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

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Prostaglandin E_2 (PGE_2) 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 PGE_2 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 PGE_2 production of rat cerebral endothelial cells (CECs). 2-Methoxycinnamaldehyde dose-dependently inhibited interleukin (IL)-1β-induced PGE_2 production in CECs with IC_{50} 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 E_2

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 E_2 (PGE_2), 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 PGE_2 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-kappaB transcriptional activity[7] and shown to be a strong inhibitor on aminopyrine *N*-demethylation in rat liver microsomes.[8] However, whether 2-methoxycinnamaldehyde has antipyretic effect remains unclear.

Therefore, in the present study, we tested the effect of 2-methoxycinnamaldehyde on PGE_2 production of rat cerebral endothelial cells (CECs) with interleukin (IL)-1β inducement to provide some pharmacological evidence for clinical use of Guizhi-Tang in fever.[9] IL-1β is a principle component of endogenous pyrogens, and it has been generally recognized that IL-1β released from cells peripherally produces fever by signaling the brain *via* various routes. For this purpose, CECs were cultured and incubated in M199 medium containing IL-1β in the presence or absence of 2-methoxycinnamaldehyde. CECs were selected due to its relevance to immune-brain signaling mediated by PGE_2.[10—12] After incubation, the medium was collected and the amount of PGE_2 was measured by enzyme-linked immunosorbent assay (ELISA). COX mRNA expression, COX protein expression and COX activity were also analyzed by real-time reverse transcription-polymerase chain reaction (RT-PCR), western blot and ELISA respectively. To better understand the specificity of these effects of 2-methoxycinnamaldehyde in Guizhi-Tang, 2-methoxycinnamic acid, another structually related compound isolated from Guizhi-Tang, was also examined for comparison. The present results show that 2-methoxycinnamaldehyde dose-dependently inhibits IL-1β-induced PGE_2 production through the inhibition of COX-2 protein and COX-2 activity in CECs, while 2-methoxycinnamic acid does not influence the PGE_2 production in CECs with IL-1β inducement.

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 acquired 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.). PGE_2 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.

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(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.[13] 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% CO_2 incubator. After confluence with 2 weeks, the cells were rinsed with phosphate buffered saline (PBS), and subsequently trypsinized with 0.25% trypsin in PBS. Experiments were performed with cells from passages three to five.

Immunochemistry The CECs were grown to confluence on chamber slides and fixed in cold acetone for 15 min, rehydrated with PBS. Cells were then incubated in 0.3% hydrogen peroxide–methanol for 15 min to block endogenous peroxidase activity, and in 20% bovine serum albumin (BSA) in PBS for 20 min to block non-specific staining. Cells subsequently were incubated for 90 min at room temperature with antibody against von Willebrand factor. The slides were rinsed twice with PBS containing 1% BSA; cells were then incubated for 30 min with biotinylated secondary antibodies. Next, they were incubated in avidin–biotin–peroxidase complex for 45 min. Peroxidase activity was visualized by the diaminobenzidine reaction according to the manufacturer's instructions. Primary antibody was omitted in negative control slides.

Treatment of CECs The CECs were sub-cultured into six-well cell culture plates and maintained until sub-confluence. The medium was then replaced by a serum-free culture medium for 24 h prior to the addition of IL-1β or other reagents. For assay of protein and activity of COX and PGE_2 experiments, the cells were incubated with various concentrations of IL-1β for 12 h, or 30 ng/ml IL-1β for different times. Then CECs were incubated in the serum-free medium containing 30 ng/ml IL-1β in the presence or absence of different concentrations of 2-methoxycinnamaldehyde or 2-methoxycinnamic acid for 12 h. For assay of COX mRNA expression experiment, the cells were incubated with various concentrations of IL-1β for 4 h, or 30 ng/ml IL-1β for different times. Then CECs were incubated in the serum-free medium containing 30 ng/ml IL-1β in the presence or absence of different concentrations of 2-methoxycinnamaldehyde or 2-methoxycinnamic acid for 4 h.

