

Cinnamaldehyde reduces IL-1 β -induced cyclooxygenase-2 activity in rat cerebral microvascular endothelial cells

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Abstract

Cinnamaldehyde is a principle compound isolated from Guizhi-Tang, which is a famous traditional Chinese medical formula used to treat influenza, common cold and other pyretic conditions. The aim of the present study was to investigate the effects of cinnamaldehyde on expression and activity of cyclooxygenase (COX) and prostaglandin E₂ (PGE₂) in rat cerebral microvascular endothelial cells (RCMEC). RCMEC were cultured, and identified by immunohistochemistry for von Willebrand factor in cytoplasm of the cells. Then cells were incubated in M199 medium containing interleukin (IL)-1 β in the presence or absence of cinnamaldehyde. After incubation, the medium was collected and the amount of PGE₂ was measured by enzyme-linked immunosorbent assay (ELISA). The cells were harvested, mRNA expression and activity of COX were analyzed by real-time reverse transcription-polymerase chain reaction (RT-PCR) with SYBR Green dye and ELISA respectively. Positive immunostaining for von Willebrand factor was present diffusely in the cytoplasm of >95% RCMEC. IL-1 β increased the mRNA expression and activity of COX-2, and production of PGE₂ in a dose- and time-dependent manner in RCMEC, while mRNA and activity of COX-1 were not significantly altered. Cinnamaldehyde significantly decreased IL-1 β -induced COX-2 activity and PGE₂ production in a dose-dependent manner, while it showed no inhibitory effect on IL-1 β -induced COX-2 mRNA expression in cultured RCMEC. In conclusion, cinnamaldehyde reduces IL-1 β -induced COX-2 activity, but not IL-1 β -induced COX-2 mRNA expression, and consequently inhibits production of PGE₂ in cultured RCMEC.

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1. Introduction

Guizhi-Tang, one of the most famous traditional Chinese medical formulas, has been widely used to treat influenza, common cold and other pyretic conditions. Chemical studies have shown that it includes saponins, polysaccharide and homoisoflavonoidal compounds (Zhao and Dang, 1997), but its anti-pyretic active components have not been adequately elucidated. Cinnamaldehyde was one of principle compounds isolated from Guizhi-Tang, which have traditionally been used to preserve foods, as well as to enhance flavor and odor. Antimicrobial effects of cinnamaldehyde have been described early. For example, cinnamaldehyde has been reported to inhibit the growth of *Clostridium botulinum* (Bowles and Miller,

1993), *Staphylococcus aureus* (Bowles et al., 1995), *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium (Helander et al., 1998). Recently, cinnamaldehyde has been found to inhibit cyclooxygenase (COX)-2 activity with IC₅₀ value of 245 μ M in vitro (Huss et al., 2002) and shown to be effective in inducing apoptotic cell death in a number of human cancer cells through activation of the proapoptotic Bcl-2 family proteins and mitogen-activated protein kinases (MAPK) pathway (Wu et al., 2005). In addition, as a selective transient receptor potential A1 activator, cinnamaldehyde has been reported to evoke significant spontaneous pain and induce heat and mechanical hyperalgesia, cold hypoalgesia and a neurogenic axon reflex erythema by applying on the forearm in study participants (Namer et al., 2005). However, whether cinnamaldehyde has antipyretic effect remains unclear.

Prostaglandin E₂ (PGE₂), a COX-derived metabolite of arachidonic acid, is a well-defined mediator of fever (Rotondo

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et al., 1988; Sehic et al., 1996). Increased mRNA expression and activity of COX or PGE₂ production have been found in fever (Li et al., 2001; Ivanov and Romanovsky, 2004). Interleukin (IL)-1 β is a principle component of endogenous pyrogens, and it has been generally recognized that IL-1 β released from cells peripherally produces fever by signaling the brain via various routes (Blatteis and Sehic, 1997).

