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Protective Effect of Baicalein Extracted from Scutellaria baicalensis against Lipopolysaccharide-Induced Glomerulonephritis in Mice

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Abstract: This study was to evaluate the therapeutic effect of baicalein, extracted from Scutellaria baicalensis, on lipopolysaccharide (LPS)-induced glomerulonephritis in mice. For this purpose, bacterial LPS (2.5 mg/kg/day) was injected intraperitoneally (i.p.) for 14 days to induce glomerulonephritis; then mice were treated with baicalein (150 mg/kg/day) by oral gavage for 14 days. In comparison to samples from mice receiving only LPS injection, oral administration of baicalein significantly attenuated the rise of serum levels of blood urea nitrogen and creatinine, proteinuria, kidney wet-to-dry weight ratio as well as renal glomerular cell proliferation induced by LPS. Moreover, treatment with baicalein decreased the urine NOx (nitrite+nitrate) concentration and serum levels of pro-inflammatory cytokine including tumor necrosis factor-α, interleukin-1β and interleukin-6 accompanied by suppression of inducible nitric oxide synthase and cyclooxygenase-2 expression and prostaglandin E2 production in renal tissue relative to LPS-injected alone mice. Similarly, the LPS-induced increase of urine 8-iso-prostaglandin F2α, a marker of reactive oxygen species, was also markedly inhibited by baicalein. The present results demonstrate that baicalein possesses anti-inflammatory and antioxidant properties that may be of benefit against the deleterious actions of LPS in glomerulonephritis.

Key words: Baicalein, lipopolysaccharide, glomerulonephritis, inflammation, nitric oxide

INTRODUCTION

Sepsis is generally considered a systemic inflammatory disease and still represents the leading cause of renal injury (Foster, 2008). Clinical evidence has shown that glomerulonephritis (GN) is a common cause of chronic kidney disease and end stage renal failure. The glomerulonephritis (GN) is defined as inflammation and cell proliferation in the glomeruli accompanied by common clinical finding including hematuria, proteinuria, edema and renal dysfunction (Foster, 2008). In response to endotoxin (lipopolysaccharide, LPS), a constituent of gram-negative bacteria wall, the pathophysiological cascade of GN is triggered by a variety of factors including proinflammatory cytokines. Among these cytokines, tumor necrosis factor-α (TNF-α) is proposed to play an early and crucial role in the cascade (Jarsen et al., 1996) and neutralizing TNF-α exhibited a protective effect against LPS-induced renal failure (Knotek et al., 2001).

The inducible nitric oxide synthase (iNOS)-derived NO overproduction stimulated by LPS and TNF-α plays a critical role in the pathogenesis of septic shock and multiple organ injury (Yang et al., 2002). Similarly, the cyclooxygenase-2 (COX-2)-derived overproduction of prostaglandin E2 (PGE2), an arachidonic acid metabolite, often found in LPS-treated animals is also an important mediator causing inflammatory responses (Ejima et al., 2003). Previous studies have shown that suppression of iNOS or COX-2-mediated process leads to an attenuation of LPS-induced renal dysfunction and renal injury (Wang et al., 2000, 2006). In addition, the glomerular Reactive Oxygen Species (ROS) also plays a key role in renal diseases by its cytotoxic effect on mesangial and glomerular endothelial cells (Rodrigo and Bosc, 2006). Thus, the agents that inhibit induction of iNOS and
COX-2, as well as production of proinflammatory cytokines and ROS may prevent and treat endotoxemic GN.

Bacacalein (5, 6, 7-trihydroxyflavone) (Fig. 1), a flavonoid, isolated from the root of Scutellaria baicalensis, is often used as herbal medicine in many Asian countries due to its beneficial value, including antibacterial, antiviral, antiinflammatory and antioxidative actions (Min, 2009; Cheng et al., 2007). Furthermore, previous studies have reported that bacacalein suppressed LPS-induced iNOS and COX-2 expression and TNF-α formation in RAW 264.7 macrophages (Cheng et al., 2007; Woo et al., 2006). On the basis of these properties, we postulated that bacacalein may protect against endotoxemic GN. However, there is no report on the effect of bacacalein on endotoxemic GN. Therefore, this study was designed to investigate whether bacacalein protects against LPS-induced GN.