Measurement of PGE_2 Production After incubation, the medium was collected for measurement of PGE_2. PGE_2 was determined by ELISA, and procedures were followed as indicated in kit instructions. The absorbance was then measured at 490 nm by an enzyme immunoassay instrument (Bio-Rad Model 550, Hercules, CA, U.S.A.).

Measurement of COX Activity CECs from the above culture were scraped and spun down at 1500×g in a microfuge 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 microfuge 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.

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.

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 quantitative 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-1-forward 5′-GGCGTTGCTCATCCATCTACTC-3′ and reverse 5′-AGCATCTGTGAGCAGTACCGG-3′ (116 bp); COX-2-forward 5′-TTTGTTGAGTCATTCACCAGACAG-AT-3′ and reverse 5′-ACGATGTGTAAGGTTTCAGGGA-GAAG-3′ (169 bp); GAPDH-forward 5′-TGAACGGGAA-GCTCACTGG-3′ and reverse 5′-GAGCTTCACAAAGT-TGTCATTGAG-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

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-1β on PGE_2 Production, COX mRNA, COX Protein, and COX Activity To determine the time-dependent effect of IL-1β, CECs were incubated with IL-1β (30 ng/ml) for different times. ELISA results indicated that IL-1β 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

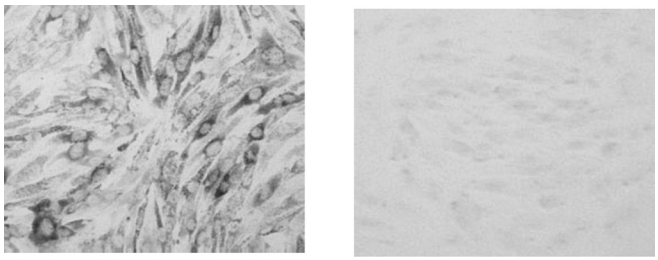

Fig. 1. Characterization of CECs

(A) CECs at passage three cultured on gelatin-coated chamber slides stain positively with antibody to von Willebrand factor; (B) von Willebrand factor antibody was omitted in negative control slides, which remained unstained (200×).

