2-Methoxycinnamaldehyde Reduces IL-1β-Induced Prostaglandin Production in Rat Cerebral Endothelial Cells

Jian-You GUO,a,b Hai-Ru HUO,a,* a,b Yuan-Xiao YANG,c Cang-Hai LI,d Hong-Bin LIU,a Bao-Sheng ZHAO,a Lan-Fang LI,a Yue-Ying MA,a Shu-Ying GUO,a and Ting-Liang JIANGa

*a Tang Center for Herbal Medicine Research, Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences; Beijing 100700, P.R. China; b Institute of Psychology, Chinese Academy of Sciences; Beijing 100101, P.R. China: and c Zhejiang Chinese Medical University; Hangzhou 310053, P.R. China.

Received March 19, 2006; accepted August 10, 2006

Prostaglandin E2 (PGE2) works as a common final mediator of the febrile. Guizhi-Tang, one of the most famous traditional Chinese medical formula used to treat influenza, common cold and other pyretic conditions, was previously reported to reduce the production of PGE2 in rats. 2-Methoxycinnamaldehyde is a principle compound isolated from Guizhi-Tang. The aim of the present study was to investigate the effects of 2-methoxycinnamaldehyde on PGE2 production of rat cerebral endothelial cells (CECs). 2-Methoxycinnamaldehyde dose-dependently inhibited interleukin (IL)-1β-induced PGE2 production in CECs with IC50 values of 174 μM. IL-1β stimulation increased the protein, activity and mRNA expression of cyclooxygenase (COX)-2 but not COX-1. 2-Methoxycinnamaldehyde reduced IL-1β-induced protein and activity of COX-2, but did not influence the COX-2 mRNA expression. Our results show that prostaglandin production in CECs during stimulated conditions is sensitive to inhibition by 2-methoxycinnamaldehyde and suggest that 2-methoxycinnamaldehyde may reduce COX-2 protein level and activity but not COX-2 mRNA.

Key words 2-methoxycinnamaldehyde; rat cerebral endothelial cell; interleukin-1β; cyclooxygenase; prostaglandin E2

Guizhi-Tang, one of the most famous traditional Chinese medical formula, has been widely used to treat influenza, common cold and other pyretic conditions. Prostaglandin E2 (PGE2), a cyclooxygenase (COX)-derived metabolite of arachidonic acid, is a well-defined mediator of fever.1,2) Guizhi-Tang was previously reported to reduce the production of PGE2 in rats.3,4) Chemical studies have shown that it includes saponins, polysaccharide and homoiso flavonoidal compounds,5) but its anti-pyretic active components have not been adequately elucidated. 2-Methoxycinnamaldehyde was one of principle compounds isolated from Guizhi-Tang. Antimicrobial effects of 2-methoxycinnamaldehyde have been described early. For example, 2-methoxycinnamaldehyde has been reported to inhibit the growth of Aspergillus parasiticus and sterigmatocystin.6) Recently, 2-methoxycinnamaldehyde has been discovered to have an inhibitory effect on LPS-induced NF-κB transcriptionsal activity7) and shown to be a principle compound isolated from Guizhi-Tang. The aim of the present study was to investigate the effects of 2-methoxycinnamaldehyde on PGE2 production of rat cerebral endothelial cells (CECs). 2-Methoxycinnamaldehyde dose-dependently inhibited interleukin (IL)-1β-induced PGE2 production in CECs with IC50 values of 174 μM. IL-1β stimulation increased the protein, activity and mRNA expression of cyclooxygenase (COX)-2 but not COX-1. 2-Methoxycinnamaldehyde reduced IL-1β-induced protein and activity of COX-2, but did not influence the COX-2 mRNA expression. Our results show that prostaglandin production in CECs during stimulated conditions is sensitive to inhibition by 2-methoxycinnamaldehyde and suggest that 2-methoxycinnamaldehyde may reduce COX-2 protein level and activity but not COX-2 mRNA.

