Effects of Gentianine on the Production of Pro-inflammatory Cytokines in Male Sprague-Dawley Rats Treated with Lipopolysaccharide (LPS)

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This study was undertaken to elucidate the mechanism of anti-inflammatory action of gentianine, a constituent of Gentiana Macrophylla. The effects of gentianine on lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines were investigated in male Sprague-Dawley rats. For the first time, we found that oral administration of gentianine (10—100 mg/kg) suppressed the increases in tumor necrosis factor-α (TNF-α) (ED50: 37.7 mg/kg) and interleukin (IL)-6 (ED50: 38.5 mg/kg) in the sera from the rats challenged with bacterial LPS (100 μg/kg; i.p.). However, LPS induced production of other interleukins, such as IL-α, was not significantly altered by gentianine. These results suggest that the potential anti-inflammatory action of gentianine might be at least partly based on the suppressed production of TNF-α and IL-6.

Key words gentianine; anti-inflammatory mechanism; tumor necrosis factor (TNF)-α; interleukin (IL)-6; lipopolysaccharide (LPS)

The crude extract of Gentiana Macrophylla Radix, one of the Gentiana species, has been used for the treatment of inflammatory diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) in oriental medicine. Gentianine is one of the major components of Gentiana Macrophylla Radix and several research groups reported its various pharmacological activities such as anti-ulcerogenic activity,1 anti-inflammatory activity2 and anti-analgesic activity.3,4 To elucidate the mechanism of the anti-inflammatory action of gentianine, its effects on the cytokine production were investigated in male Sprague-Dawley rats (SD-rats) treated with lipopolysaccharide (LPS).

Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 are produced and play critical roles in the inflammation processes. Among these pro-inflammatory cytokines, TNF-α has been highlighted recently as a main mediator in the inflammatory diseases mechanism. In placebo-controlled clinical trials, TNF-α antagonists were proven highly effective in the treatment of some inflammatory diseases. TNF-α may have a significant role in pathogenesis of several inflammatory diseases such as rheumatoid arthritis,5 psoriatic arthritis,6 juvenile rheumatoid arthritis,7 and Crohn’s disease.8,9

Bacterial LPS binds to CD14 on the monocyte membrane and stimulates the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α that are important signaling molecules in initiating and coordinating a large range of immune responses against invading pathogens.9 Based on the facts that gentianine possesses anti-inflammatory effects and inflammatory cytokines play a key role in inflammation, we assumed that gentianine might have inhibitory effects on the production of pro-inflammatory cytokines. Therefore, gentianine was examined in vitro and in vivo for its effects on the production of TNF-α and IL-6, pro-inflammatory cytokines, which are involved in the pathogenesis of inflammatory diseases.

MATERIALS AND METHODS

Experimental Animals and Materials Male SD-rats weighing between 280 g and 350 g were used for the study. Three rats were housed in suspended polypropylene cages with wire grid floors and all animals had free access to tap water and pellet food (Agribranps, Purina, Korea). The room temperature and relative humidity were set at 21°C and 50%, respectively. Prior to the experiment, the rats were deprived of food for 18 h, but not of water. The study has been carried out according to the “Principles of laboratory animal care” (NIH Publication no. 85-23, revised 1985).

LPS, serotype 0111:B4, derived from Escherichia coli was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Microarray kit was purchased from RayBiotech, Inc. (Norcross, GA, U.S.A.) and enzyme linked immunosorbent assay (ELISA) kits were obtained from R & D systems (Minneapolis, MN, U.S.A.). All other chemicals were of the highest grade from commercial sources.

To secure enough sample amounts required for this study, gentianine was synthesized by heating 4-methyl-5-vinylmico- tinic acid with aqueous formalin in the presence of sodium bicarbonate, followed by acid treatment.9 Purification was performed by medium pressure liquid chromatography (40% ethylacetate/hexanes) and re-crystallization (ethylacetate/hexanes: 4:6) to give white crystalline: mp 80—81°C (lit. mp 80—81°C; IR (CHCl3) 1720 and 1621 cm−1; MS m/e 175 (M+) Figure 1 shows the chemical structure of gentianine.