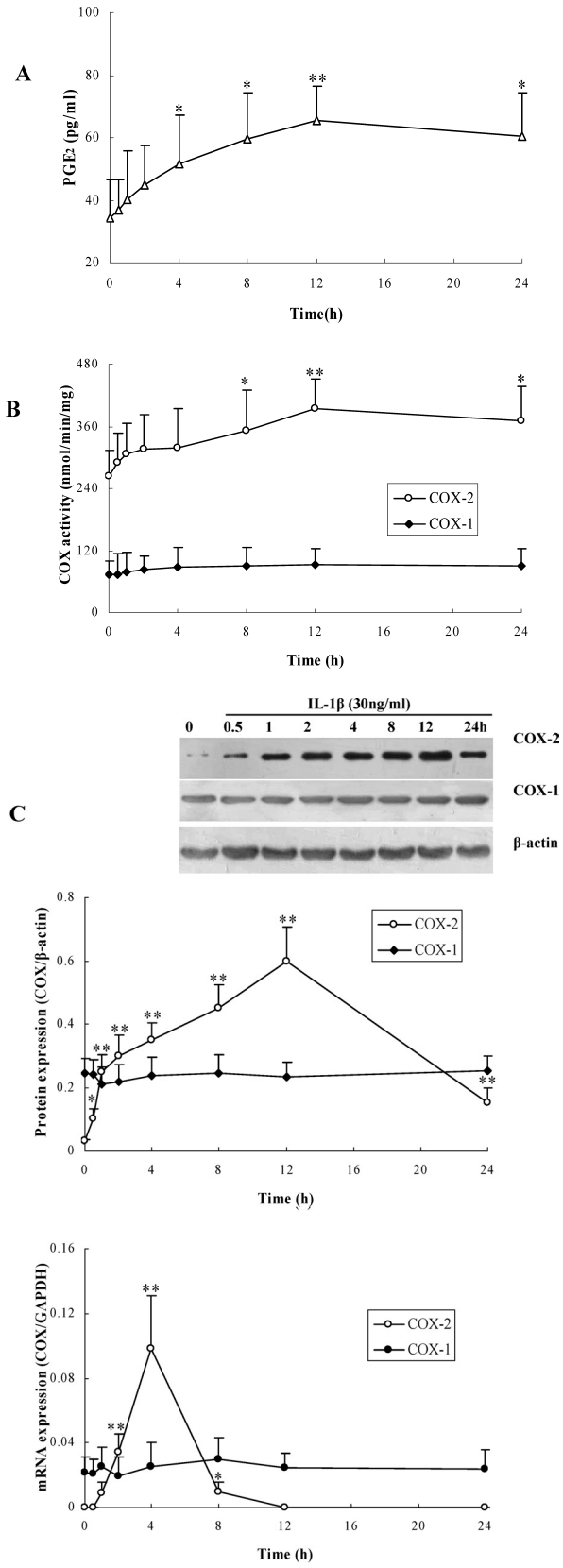

Fig. 2. Time Course of PGE_2 Production, COX Activity, COX Protein and COX mRNA Expression in CECs Stimulated by IL-1β

CECs were incubated with 30 ng/ml IL-1β for different times. After incubation, the medium was collected and the amount of PGE_2 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) PGE_2 production; (B) COX activity; (C) COX protein; (D) COX mRNA expression. Statistical significance: $^{**}p<0.01$, $^{*}p<0.05$ *vs.* non-IL-1β.

