Tetrandrine Inhibits Proinflammatory Cytokines, iNOS and COX-2 Expression in Human Monocytic Cells

Shu-Jing Wu* and Lean-Teik Ng*.*

*Department of Health and Nutrition, Chia-Nan University of Pharmacy and Technology; Tainan, Taiwan; and
*Department of Biotechnology, Tajen University; Pingtung, Taiwan. Received May 11, 2006; accepted October 11, 2006

Tetrandrine (TET), a bis-benzylisoquinoline alkaloid isolated from the dried root of Hang-Fang-Chi (Stephania tetrandra S. Moore), is traditionally used in China for treating inflammation, hypertension and silicosis. In this study, our aim was to examine the anti-inflammatory mechanism of TET through measuring the inducible nitric oxide synthase (iNOS), cyclooxygenase-1, and -2 (COX-1 and COX-2) expression, cytokines (TNF-α, IL-4 and IL-8) formation, nitric oxide (NO) release and prostaglandin E2 (PGE2) generation in lipopolysaccharide (LPS)-induced human monocytic (THP-1) cells. Results showed that TET remarkably suppressed the LPS (1 μg/ml) induction of NO release and PGE2 generation. It also significantly attenuated the LPS-induced transcription of proinflammatory cytokines (TNF-α, IL-4 and IL-8) in a dose-dependent manner. Furthermore, TET at 100 μM significantly blocked the LPS induction of iNOS and COX-2 expression, but not the COX-1. Taken together, these results suggest that TET exerts anti-inflammatory effects probably through the suppression of COX-2 and iNOS expression.

Key words: tetrandrine; cytokine; inducible nitric oxide synthase (iNOS); COX-2; THP-1 cell

Chronic inflammations and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. Among the proinflammatory enzymes, the inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX), which are responsible for increasing the levels of nitric oxide (NO) and prostaglandins (PGE2), are known to be involved in various chronic diseases including multiple sclerosis, Parkinson’s and Alzheimer’s diseases, and colon cancer.1 Lipopolysaccharide (LPS), which is a component of the cell wall of gram-negative bacteria, is known to activate a number of cellular signals of human monocytic cells during inflammation.2

NO is synthesized by many cell types involved in immune and inflammatory response. In the THP-1 cells, inducible NOS (iNOS) triggered the production of NO after LPS-stimulation.3 Cyclooxygenase (COX) is a key enzyme in regulating the formation of PGE2 from arachidonic acid. There are two isoforms of cyclooxygenase, namely cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is in a constitutively expressed form in normal physiologic functions, whereas COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin LPS.4,5 COX-2 is responsible for the elevated production of PGE2 during inflammation.6 After lactoferrin and β-amyloid treatment, transcription of most genes for inflammatory proteins such as TNF-α, IL-4, IL-6, and IL-8 was inhibited in LPS-induced human monocytic cells.7,8

Tetrandrine (TET), a bis-benzylisoquinoline alkaloid, is isolated from the roots of Stephania tetrandrae S. Moore. It is traditionally used in China for treating patients with arthritis, arrhythmia, hypertension, inflammation and silicosis.9—11 Previous reports indicated that TET possesses anticancer,12 immunosuppressive,13 and free radical scavenging14 activities. Although its mechanism of anti-inflammation remains unclear, TET has been shown to exhibit anti-inflammatory effect on mouse ear edema.15 In this study, our aim was to investigate the anti-inflammatory mechanism of TET through measuring the inducible nitric oxide synthase (iNOS), cyclooxygenase-1 and -2 (COX-1 and COX-2) expression, cytokines (TNF-α, IL-4 and IL-8) formation, nitric oxide (NO) release and prostaglandin E2 (PGE2) generation in lipopolysaccharide (LPS)-induced THP-1 cells.

MATERIALS AND METHODS

Chemicals Dimethylsulfoxide (DMSO), penicillin, streptomycin, anti-β-actin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT), lipopolysaccharide (LPS; from E. coli strain O55:B5) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from GIBCO BRL (Gaithersburg, MD, U.S.A.). The ELISA kit used for the determination of prostaglandin E2 (PGE2) was purchased from Alexis Biochemicals (Ann Arbor, MI, U.S.A.). Human TNF-α, IL-4 and IL-8 ELISA kits were obtained from Raybiotech Inc. (Norcross, GA, U.S.A.). Polyclonal antibodies for COX-1, COX-2 and iNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-mouse IgG antibody was obtained from Promega (Madison, WI, U.S.A.). All other chemicals and reagents used were of analytical grade.

Test Drug Tetrandrine (TET), with purity greater than 98%, was purchased from Fluka (St. Gallen, Switzerland). It was first dissolved in DMSO and then diluted with distilled water to obtain a stock solution of concentration 1 mM. For each experiment, the stock solution was further diluted with distilled water to desired concentrations (10, 50, 100 μM). The final DMSO concentration in all experiments was controlled at 0.1%.