**MATERIALS AND METHODS**

**Materials:** Bacacalein was synthesized by Professor Wen-Hsin Huang in July, 2007 according to the protocol described previously (Huang et al., 2003). The synthetic bacacalein was further purified by column chromatography with silica gel in acetone/hexane (9/1). The purity was identified by HPLC and the purity of synthetic bacacalein was over 98%, comparable to commercial bacacalein. Bacacalein suspended in vehicle (0.5% carboxymethylcellulose) was used to treat animals by oral administration. All the chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

**Animal experimental protocol:** The study was approved by the Ethical Committee of Animal Experiments of National Defense Medical Center. C57BL/6 male mice aged 8-10 weeks, weighting between 25-30 g, were used throughout the study. Mice were maintained on a standard rodent chow and filtered tap water on an ad libitum manner. To establish GN animal model, the mice were injected intraperitoneally (i.p.) with LPS (Escherichia coli serotype O26:B6, 2.5 mg/kg/day) dissolved in sterile saline for 14 days. In our preliminary test, different doses of bacacalein ranging from 50-150 mg kg^{-1} were administrated to evaluate their effects on renal function and kidney wet-to-dry weight ratio. Present results showed that 50 or 100 mg kg^{-1} bacacalein had no significantly protective effect in this endotoxemic GN model. Thus, a dose of 150 mg kg^{-1} bacacalein was chosen for this study. The animals were divided into three experimental groups. (1) Normal group. Mice were injected with the same volume of saline only without LPS and were given vehicle (0.5% carboxymethylcellulose) alone by oral gavage for 14 days. (2) LPS-treated group. Mice were injected with LPS for 14 days and followed by receiving vehicle (0.5% carboxymethylcellulose) by oral gavage for 14 days. (3) Bacacalein-treated group. After a successive injection with LPS for 14 days, the mice were treated with bacacalein (150 mg/kg/day) by oral gavage for 14 days. After finishing the treatment, samples were taken from these mice for subsequent tests.

**Measurement of biochemical parameters and kidney edema:** After finishing the treatment as the experimental protocol, a blood sample was collected from the mice and centrifuged (2000 g for 3min) to separate serum and the serum was stored at -80°C until assay. The levels of serum blood urea nitrogen (BUN), creatinine and urine albumin used as indicators of renal function were commercially measured. After the weight of wet right kidney was measured, the same kidney was dried in an oven for 48 h to remove any gravimetrically detectable water and weighed it again to calculate the kidney wet-to-dry weight ratio, an indicator of renal edema.

**Measurement of cytokines, PGE, and urine 8-isoprostaglandin F,α:** The serum levels of TNF-α, interleukin-1β (IL-1β) interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured by ELISA Kits (R and D Systems, Inc., Minneapolis, MN), respectively. The level of PGE, and 8-isoprostaglandin F,α was measured by ELASA kit (Cayman Chemical).

**Measurement of urine NOx (nitrite+nitrate) level:** Aliquots of 30 μL sample were deproteinated with 100 μL 95% alcohol for 30 min (4°C) and samples were centrifuged for 6 min at 12,000 g. The supernatant (6 μL) was injected into a collection chamber containing 5% VCl. In this strong reducing environment, both nitrate and nitrite were converted to NO. A constant stream of helium gas was used to carry the output into a NO analyzer (Sievers 280NOA; Sievers Instruments Inc., Boulder, CO, USA), where the NO reacts with ozone (O_3).
resulting in the emission of light. Light emission is proportional to the NO formed. Standard amounts of sodium nitrate were used for calibration.

**Immunohistochemical staining:** Paraffin sections of kidney tissue were deparaffinized and the endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature. Sections were washed in 0.01 M Phosphate-Buffered Saline (PBS), pH 7.2, containing 10% normal goat serum and incubated overnight at 4°C with rabbit anti-\( \text{iNOS} \) or anti-COX-2 antibody. Horseradish-conjugated goat anti-rabbit Ig antibody was used at 1:200 after washing. Immune complexes were detected with a solution of 3, 3-diaminobenzidine (0.2 mg mL\(^{-1}\)) and hydrogen peroxide in 0.05 M Tris-HCl buffer. Sections were counterstained with methyl green. In negative control sections, rabbit antiserum against ovalbumin (1 : 500) was used as an irrelevant antibody.

**Histopathological studies:** The kidney tissues were removed, placed in formalin and embedded in wax according to a standard protocol. Four micrometer sections were cut and stained with hematoxylin and eosin for light microscopy and the histological alteration was evaluated by a pathologist who was blind to the experimental conditions.