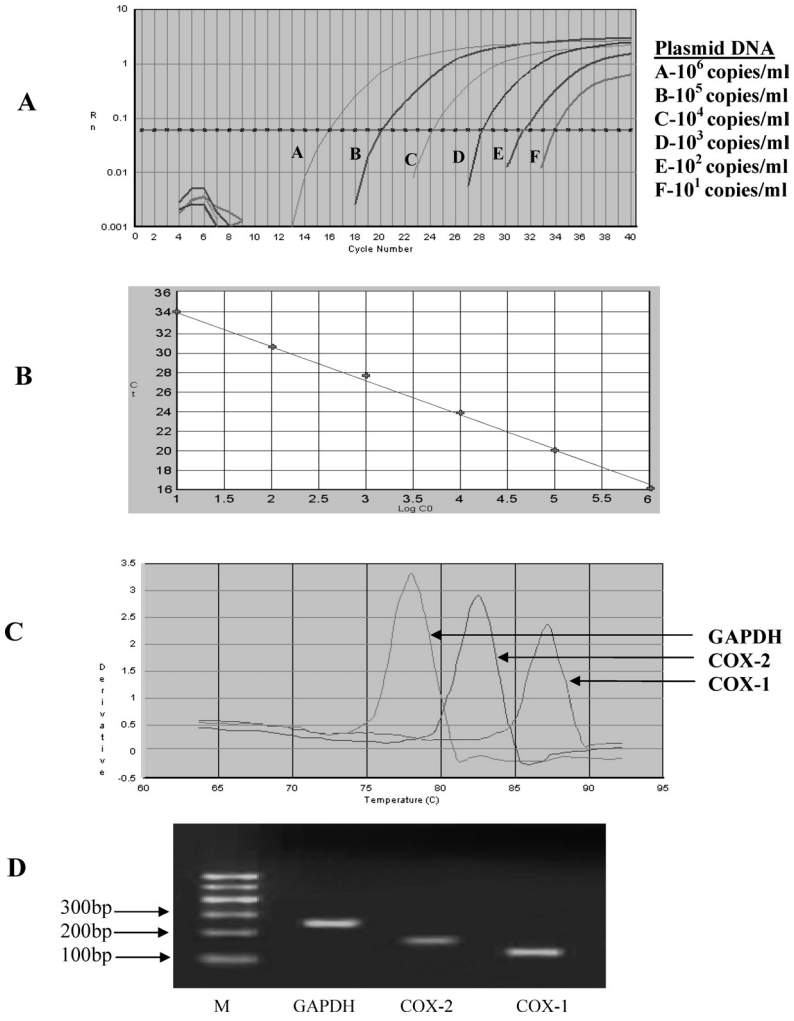

Fig. 3. Real-Time Quantiative PCR Analysis

(A, B) Linear stand curve from plasmid DNA concentrations of GAPDH; (C) melting curves of the amplification products from COX-1, COX-2 and GAPDH; (D) gel electrophoretic analysis. Gene mRNA-specific DNA bands were identified by analyzing the real-time PCR products on 2% agarose gel. M, molecular marker; predicted lengths of the PCR products were 260 bp (GAPDH), 169 bp (COX-2), 116 bp (COX-1).

COX-2 activity were increased significantly by IL-1β as early as 8 h ($p<0.05$), reached a maximum by 12 h of incubation ($p<0.01$) 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, CECs were incubated with various concentrations of IL-1β for 4 h or 12 h. 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-

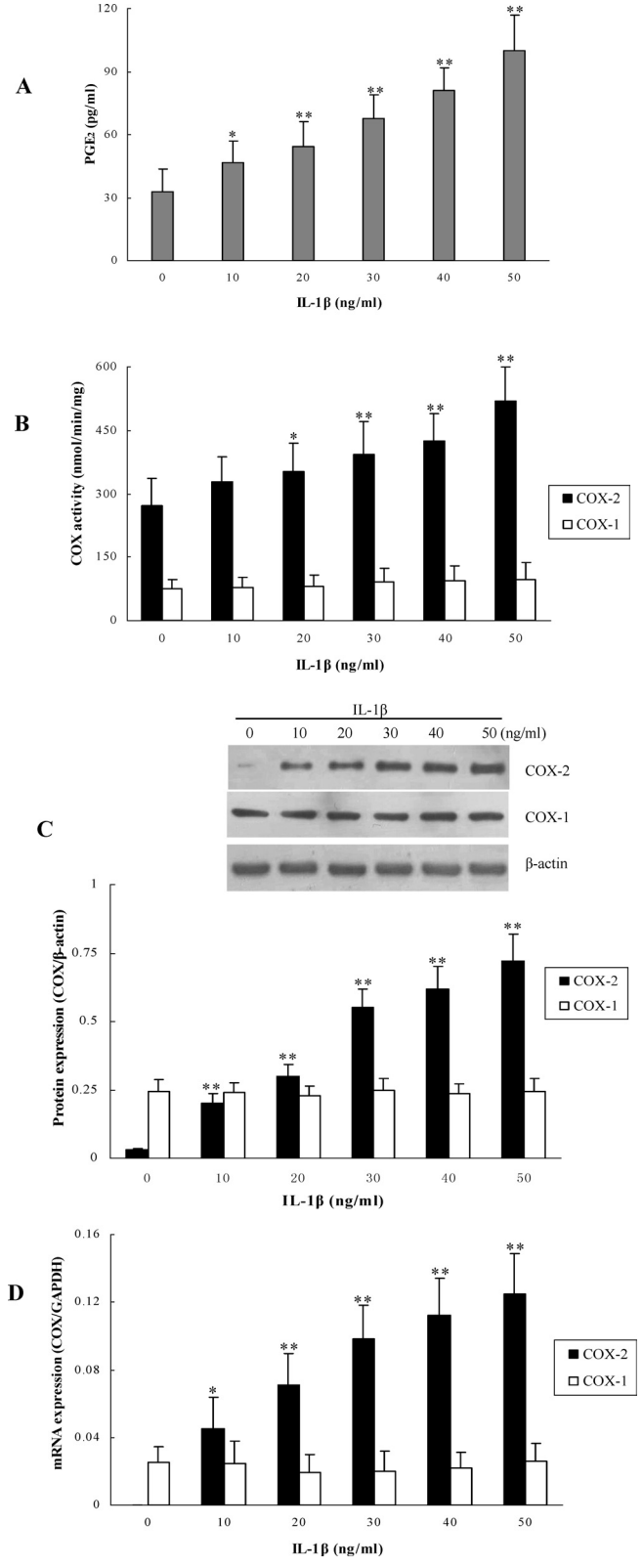

Fig. 4. The Dose–Response Analysis of IL-1β on PGE_2 Production, COX Activity, COX Protein and COX mRNA Expression in CECs Stimulated by IL-1β