Cell Culture and Drug Treatment THP-1, a promonocytic cell line, was obtained from the American Type Culture Collection (ATCC No. TIB-202, Manassas, VA, U.S.A.). Cells were grown in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. They were maintained at 37 °C in a humidified atmosphere of 5% CO2.

MTT Assay for Cell Viability Cytotoxicity studies were performed in 96-well plates. THP-1 cells were cultured at...
1×10^5 cells per well containing 100 µl of RPMI-1640 medium. After an overnight incubation, the test drug was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 µl of FBS-free medium containing MTT (5 mg/ml). After 4 h of incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO. The optical density was measured at 550 nm.

Nitrite Determination After incubating the cells with either LPS (1 µg/ml) or LPS plus various concentrations of TET (10, 50, 100 µg/ml) for 24 h, the supernatant was removed from the cultures. The nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 µl of each supernatant was mixed with the same volume of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethenediamine dihydrochloride in water], and then incubated at room temperature for 10 min. The absorbance was measured at 550 nm using an ELISA reader (Anthos 2010, Austria). The amount of nitrite produced was determined by using a sodium nitrite standard curve.

PGE2 Assay The prostaglandin E2 (PGE2) level in the cultured medium was quantified using ELISA kits and conducted according to the manufacturer instructions.

Measurement of Cytokines Proinflammatory cytokines (TNF-α, IL-4 and IL-8) were measured using ELISA kits and carried out according to the manufacturer instructions.

Western Immunoblot Analysis Western blot analysis of COX-1, COX-2 and iNOS were carried out by employing the respective antibodies. Cellular proteins from the control, LPS (1 µg/ml) alone or LPS plus TET (100 µg/ml) treated samples were isolated in lysis buffer (100 mM Tris–HCl, pH 7.5, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and 120 mM sodium chloride) containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 1 µg/ml aprotinin (Sigma Chemical Co., St. Louis, MO, U.S.A.). The samples were then centrifuged at 10000×g for 10 min. Equal amounts of lysate protein (50 µg/lane) were then loaded onto SDS-polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). After inhibiting the nonspecific binding sites with 5% (w/v) skim milk in 0.1% (v/v) Tween 20 containing PBS (PBST) for 1 h at room temperature, the membrane was incubated for 1 h with the specific primary antibodies, namely anti-COX-1 (1:500), anti-COX-2 (1:500), anti-iNOS (1:500), and anti-β-actin (1:5000). Antibody recognition was detected with an anti-mouse secondary antibody linked to a horseradish peroxidase. Antibody-bound proteins were detected by the ECL western blotting analysis system (Amer sham, Aylesbury, U.K.). The expression of β-actin was used as a control.

Statistical Analysis Data were presented as means± standard deviations (S.D.) of three independent experiments. Values were evaluated by one way ANOVA, followed by Duncan’s multiple range tests using the Statistical Analysis System (SAS Institute, Cary, NC, U.S.A.). Control and treatment groups were compared among themselves using Student’s t-test. Differences were considered significant when p-value was <0.05.

RESULTS

Effect of TET on THP-1 Cell Viability In order to determine whether TET causes toxicity on THP-1 cells, the MTT assay was performed. Figure 1 showed that THP-1 cell numbers were affected after 24 h of treatment with LPS (1 µg/ml) in the presence or absence of TET at 10 µM. However, TET at 50 µM and 100 µM was found to fully protect against LPS-induced cytotoxicity in THP-1 cells.

Effect of TET on NO Production To investigate the effect of TET on NO generation, we measured the accumulation of nitrite, the stable end product of NO, in the culture media using Griess reagent. THP-1 cells were induced with LPS (1 µg/ml) for 24 h to evoke nitric oxide (NO) production. As shown in Table 1, co-incubation of cells with LPS and TET (10, 50, 100 µM) resulted in a dose-dependent reduction of NO production. At 100 µM TET, the concentration of NO in the medium was found to return to the level similar to that of the un-stimulated control.

Effect of TET on PG E2 Synthesis PGE2 release indicates an inflammatory response of cells. To examine the effect of TET on PGE2 production in THP-1 cells, cells were stimulated with 1 µg/ml LPS alone or LPS plus TET for 24 h. Results showed a dramatic increase in PGE2 production (42.67±3.68 ng/ml) in the LPS-stimulated cells when compared to the control (5.60±0.45 ng/ml) (Table 1). Co-treatment of cells with LPS plus TET (10, 50, 100 µM) resulted in a decrease of PGE2 production in a dose-dependent manner. At 100 µM, TET significantly reduced the level of PGE2 to that of the control group.

Effect of TET on Cytokines Release To determine the
effect of TET on the production of proinflammatory cytokines (i.e. TNF-α, IL-4 and IL-8), THP-1 cells were treated with control (0.1% DMSO) and TET (10, 50, 100 μM) in the presence or absence of LPS (1 μg/ml) for 24 h, and the cytokine levels were measured in the media by an ELISA reader. LPS-treated cells showed a dramatic increase in TNF-α, IL-4 and IL-8 production (Table 2). However, co-treatment of cells with LPS plus TET significantly suppressed the production of these proinflammatory cytokines, and the reduction was in a dose-dependent manner. At 50 and 100 μM, the levels of TNFα, IL-4 and IL-8 were not statistically different from the control group.