MATERIALS AND METHODS

Materials 2-Methoxycinnamaldehyde and 2-methoxycinnamic acid (99.8%) were provided by School of Pharmaceutical Sciences in Peking University. Culture media, serum and buffers for cell culture were obtained from Invitrogen Inc. (Carlsbad, CA, U.S.A.). Endothelial cells growth factor (ECGF) was purchased from Roche Inc. (Basel, Switzerland). Antibody for von Willebrand factor was purchased from Dako Co. (Santa Barbara, CA, U.S.A.). IL-1β was obtained from PeproTech Inc. (Rocky Hill, NJ, U.S.A.). PGE2 ELISA kit was purchased from Shanghai Sun Biomedical Co. (Shanghai, China). Antibodies against COX-1, COX-2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Anti-β-actin antibody was from Sigma Inc. (St. Louis, MO, U.S.A.). Enhanced chemiluminescence detection (ECL) was purchased from PerkinElmer Co. (Boston, MA, U.S.A.). COX activity assay kit and chemiluminescent COX inhibitor screening assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Reverse Transcription Reagents and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Branchburg, NJ, U.S.A.). TRIZol and electrophoresis reagents were from Takara Co.
(Tokyo, Japan). All reagents for cell culture were of tissue culture grade, and for RNA extraction, reagents were of molecular biology grade.

**Cell Culture** Endothelial cells were isolated from rat microvessels and cultured as described previously. Briefly, rat cerebral cortices were cut into small pieces, homogenated and filtered through sieves. The remnant microvessels on sieves were collected and digested in 0.1% collagenase II. The digested microvessels were centrifuged and the pellet was resuspended in M199 medium supplemented with 150 ng/ml ECGF, 25% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. The microvessel suspension was plated on tissue culture flask and incubated at 37 °C in a 5% CO₂ incubator. After confluence with vessel suspension was plated on tissue culture flask and incubated at 490 nm by an enzyme immunoassay instrument (Bio-Rad Model 550, Hercules, CA, U.S.A.).

**RNA Extraction and cDNA Synthesis** The total RNA from different experimental conditions was obtained by TRIzol method. The concentration of RNA was determined by an absorbance at 260 nm and RNA was reverse transcribed to cDNA using the Taqman Reverse Transcription Reagents (Applied Biosystems). Reverse transcription was performed at 48 °C for 30 min followed by RT inactivation at 95 °C for 5 min (Perkin-Elmer GeneAmp 9600, Foster City, CA, U.S.A.). cDNA was analyzed immediately or stored at −20 °C until use.

**Real-Time Quantitative PCR** Real-time qualitative PCR analyses for COX and GAPDH were performed in 96-well plates using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems). PCR were performed with the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol, using the following oligonucleotide primers: COX-2-forward 5'-GGCGTTGCTCATCCATCTACTC-3' and reverse 5'-AGCATCTGTGAGCAGTACCGG-3' (169 bp); GAPDH-forward 5'-ACGATGTGTAAGGTTTCAGGGA-TGAACGGGAA-3' and reverse 5'-TGGTCATTGAG-3' (260 bp). The basic protocol for real-time PCR was an initial incubation at 95 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and finally cooling to 40 °C. All samples were run in triplicate, the relative expression values were normalized to the mean of β-actin.

**Western Blot Analysis** After treatment, CECs lysates were prepared using lysis buffer (50 mM Tris–HCl, pH 8.0; 20 mM EDTA; 1% SDS; and 100 mM NaCl). Protein concentration was measured using a protein assay according to the manufacturer’s procedure. Lysate samples were applied to 10% SDS–polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes for 3 h at 60 V constant voltage at 4 °C. After blocking with 5% nonfat dry milk in a buffered solution (10 mM Tris–HCl, pH 7.5; 100 mM NaCl; and 0.1% Tween 20) at 4 °C overnight, the membrane was exposed to the primary antibody for 3 h at room temperature on a shaker. Membranes were washed with the buffer solution and incubated with secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Immunoreactivity was visualized by ECL COX-2 and COX-1 protein expression were quantified by densitometry using the Scion Image Beta 4.02 software and are shown as density relative to β-actin.