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 PGE_2 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) PGE_2 production; (B) COX activity; (C) COX protein (D) COX mRNA expression. Statistical significance: $^{**}p<0.01$, $^{*}p<0.05$ *vs.* non-IL-1β.

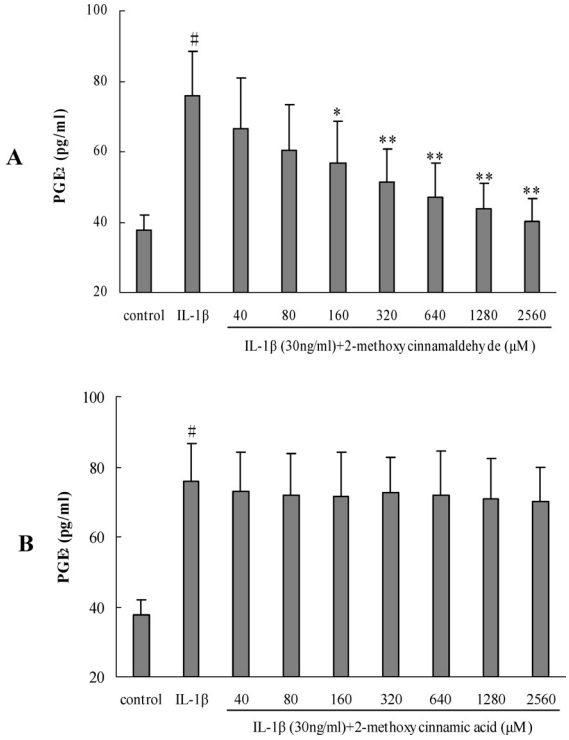

Fig. 5. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-Methoxycinnamic Acid on PGE_2 Production Induced by IL-1β

CECs were incubated with indicated concentrations of test materials for 12 h in the presence 30 ng/ml IL-1β. After incubation, the medium was collected and the amount of PGE_2 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.

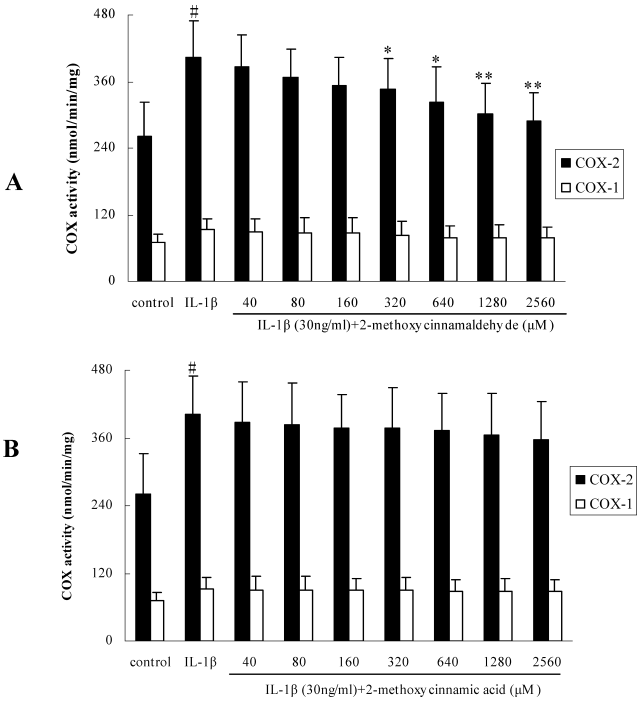

Fig. 6. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-Methoxycinnamic Acid on COX Activity Induced by IL-1β