**Effect of TET on iNOS, COX-1 and COX-2 Expression**

To further investigate the important role of TET on anti-inflammation, THP-1 cells were induced with control (0.1% DMSO), 1 μg/ml LPS alone, 100 μM TET alone or 1 μg/ml LPS plus 100 μM TET for 24 h. Western blotting analysis displayed that iNOS, COX-1 and COX-2 were up-regulated after LPS induction. Co-treatment of cells with LPS plus 100 μM TET significantly suppressed iNOS and COX-2 expression, but the protein level of COX-1 was not affected (Fig. 2).

**DISCUSSION**

The present study demonstrated that TET is an effective inhibitor of LPS-induced NO generation, cytokines (TNF-α, IL-4 and IL-8) secretion, PGE₂ production, iNOS and COX-2 expression in THP-1 cells. Inflammation is a complex process, which involves numerous mediators of cellular and plasma origins. Studies indicated that β-amyloid peptides and hydrolyzed olive vegetation water exhibited anti-inflammatory activities in human THP-1 cells. In this study, we showed that TET possessed potent anti-inflammatory activity on the same cells.

A high level of NO produced by iNOS has been defined as a cytotoxic indicator in inflammation and endotoxemia. NO and cytokines stimulate the expression of iNOS and COX-2, which lead to an enhanced formation of PGE₂. These molecules have been implicated as important mediators in the process of inflammation. Harpagoside remarkably inhibited the NO production in LPS-treated cells. In this study, we showed that LPS induced a dramatic increase in NO and PGE₂ production in THP-1 cells. However, co-treatment of cells with LPS plus TET significantly reduced both the NO and PGE₂ levels. Interestingly, the levels of these mediators at 100 μM TET were no difference from the control group, suggesting that TET is an effective inhibitor of LPS-induced NO generation and PGE₂ production. The suppression of TET on PGE₂ release could be through the inhibition of COX-2 enzyme activity, and its inhibition on NO production could be resulted from the suppression of iNOS expression.

In the search of new anti-inflammatory drugs, recent strategies have been concentrated on a selective inhibition of COX-2 enzymatic activity without altering COX-1 activity. Interestingly, we demonstrated that TET possesses inhibitory effect on COX-2 but not COX-1 expression. At present, NSAIDs are among the most widely used therapeutic agents for inflammation and pain, however, they are believed to possess potential gastrointestinal and renal side effects resulting from the inhibition of the housekeeping enzyme COX-1 activity. With its selective inhibition on COX-2 activity, the use of TET for preventing inflammatory related diseases could avoid such adverse effects.

Inhibitors of iNOS and COX-2 have been considered as potential effective therapeutic for preventing inflammatory response and diseases. Studies have shown that parthenolide effectively inhibited 12-O-tetradecanoylphorbol-13-acetate-mediated induction of iNOS expression and NO production in THP-1 cells. Curcumin, a natural flavonoid found in *Curcuma* species, inhibited phorbol ester-mediated induction of iNOS as well as LPS-stimulated iNOS expression. Capsaicin, a major ingredient of hot pepper, markedly inhibited PGE₂ release by inhibiting the catalytic activity of the COX-2 enzyme. Wagonin was shown to abolish the induction of both iNOS and COX-2 after LPS treatment. Thus, these potential inhibitors of iNOS and COX-2 were considered to be effective for preventing inflammatory diseases. In this study, TET significantly suppressed the expression of COX-2 but not the COX-1. TET also significantly inhibited the expression of iNOS. These results therefore suggest that TET is a potential candidate for preventing inflammatory diseases.

Studies have shown that proinflammatory cytokines such as TNF-α, IFN-γ, IL-4, IL-6 and IL-8 were involved in the development of various inflammatory lesions. These cytokines were also shown to mediate the different phases of...
inflammation in tissues. Thus, drugs that inhibit these cytokines production may play an important role in the control of inflammation. Studies have indicated that lactoferrin and β-amyloid peptides inhibited cytokines (TNF-α, IL-4, IL-6 and IL-8) production in LPS-stimulated THP-1 cells. In this study, TET showed inhibitory effect on TNF-α, IL-4 and IL-8 expression in the same cells induced by LPS. This finding further supports that TET possesses potent anti-inflammatory activity.

In conclusion, we have demonstrated that TET possesses potent anti-inflammatory activity. It prevented the cytokines formation, NO generation, PGE2 production, iNOS and COX-2 expression but has no effect on COX-1 in LPS-treated THP-1 cells. These findings suggest that TET exerts anti-inflammatory effects probably through the suppression of COX-2 and iNOS expression. The present study also provides further evidence to support the traditional anti-inflammatory claim of _Stephania tetrandrae_, and its bioactive constituent “TET”.

REFERENCES