**Measurement of COX Activity** CECs from the above culture were scraped and spun down at 1500×g for 10 min at 4 °C and washed once with saline. Then cells were suspended in cold buffer (0.1 M Tris–HCl, pH 7.8; 1 mM EDTA) and sonicated in an ice bath for 3×4 s by an ultrasonic sonicator. The crude homogenate was centrifugation at 10000×g for 10 min in a tabletop microtube and supernatant was collected. The protein content was measured with a protein assay kit, then the COX activity was determined by ELISA as indicated in COX activity assay kit instructions. The kit includes arachidonic acid and isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity. The ELISA reactions were initiated by adding the arachidonic acid solution, the COX-1 activity was assayed with using of COX-2 specific inhibitor, while the COX-2 activity was determined with using of COX-1 specific inhibitor. The direct effects of 2-methoxycinnamaldehyde and 2-methoxycinnamic acid on COX activity were also measured with the chemiluminescent COX inhibitor screening assay kit.
expression value of GAPDH.

To quantify the results obtained by real-time PCR, we used the plasmids containing cDNA as standard. The cDNA of interest was amplified by RT-PCR using the same primers as for real-time RT-PCR. The PCR products were cloned into pGEM-T easy vector (Invitrogen) and confirmed by sequencing. The purified recombinant plasmid DNA was quantified by UV spectrophotometer and then serially diluted in double-distilled water to serve as standard for numerical quantification. The standard curve was prepared for each target cytokines and while GAPDH was used as housekeeping gene in this study. The PCR products were sequenced to verify the analytical specificity using standard sequencing procedures. Melting curve analysis was also performed after PCR amplification.

Statistics All values were given as means±S.D. Experiments were repeated two to four times and the data were pooled. The results were analyzed by Kruskal–Wallis test, followed by Dunn’s multiple comparisons test between groups. A difference with \( p<0.05 \) was considered significant.

RESULTS

Characterization of the Cultured CECs The microvessels isolated according to the procedure described in the method were pure, without obvious neuronal or glial cells. The endothelial cells started to migrate out from the vessels on day 2 and grew to confluency within 7—10 d. Primary endothelium culture displayed typical “cobble stone” morphology. Positive immunostaining for von Willebrand factor, a marker for all endothelial cells, was found to be present diffusely in the cytoplasm of cultured CECs (Fig. 1A). Based on morphology, >95% of the population were endothelial cells. Von Willebrand factor antibody was omitted in negative control slides, which consistently remained unstained (Fig. 1B).

Dose- and Time-Dependent Effect of IL-1\( \beta \) on PGE\(_2\) Production, COX mRNA, COX Protein, and COX Activity To determine the time-dependent effect of IL-1\( \beta \), CECs were incubated with IL-1\( \beta \) (30 ng/ml) for different times. ELISA results indicated that IL-1\( \beta \) increased the PGE\(_2\) production and COX-2 activity in a time-dependent manner, while COX-1 activity was not significantly altered during the incubation period (Figs. 2A, B). The PGE\(_2\) production and
COX-2 activity were increased significantly by IL-1β as early as 8 h ($p<0.05$) and then declined thereafter. Western blot results showed that IL-1β induced COX-2 protein in a time-dependent manner, while COX-1 protein was not significantly altered during the incubation period (Fig. 2C). COX-2 protein expression was increased significantly by IL-1β as early as 0.5 h ($p<0.05$), reached a maximum by 12 h of incubation ($p<0.01$) and then declined thereafter. Real-time quantitative PCR was performed to amplify COX-1 and COX-2 mRNAs, and the standard curve was run for each target gene (for example, GAPDH in Figs. 3A, B). Melting curve analysis confirmed that there was no primer dimer in the PCR products (Fig. 3C). For each of the primer sets, non-specific amplification was visualized after electrophoresis and ethidium bromide staining of agarose gels (Fig. 3D). The expression of COX-2 mRNA was induced and became detectable after 1 h and reached a maximum induction by 4 h of incubation. Then it declined and became undetectable by 12 and 24 h after incubation with IL-1β. While COX-1 mRNA was present and remained constant during the incubation period (Fig. 2D).