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-methoxycinnamaldehyde decreased IL-1β-induced COX-2 activity in a dose-dependent manner, the IC_{50} value of 2-methoxycinnamaldehyde 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-methoxycinnamaldehyde on COX-2 activity was further clarified. Consistent with the above observation, *in vitro* tests showed that 2-methoxycinnamaldehyde decreased COX-2 activity in a dose-dependent manner with IC_{50} 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-methoxycinnamaldehyde 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-methoxycinnamaldehyde and 2-methoxycinnamic acid decreased IL-1β-induced COX-2 protein in a dose-dependent manner. However, 2-methoxycinnamaldehyde significantly reduced IL-1β-induced COX-2 protein as the concentration reached 160 μM compared with 2-methoxycinnamic acid of 2560 μM. 2-Methoxycinnamaldehyde 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-methoxycinnamaldehyde 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-methoxycinnamaldehyde and 2-methoxycinnamic acid showed no significant inhibitory effect on IL-1β-induced COX-1 and COX-2 mRNAs.

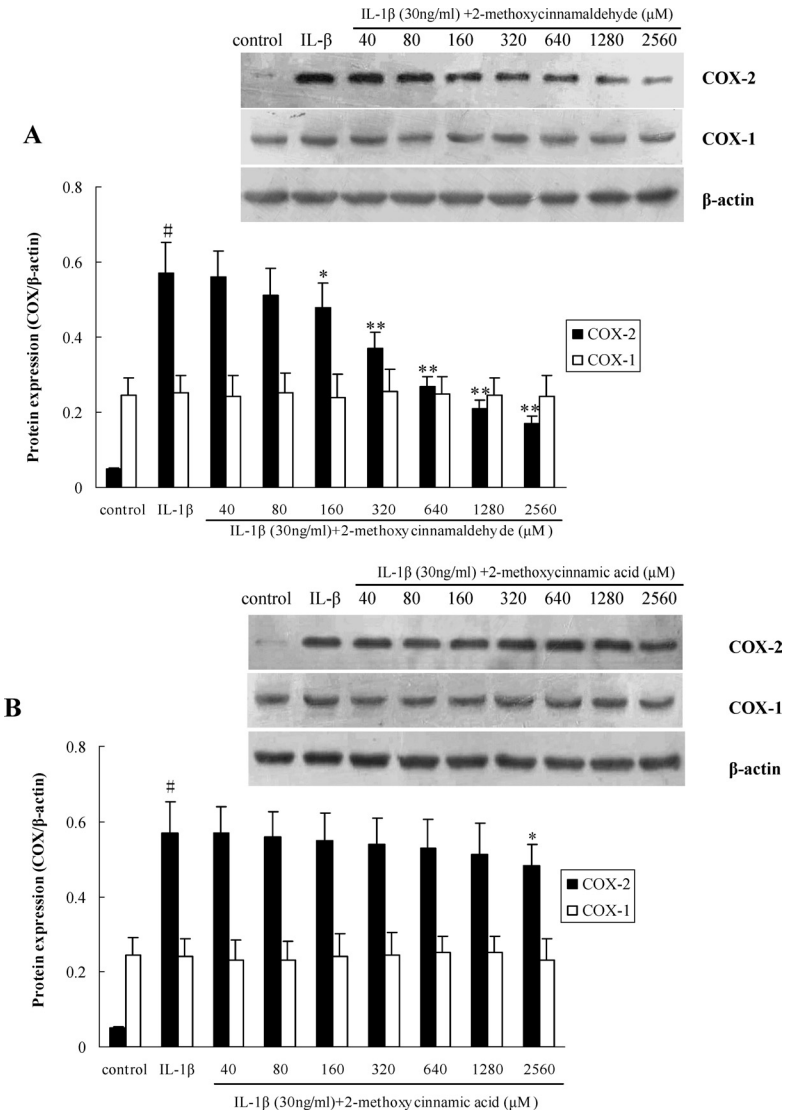

Fig. 7. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-Methoxycinnamic Acid on COX Protein Induced by IL-1β

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-Methoxycinnamaldehyde; (B) 2-methoxycinnamic acid. Statistical significance: $^{\#}p<0.01$ *vs.* control; $^{**}p<0.01$, $^{*}p<0.05$ *vs.* IL-1β.