**Statistical analysis:** Data were expressed as Means±SE of the Mean (SEM) All results were analyzed by one-way ANOVA followed by a multiple comparison test (Scheffe test). A p-value less than 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Baicalein improved renal function and kidney wet-to-dry weight ratio in endotoxemic mice:** Injection of LPS (2.5 mg/kg/day, i.p.) for 14 days induced a renal dysfunction reflected by increased serum BUN and creatinine concentrations, proteinuria and kidney wet-to-dry weight ratio. Oral administration of baicalein (150 mg kg\(^{-1}\)) for 14 days significantly improved these alterations (Fig. 2a-c, 3) compared to those in the LPS-treated group.

![Graphs showing BUN, Serum Creatinine, and Urine Protein levels](image)

**Fig. 2:** (a-c) Effect of baicalein on serum BUN, creatinine and urine protein levels in LPS-treated mice. The mice were injected with LPS (2.5 mg/kg/day, i.p.) for 14 days followed by treatment with baicalein (150 mg/kg/day, p.o.) for 14 days and the blood and urine were taken for measurement of these parameters to evaluate renal function. Each data was expressed as Mean±SEM (n = 5). *p<0.05, **p<0.01 vs. LPS-treated group.
Biacelein inhibited serum cytokine concentrations in endotoxemic mice: Injection of LPS for 14 days induced a marked increase in serum TNF-α, IL-1β, and IL-6 levels compared to that in normal group. Oral administration of biacelein for 14 days significantly decreased the increased cytokine concentrations compared to those in the LPS-treated group (Fig. 4a-c).

Biacelein reduced urine 8-iso-prostaglandin F₂α level in endotoxemic mice: After injection of LPS for 14 days the concentration of urine 8-iso-prostaglandin F₂α was markedly increased compared to that in normal group and this enhancement was significantly attenuated by treatment with biacelein (Fig. 5).

Biacelein inhibited urine NOx level and renal PGE₂ formation in endotoxemic mice: The urine NOx level and PGE₂ formation in renal tissue were markedly increased after injection of LPS for 14 days. Treatment with biacelein significantly inhibited the rise of urine NOx and renal PGE₂ formation compared to that in LPS-treated group (Fig. 6a, b).

Fig. 3: Effect of biacelein on kidney edema in LPS-treated mice. The mice were injected with LPS (2.5 mg/kg/day, i.p.) for 14 days followed by treatment with biacelein (150 mg/kg/day, p.o.) for 14 days and the kidneys were photographed and measured the kidney wet-to-dry weight ratio. Each data was expressed as Mean±SEM (n = 5). *p<0.05 vs. normal mice. *p<0.01 vs. LPS-treated group.

Fig. 4: (a-c) Effect of biacelein on serum TNF-α, IL-1β, and IL-6 levels in LPS-treated mice. The mice were injected with LPS (2.5 mg/kg/day, i.p.) for 14 days followed by treatment with biacelein (150 mg/kg/day, p.o.) for 14 days and the blood was taken for measurement of cytokines. Each data was expressed as Mean±SEM (n = 5). *p<0.05 vs. LPS-treated group.
Fig. 5: Effect of baicalein on urine 8-iso-prostaglandin F$_{2\alpha}$ level in LPS-treated mice. The mice were injected with LPS (2.5 mg/kg/day, i.p.) for 14 days followed by treatment with baicalein (150 mg/kg/day, p.o.) for 14 days and the urine was taken to measure 8-iso-prostaglandin F$_{2\alpha}$ level. Each data was expressed as Mean±SEM (n = 5). **p<0.01 vs. LPS-treated group.

**Baicalein inhibited iNOS and COX-2 expression in renal:** The iNOS and COX-2 expression in renal tissue evaluated by immunohistochemical staining was enhanced by LPS and the increased expression of iNOS and COX-2 was reduced by baicalein (Fig. 7).

**Baicalein improved renal histological alterations in endotoxemic mice:** Histological examinations showed that there was an appearance of mesangial cell proliferation and synthesis of extracellular matrix proteins in LPS-treated group, which was markedly ameliorated by treatment with baicalein (Fig. 8).