LPS Induced Production of Cytokines in Rats Treated with Gentianine Gentianine was dissolved in distilled water and administered orally with doses ranging between 3—100 mg/kg. Control rats received distilled water as a vehicle. Two hours after the gentianine administration, rats were challenged with LPS, serotype 0111:B4, derived from Es-

Fig. 1. The Structure of Gentianine
cherichia coli. Animals were intraperitoneally administered with either LPS (100 μg/kg) or 0.89% NaCl at an injection volume of 1 ml/kg body weight. Two hours after the challenge, rats were sacrificed by decapitation. The dose and route of LPS administration adopted in the study produce quantifiable increase in circulating concentrations of pro-inflammatory cytokines IL-6 and TNF-α. Following the decapitation, trunk blood was collected into plastic tubes and stored on ice prior to centrifuge at 800 × g at 4 °C for 15 min. After centrifugation, aliquots of serum were removed for the determination of TNF-α and IL-6. The removed sera were frozen immediately on dry ice and later transferred to a −80 °C freezer where they remained until the assays were performed.

LPS Induced Production of Cytokines in Whole Blood Treated with Gentianine Human whole bloods were obtained from 3 healthy volunteers (male, age of 26—30) after getting informed consent. Heparinized whole blood was pre-incubated with either vehicle or gentianine at 37 °C for 10 min. LPS (1 μg/ml) was then added to the cultures and incubated for another 12 h. After incubation, the incubation mixtures were centrifuged at 1500 × g at 4 °C for 10 min.

The supernatants obtained were applied to the assay of the produced cytokines.

Detection of Cytokines in the Sera Using Antibody Microarray Kit Rat cytokine array system was used as a primary screening tool to detect the cytokine production. Rat cytokine array was purchased from RayBiotech, Inc. (Norcross, GA, U.S.A.). The cytokine assay was performed according to the standard protocol suggested by the manufacturer. In brief, cytokine array membranes were treated with 5% BSA, and then the membranes were incubated with one milliliter of rat serum followed by further incubation with HRP conjugated immunoglobulin G. The signals were visualized with enhanced chemoluminescence (ECL).

Assay of IL-6 and TNF-α in the Sera Serum concentrations of TNF-α and IL-6 were measured by using commercially available ELISA kits (R & D systems, Minneapolis, MN, U.S.A.); and absorbance was read at 450 nm on ELISA plate reader (Molecular Devices, SPECTRA max PLUS 384, U.S.A.). Results were expressed as ng TNF-α/ml serum or pg IL-6/ml serum.

Statistical Analysis of Data All data were expressed as mean ± S.E.M. Statistical significances among groups were tested using Sigma Stat (Jandel Co., San Rafael, CA, U.S.A.) by one-way analysis of variance (ANOVA) followed by Dunnett’s test. Differences were considered significant when p was less than 0.05. All experiments were done at least three times, each time with three independent observations.

RESULTS AND DISCUSSION

The mechanism of anti-inflammatory action of gentianine has not yet been elucidated. Thus, we investigated, in the present study, the effects of gentianine on the production of pro-inflammatory cytokines (TNF-α and IL-6) in male SD-rats treated with LPS.

Micro spot antibody array was performed to detect the cytokines, of which production was changed in the sera from the rats treated with gentianine followed by LPS challenge. As shown in Fig. 2, LPS markedly increased the production of two pro-inflammatory cytokines (TNF-α and IL-6) and those increased production was specifically inhibited by gentianine. Based on this result, we decided to investigate the effects of gentianine on the production of TNF-α and IL-6.

TNF-α is a central mediator that plays a critical role in the regulation of inflammatory processes. It is chiefly synthesized and secreted by macrophages in response to pro-inflammatory stimuli such as bacterial LPS. TNF-α functions its biological activities by binding to the cell surface receptors, TNF receptor I and TNF receptor II. TNF-α is associated with development of serious infectious diseases and certain inflammatory diseases. It is well known that TNF-α is able to stimulate the production of other cytokines. These facts suggest that TNF-α plays a central role in the cascade of cytokine production. This theory is supported by the fact that administration of anti-TNF-α antibodies is effective in lowering the levels of other cytokines in LPS-infuced inflammation.