To determine the concentration dependent effect of IL-1β in CECs, ELISA results indicated that IL-1β increased the PGE$_2$ production and COX-2 activity in a concentration dependent manner for 12 h, while COX-1 activity was not significantly altered during the incubation period (Figs. 4A, B). Western blot results showed that IL-1β induced the COX-2 protein in a concentration dependent manner for 12 h, while COX-1 protein was not significantly altered (Fig. 4C). Real-time quantitative PCR results indicated that IL-1β increased the COX-2 mRNA in a concentration dependent manner for 4 h, while COX-1 mRNA remained constant (Fig. 4D).

**Effect on PGE$_2$ Production Stimulated by IL-1β in CECs**

To determine whether 2-methoxycinnamaldehyde or 2-methoxycinnamic acid affect the IL-1β-induced PGE$_2$ production, the cells were incubated with various concentrations of test materials for 12 h in the presence of 30 ng/ml IL-1β. ELISA results indicated that 2-methoxycinnamaldehyde decreased IL-1β-induced PGE$_2$ production in a dose-dependent manner, the IC$_{50}$ value was 174 μM (Fig. 5A). In contrast, 2-methoxycinnamic acid did not inhibit IL-1β-induced PGE$_2$ production (Fig. 5B).

**Effect on COX Activity Stimulated by IL-1β in CECs**

To determine whether 2-methoxycinnamaldehyde or 2-
Fig. 4. The Dose–Response Analysis of IL-1β on PGE₂ Production, COX Activity, COX Protein and COX mRNA Expression in CECs Stimulated by IL-1β
CECs were incubated with various concentrations of IL-1β for 4 h or 12 h. After incubation, the medium was collected and the amount of PGE₂ was determined by ELISA. The cells were harvested, COX activity, protein and mRNA expression were analyzed by ELISA, western blot and real-time quantitative PCR respectively. (A) PGE₂ production; (B) COX activity; (C) COX protein; (D) COX mRNA expression. Statistical significance: **p<0.01, *p<0.05 vs. non-IL-1β.

Fig. 5. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-Methoxycinnamic Acid on PGE₂ Production Induced by IL-1β
CECs were incubated with indicated concentrations of test materials for 12 h in the presence of 30 ng/ml IL-1β. After incubation, the medium was collected and the amount of PGE₂ was determined by ELISA. (A) 2-Methoxycinnamaldehyde; (B) 2-methoxycinnamic acid. Statistical significance: #p<0.01 vs. control; **p<0.01, *p<0.05 vs. IL-1β.

Fig. 6. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-Methoxycinnamic Acid on COX Activity Induced by IL-1β
CECs were incubated with indicated concentrations of test materials for 12 h in the presence of 30 ng/ml IL-1β. The cells were harvested, COX activity was analyzed by ELISA. (A) 2-Methoxycinnamaldehyde; (B) 2-methoxycinnamic acid. Statistical significance: #p<0.01 vs. control; **p<0.01, *p<0.05 vs. IL-1β.
methoxycinnamic acid affect the IL-1β-induced COX activity, CECs were incubated with various concentrations of test materials for 12 h in the presence of 30 ng/ml IL-1β. After incubation, the COX activity was measured by ELISA. As shown in Fig. 6A, 2-methoxy-cinnamaldehyde decreased IL-1β-induced COX-2 activity in a dose-dependent manner, the IC50 value of 2-methoxy-cinnamaldehyde in inhibiting IL-1β-induced COX-2 activity was 378 µM, while COX-1 activity was not significantly altered during the incubation period. 2-Methoxycinnamic acid did not inhibit IL-1β-induced COX activity (Fig. 6B).