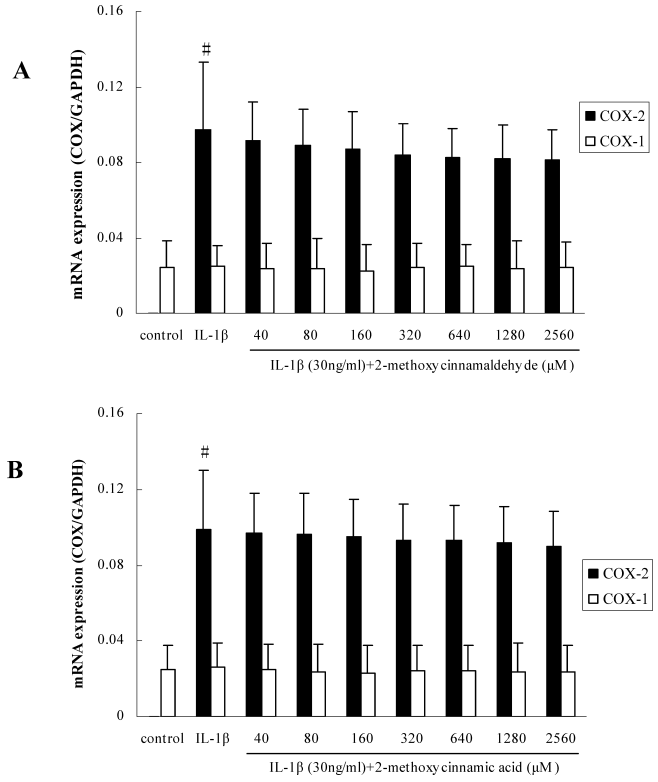

Fig. 8. Effect of Different Doses of 2-Methoxycinnamaldehyde and 2-Methoxycinnamic Acid on COX mRNA Expression Induced by IL-1β

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 expression 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 mechanisms evoked by the entry into the body of such pathogens. The conventional view of the steps that lead to fever production is that they begin with the biosynthesis of pyrogenic cytokines 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 inflammatory agents. Three routes are considered for these cytokines to gain access to the brain: (1) *via* afferent fibres that travel mostly through the vagus nerve and make their first synapse in the nucleus of the solitary tract[14,15]; (2) *via* circumventricular organs, such as the organum vasculosum laminae terminalis and the subfornical organ, which lack a blood–barrier[16]; 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, PGE_2 will be released into the hypothalamus and binds to EP3 receptors on the cells in the hypothalamic thermoregulatory center; this is followed by PGE_2-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 PGE_2 production of CECs with IL-1β inducement. PGE_2 works as a common final mediator of the febrile. CECs, a site of the blood–brain barrier *in vivo*, regulate a number of physiological 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 injection of IL-1β in rats can induce these enzymes in brain endothelial 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 PGE_2 production of CECs in a time and dose-dependent manner. 2-Methoxycinnamaldehyde dose-dependently reduced IL-1β-induced PGE_2 production with IC_{50} value of 174 μM, while no significant inhibition was observed for 2-methoxycinnamic acid. These findings suggest IL-1β is a potent inducer of prostaglandin biosynthesis in cultured CECs and strongly support the opinion that PGE_2 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 constitutive isoform COX-1 is responsible for the production of prostaglandins involved in prostanoid-mediated physiological functions. COX-2, the inducible form of the enzyme, can undergo 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, non-specific amplification was not visualized after electrophoresis and ethidium bromide staining of agarose gels. This result indicated that real-time PCR conditions 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 activity, 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 indicates 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 posttranscriptional level. 2-Methoxycinnamic acid reduced IL-1β-induced COX-2 protein at high concentration, but did not inhibit IL-1β-induced activity and mRNA expression of COX-2 in cultured CECs.

In conclusion, 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. 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.

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