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<th>Inhibition-doses</th>
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<tr>
<td></td>
<td>TNF-α</td>
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<td>ED_{10}</td>
<td>14.48</td>
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<td>ED_{50}</td>
<td>37.658</td>
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<td>ED_{90}</td>
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Table 1. Doses of Gentianine for the Inhibition of the Production of TNF-α and IL-6 in Male SD-Rats Treated with LPS
TNF-α is also known as a crucial factor in immune responses. TNF-α production in rat blood peaked 1 h after LPS-challenge, but decreased rapidly thereafter (data not shown). Figure 3 showed that gentianine was found to inhibit LPS-induced TNF-α secretion in a dose-dependent manner.

In addition, IL-6 has been also known as an important cytokine concerned to inflammation, immuno-regulation and hematopoiesis. Continuous overproduction of IL-6 also plays an important role in the pathogenesis of some inflammatory diseases such as RA.

In this study, most abundant IL-6 production was observed at 2—4 h after LPS challenge (data not shown). It was confirmed that a LPS challenge increased the production of TNF-α and IL-6, circulating pro-inflammatory cytokines,15,16) and LPS-induced production of TNF-α (Fig. 3) and IL-6 (Fig. 4) was markedly inhibited by gentianine in vitro and in vivo. The inhibition doses are shown in Table 1: ED50 for TNF-α and IL-6; 37.65 mg/kg and 38.52 mg/kg respectively. But the production of other inflammatory cytokines including IL-1 was not affected by gentianine in LPS-injected rats (Fig. 2). This differential effect is not yet clearly understood. But we could make an assumption that IL-1 might be regulated differently from TNF-α and IL-6 in the present experimental condition. This assumption could be supported at least in part by the following three reports; Erroi A. et al. confirmed that IL-1 production was differentially regulated from that of TNF-α and IL-6 in LPS tolerant mice.17) And O’Brien-Ladner A. R. et al. also reported that iron differentially regulated human alveolar macrophage-derived IL-1 and TNF-α. They found that the addition of deferoxamine, a chelator of iron, increased the release of IL-1, but not TNF-α, from alveolar macrophages from smokers and nonsmokers.18) In addition, Meldrum D. R. et al. reported that tissue-specific protein kinase C isoforms differentially mediate macrophage TNF-α and IL-1 production.19)

Unregulated activities of inflammatory mediators can lead to development of serious inflammatory diseases. For example, enhanced TNF-α synthesis is associated with the development of rheumatoid arthritis (RA), psoriatic arthritis and inflammatory bowel disease.20) And NO production by iNOS may reflect the degree of inflammation and provides a measure to assess the effect of drugs on the inflammatory process.21)

It was also reported that IL-1β, TNF-α and inducible nitric oxide synthase (iNOS) are highly expressed in synovial cells, infiltrating leukocytes and endothelial cells in RA. IL-1 and TNF-α play a central role in many inflammatory processes and appear to be involved in the catabolic process of OA.8) These cytokines also enhance the production of 5-LOX products and PGE2. The TNF-α inhibition by antibodies to TNF-α itself or its receptors in these inflammatory diseases has been remarkably successful. In addition, the blockade of IL-6 may also provide a new therapeutic strategy for RA.22) For example, Atlizumab is a recombinant humanized anti-human IL-6 receptor antibody that blocks IL-6 binding on its receptor. However, the current injectable protein therapies have been associated with risks and limitations. Therefore, an oral therapy with small molecule could either replace the injectables or provide better disease control when used alone or in conjunction with existing therapies.23)

Fig. 3. LPS-Induced TNF-α Production in Rats Treated with Gentianine

Fig. 4. LPS Induced IL-6 Production in Rats Treated with Gentianine

Fig. 5. LPS Induced Production of TNF-α in Whole Blood Treated with Gentianine
whole blood with gentianine significantly reduced the production of TNF-α (Fig. 5). And IL-6 production seemed to be slightly inhibited by gentianine, but it was not statistically significant (data not shown). In vitro effects of gentianine on cytokine production were somewhat different from its in vivo effects. The difference between in vivo and in vitro effects should be further investigated. In addition, the effects on the products of arachidonic acid metabolism should be also investigated as an additional mechanism of anti-inflammatory action of gentianine.

In summary, the experimental data provide evidences that gentianine might express anti-inflammatory action at least partly through the prevention of the immune cells including macrophages from producing TNF-α and IL-6, pro-inflammatory cytokines, in male SD-rats treated with LPS. This finding suggests that gentianine might be a small molecular potential candidate that could be developed as a drug for oral therapy of inflammatory diseases such as rheumatoid arthritis.

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REFERENCES