This study demonstrates that oral administration of baicalein protects against LPS-induced GN. This conclusion is based on the significant attenuation of the increased serum BUN and creatinine concentrations, proteinuria, as well as kidney wet-to-dry weight ratio after LPS administration in baicalein-treated group compared to those in LPS-treated group. In response to LPS, various inflammatory mediators, such as proinflammatory cytokines, ROS, NO and PGE$_{2}$, are produced, which are thought to contribute to the LPS-induced pathological symptoms in renal diseases (Wang et al., 2003). In this study, LPS-induced increase of serum levels of TNF-$\alpha$, IL-$\beta$ and IL-6 was significantly inhibited by baicalein, indicating that its protective effect in GN may be through reducing proinflammatory cytokine production. However, the serum level of IL-10, an antiinflammatory cytokine (Sanjabi et al., 2009), was not affected by baicalein (data not shown), suggesting that the beneficial effect of baicalein in GN might be not mediated by enhanced IL-10 formation; whether the effect of baicalein is a result of reduction in proinflammatory cytokines remains to be determined.

During endotoxemia, overproduction of ROS may lead to leukocyte activation, mesangial cell apoptosis and glomerular damage (Moreno et al., 2000). Therefore, the inhibition of LPS-induced urine 8-iso-prostaglandin F$_{2\alpha}$ level, a useful clinical biomarker of oxidative stress (Lim et al., 2002), by baicalein, may be also involved in the beneficial effects in endotoxemic GN. In addition, the large amount of NO produced by iNOS in response to LPS can further form highly reactive species, most notably peroxynitrite, by interaction with superoxide anion (Kukreja and Hess, 1992). Then, peroxynitrite decomposes to produce hydroxyl radical. Both hydroxyl
Fig. 7: Effect of baicalein on the expression of iNOS and COX-2 of kidney in LPS-treated mice. The mice were injected with LPS (2.5 mg/kg/day, i.p.) for 14 days followed by treatment with baicalein (150 mg/kg/day, p.o.) for 14 days and the kidneys were taken to measure iNOS and COX-2 expression by immunohistochemical staining method.

Fig. 8: Effect of baicalein on the histological changes of kidney in LPS-treated mice. The mice were injected with LPS (2.5 mg/kg/day, i.p.) for 14 days followed by treatment with baicalein (150 mg/kg/day, p.o.) for 14 days and the histological examination of the kidney was preformed.

radical and peroxynitrite results in lipid peroxidation, protein oxidation, mitochondrial dysfunction and subsequently leads to tissue damage and cell death. Thus, the inhibition of iNOS/NO pathway in renal tissue by baicalein is an important mechanism contributing to attenuation of pathogenesis of endotoxemic GN.

A lot of studies have suggested that the COX-2/PGE2-mediated pathway is involved in the renal dysfunction of GN including reduction of glomerular filtration rate and tubular function through down regulation of Organic Anion Transporters (OATs) especially OAT1 and OAT3 gene expression (Hocherl et al., 2009; De Vriese, 2003). Accordingly, the beneficial effect of baicalein on improving renal function in endotoxemic GN may be associated with the inhibition of COX-2-mediated downregulation of OATs. Once activation by LPS, the nuclear factor kappa B (NF-κB), a nuclear transcription factor, translocates into nucleus and in turn activates several inflammatory gene transcription including iNOS, COX-2 and proinflammatory cytokines (Pasparakis, 2009). Based on the results of previous study showing baicalein blocked the activation of NF-κB by inhibiting the degradation of IκBα in LPS-treated RAW 264.7 macrophages (Cheng et al., 2007), we proposed that
the antiinflammatory activities of baicalein in GN may be mediated by suppressing the activation of NF-κB.

In clinical practice, GN is characterized by the appearance of mesangial cell proliferation and synthesis of extracellular matrix proteins. It has been demonstrated that several factors including enhanced iNOS and COX-2 expression, platelet-derived growth factor (PDGF) and ROS all stimulate mesangial cell proliferation (Tokuyama et al., 2008; Sheu et al., 2005; Budisavljevic et al., 2003). Furthermore, in glomerular mesangial cells, the synthesis of 12-(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a product of 12-lipoxygenases (12-LOX), also can directly induce mesangial cell hypertrophy and matrix production by activating mitogen-activated protein kinase (MAPK) pathway (Reddy et al., 2002). Importantly, it is well known that baicalein is a potent inhibitor of 12-LOX. Therefore, the inhibition of LPS-induced glomerular cell proliferation by baicalein in endotoxemic GN may be, at least in part, due to attenuation of iNOS and COX-2 induction, free radical formation, PDGF-A expression and 12-LOX-mediated process.

CONCLUSION

The results obtained in this study first demonstrates that baicalein protects against LPS-induced GN in mice, which is probably mediated by inhibition of various proinflammatory mediator formation, iNOS and COX-2 induction.

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