The effect of 2-methoxy-cinnamaldehyde on COX-2 activity was further clarified. Consistent with the above observation, in vitro tests showed that 2-methoxy-cinnamaldehyde decreased COX-2 activity in a dose-dependent manner with IC50 value of 337 µM, while it did not influence COX-1 activity. 2-Methoxycinnamic acid also did not inhibit COX activity (data not shown).

Effect on COX Protein Stimulated by IL-1β in CECs

To determine whether 2-methoxy-cinnamaldehyde or 2-methoxycinnamic acid affect the IL-1β-induced COX protein, CECs were incubated with various concentrations of test materials for 12 h in the presence of 30 ng/ml IL-1β. After incubation, the COX protein was detected by western blot. As shown in Figs. 7A and B, both 2-methoxy-cinnamaldehyde and 2-methoxycinnamic acid decreased IL-1β-induced COX-2 protein in a dose-dependent manner. However, 2-methoxy-cinnamaldehyde significantly reduced IL-1β-induced COX-2 protein as the concentration reached 160 µM compared with 2-methoxycinnamic acid of 2560 µM. 2-Methoxy-cinnamaldehyde and 2-methoxycinnamic acid showed no significant inhibitory effect on COX-1 protein.

Effect on COX mRNA Expression Stimulated by IL-1β in CECs

To determine whether 2-methoxy-cinnamaldehyde or 2-methoxycinnamic acid affect the IL-1β-induced COX mRNA expression, CECs were incubated with various concentrations of test materials for 4 h in the presence of 30 ng/ml IL-1β. After incubation, the COX mRNAs were measured by real-time quantitative PCR. As shown in Figs. 8A and B, 2-methoxy-cinnamaldehyde and 2-methoxycinnamic acid showed no significant inhibitory effect on IL-1β-induced COX-1 and COX-2 mRNAs.

Fig. 7. Effect of Different Doses of 2-Methoxy-cinnamaldehyde and 2-Methoxycinnamic Acid on COX Protein Induced by IL-1β

CECs were incubated with indicated concentrations of test materials for 12 h in the presence or absence of 30 ng/ml IL-1β. The cells were harvested, COX protein was analyzed by western blot. (A) 2-Methoxy-cinnamaldehyde; (B) 2-methoxycinnamic acid. Statistical significance: *p<0.01 vs. control; **p<0.01, *p<0.05 vs. IL-1β.
the blood–brain interface,
2-methoxycinnamic acid. Statistical significance: #

Fig. 8. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-
Methoxycinnamic Acid on COX mRNA Expression Induced by IL-1β
CECs were incubated with indicated concentrations of test materials for 4 h in the
presence or absence of 30 ng/ml IL-1β. The cells were harvested, COX mRNA expres-
sion was measured by real-time quantitative PCR. (A) 2-Methoxycinnamaldehyde; (B)
2-methoxycinnamic acid. Statistical significance: *p<0.01 vs. control.

