translocates into the nucleus to regulate inflammatory cytokine expression (Gasparian et al., 2002; Kanarek and Ben - Neriah, 2012; Wang et al., 2016b). NF- κ B has been reported to be essential for the treatment of LPS-induced AKI (Meyer-Schwesinger et al., 2009). Studies showed that many natural compounds protected against LPS-induced AKI by inhibiting NF- κ B activation (Xu et al., 2014; Yu et al., 2015). In this study, our results showed that TNG significantly inhibited LPS-induced NF- κ B activation. TLR4 is the major receptor of LPS. TLR4 in the kidney is a potential mediator of innate activation and inflammation (Chowdhury et al., 2006). Studies showed that TLR4 mediates inflammation and injury in nephrotoxicity (Anders, 2010; Zhang et al., 2008). Inhibition of TLR4 could alleviate the injury of kidney tissues (Liu et al., 2010). To investigate the anti-inflammatory mechanism of TNG, the effects of TNG on LPS-induced TLR4 signaling pathway were detected in this study. We observed TNG significantly inhibited LPS-induced TLR4 expression in kidney tissues. These results indicated TNG inhibited LPS-induced inflammatory response by inhibiting TLR4/NF- κ B signaling pathway. Previous study showed that paeonol also had protective effects against LPS-induced kidney injury by TLR4 signaling pathway (Fan et al., 2016). However, the doses of TGN (2, 4, 8 mg/kg) used in this study were much lower than paeonol (12.5, 25, 50 mg/kg).

In conclusion, the results showed that TNG attenuated LPS-induced acute kidney injury, including reduction of BUN and creatinine production. Furthermore, TNG also inhibited LPS-induced inflammatory cytokines production. The protective effects of TNG against LPS-induced AKI were

related with the inhibition of TLR4 signaling pathway (Fig. 5).

Acknowledgement

This work was supported by Beijing Natural Science Foundation of China (Grant No.7163224) and the National Science Foundation for Postdoctoral Scientists of China(CPSF) (Grant No.2015M572776).

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Figure Legend

Fig.1 Effects of TNG on histopathological changes in kidney tissues in LPS-induced AKI mice. TNG (2, 4, 8 mg/kg) were given intraperitoneally (i.p.) 1 h after LPS treatment. 24 h after LPS challenge, Kidney tissues from each experimental group were processed for histological evaluation. Representative histological changes of kidney obtained from mice of different groups. A: Control group, B: LPS group, C: LPS+ TNG (2mg/kg) group, D: LPS+ TNG (4 mg/kg) group, E: LPS + TNG (8 mg/kg) group F: F: Tubular score (Hematoxylin and eosin staining, magnification 200 $\times$).

Fig. 2 Effects of TGN on LPS-induced TNF- α , IL-6 and IL-1 β in serum and kidney tissues. The values presented are mean $\pm$ S.E.M. (n=12 in each group). $P^{\#}<0.01$ vs. control group, $P^*<0.05$, $P^{**}<0.01$ vs. LPS group.

Fig.3 Effects of TGN on BUN and creatinine levels in serum. The values presented are mean $\pm$ S.E.M. (n=12 in each group). $P^{\#}<0.01$ vs. control group, $P^*<0.05$, $P^{**}<0.01$ vs. LPS group.

Fig. 4 TGN inhibits LPS-induced TLR4 expression and NF- κ B activation. The values presented are mean $\pm$ S.E.M. (n=12 in each group). $P^{\#}<0.01$ vs. control group, $P^*<0.05$, $P^{**}<0.01$ vs. LPS group.

Figure 1

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