DISCUSSION
The innate immune system serves as the first line of host
defense against the deleterious effects of invading infectious
pathogens. Fever is the hallmark among the defense mecha-
nisms evoked by the entry into the body of such pathogens.
The conventional view of the steps that lead to fever produc-
tion is that they begin with the biosynthesis of pyrogenic cy-
tokinines such as IL-1β, tumor necrosis factor (TNF), and IL-
6, which are elaborated and released into circulation by
mononuclear phagocytes that are activated by exogenous in-
flammatory agents. Three routes are considered for these cy-
tokinines to gain access to the brain: (1) via afferent fibres that
circle mostly through the vagus nerve and make their first
synapse in the nucleus of the solitary tract14,15), (2) via cir-
cumventricular organs, such as the organum vasculosum
laminae terminalis and the subfornical organ, which lack a
blood–barrier16,17), and (3) via interaction with cells located in
the blood–brain interface, i.e. endothelial cell.10,17)
When some of the afferent pathways are activated, PGE2 will be re-
leased into the hypothalamus and binds to EP3 receptors on
the cells in the hypothalamic thermoregulatory center; this is
followed by PGE2-induced neuronal mechanisms involving
cyclic AMP and neurotransmitters to elevate the temperature
set-point, resulting in fever.18,19)
In the present study, we first investigated the effects of 2-
methoxycinnamaldehyde and 2-methoxycinnamic acid on
PGE2 production of CECs with IL-1β inducement. PGE2
works as a common final mediator of the febrile. CECs, a site
of the blood–brain barrier in vivo, regulate a number of phys-
iological and pathophysiological processes in the brain. IL-
1β is a principle component of endogenous pyrogens, and it
has been reported to induce prostaglandin biosynthesis in
many types of endothelial cells, including human umbilical
vein endothelial cells.20,21) In addition, intraperitoneal injec-
tion of IL-1β in rats can induce these enzymes in brain en-
dothelial cells in vivo. However, study on prostaglandin
biosynthesis in cultured brain endothelial cells with IL-1β-
inducement has not been reported in the literature. Here, we
isolated and cultured of functionally active CECs, then the
confluent of endothelial cells was incubated with 2-
methoxycinnamaldehyde or 2-methoxycinnamic acid in the
presence of IL-1β. Our results show that IL-1β induced
PGE2 production of CECs in a time and dose-dependent
manner. 2-Methoxycinnamaldehyde dose-dependently re-
duced IL-1β-induced PGE2 production with IC50 value of
174 μM, while no significant inhibition was observed for 2-
metoxycinnamic acid. These findings suggest IL-1β is a po-
tent inducer of prostaglandin biosynthesis in cultured CECs
and strongly support the opinion that PGE2 may gain access
to the brain via interaction with endothelial cell.
Prostaglandins are synthesized from arachidonic acid by a
reaction catalyzed by COX. It is now well established that
COX exists as two isoforms that catalyze the same reaction
but differ in terms of regulation of expression. The constitu-
tive isofrom COX-1 is responsible for the production of
prostaglandins involved in prostanoid-mediated physiological
functions. COX-2, the inducible form of the enzyme, can un-
dergo rapid induction in response to many factors such as
bacterial lipopolysaccharides, growth factors, cytokines and
phorbol esters.22,23) Therefore, in this paper, effect of 2-
methoxycinnamaldehyde and 2-methoxycinnamic acid on
COX activity, COX protein and COX mRNA expression in
CECs with IL-1β inducement were also measured. For each of
the primer sets, nonspecific amplification was not visual-
ized after electrophoresis and ethidium bromide staining of
agarose gels. This result indicated that real-time PCR condi-
tions used in this study were suitable for the detection of
specific mRNAs expressed in CECs. As previously
observed,24,25) we also found that IL-1β induced COX-2 ac-
tivity, protein and mRNA expression in a dose- and time-
dependent manner in CECs, while COX-1 activity, protein
and mRNA expression were not significantly altered. 2-
Methoxycinnamaldehyde reduced the IL-1β-induced COX-2
activity and protein, but did not reduce IL-1β-induced COX-
2 mRNA in cultured CECs. These results indicate that 2-
methoxycinnamaldehyde may influence the COX-2 protein
synthesis translation and the molecular basis of the effect of
2-methoxycinnamaldehyde was manifested at the posttrans-
criptional level. 2-Methoxycinnamic acid reduced IL-1β-
induced COX-2 protein at high concentration, but did not in-
hbit IL-1β-induced activity and mRNA expression of COX-
2 in cultured CECs.
In conclusion, our results show that prostaglandin produc-
tion in CECs during stimulated conditions is sensitive to in-
hibition by 2-methoxycinnamaldehyde and suggest that 2-
methoxycinnamaldehyde may reduce COX-2 protein level
and activity but not COX-2 mRNA. These results suggest that
Guizhi-Tang's antipyretic property might be partly ascribable to 2-methoxycinnamaldehyde rather than 2-
methoxycinnamic acid. Other active constituents of Guizhi-Tang remained to be studied in the future.

Acknowledgments This research was supported by grant no. 90209006 from the National Natural Science Foundation of China.

REFERENCES