Therefore, in our present study, we tested the effect of cinnamaldehyde on IL-1 β -induced expression and activity of COX and PGE₂ production to provide some pharmacological evidence for clinical use of Guizhi-Tang in fever. For this purpose, rat cerebral microvascular endothelial cells (RCMEC) were cultured and incubated in M199 medium containing IL-1 β in the presence or absence of cinnamaldehyde. The cells were selected due to its relevance to immune-brain signaling mediated by PGE₂ (Cao et al., 1996; Inoue et al., 2002; Matsumura and Kobayashi, 2004). After incubation, the medium was collected and the amount of PGE₂ was measured by enzyme-linked immunosorbent assay (ELISA). The cells were harvested, mRNA expression and activity of COX were analyzed by real-time reverse transcription-polymerase chain reaction (RT-PCR) with SYBR Green dye and ELISA, respectively. The present results show that cinnamaldehyde inhibits IL-1 β -induced PGE₂ production through the inhibition of COX-2 activity in cultured RCMEC.

2. Materials and methods

2.1. Materials

Cinnamaldehyde (99%) was provided by School of Pharmaceutical Sciences in Peking University. Culture media, serum and buffers for cell culture were obtained from Invitrogen Inc. (Carlsbad, CA). Endothelial cells growth factor (ECGF) was acquired from Roche Inc. (Basel, Switzerland). Rabbit anti-human von Willebrand factor antigen was purchased from Dako Co. (Santa Barbara, CA). IL-1 β was obtained from PeproTech Inc. (Rocky Hill, NJ). PGE₂ ELISA kit was purchased from Shanghai Sun Biomedical Co. (Shanghai, China). COX ELISA kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). Reverse Transcription Reagents and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Branchburg, NJ). 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. All other materials were purchased from Sigma Co. (St. Louis, MO) except where indicated, and were of analytical grade.

2.2. Cell culture

Endothelial cells were isolated from rat microvessels and cultured as described previously (Gordon et al., 1991). 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. The digested microvessels were centrifuged and the pellet was resuspended

in M199 medium supplemented with 150ng/ml ECGF, 25% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin. The microvessel suspension was plated on tissue culture flask and incubated at 37°C in a 5% CO₂ incubator. After confluence for 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 3 to 5.

2.3. Immunocytochemistry

The RCMEC 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, which consistently remained unstained.

2.4. Treatment of RCMEC

The RCMEC 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-1 β or other reagents. For assay of PGE₂ and COX activity experiments, the cells were incubated with various concentrations of IL-1 β for 12 h, or 30 ng/ml IL-1 β for different times. Then RCMEC were incubated in the serum-free medium containing 30 ng/ml IL-1 β in the presence or absence of different concentrations of cinnamaldehyde for 12 h. For assay of COX mRNA expression experiment, the cells were incubated with various concentrations of IL-1 β for 4 h, or 30 ng/ml IL-1 β for different times. Then RCMEC were incubated in the serum-free medium containing 30 ng/ml IL-1 β in the presence or absence of different concentrations of cinnamaldehyde for 4 h.

2.5. Assay of prostaglandin E₂

After incubation, the medium was collected for measurement of PGE₂. PGE₂ 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).

2.6. RNA extraction and real-time RT-PCR

COX mRNA was measured by real-time RT-PCR as previously described (Stirone et al., 2003; Veistinen et al., 2002). The total RNA from different experimental conditions

was obtained by TRIzol method. The concentration of RNA was determined by an absorbance at 260nm and RNA was reverse transcribed to cDNA using the Taqman® Reverse Transcription Reagents (Applied Biosystems). Reverse transcription was performed at 48°C for 30min followed by RT inactivation at 95°C for 5min (Perkin-Elmer GeneAmp 9600, Foster City, CA). cDNA was analyzed immediately or stored at –20°C until use. The details of all oligonucleotide primer sequences, predicted product lengths and gene bank accession by amplification in real-time PCR are listed in Table 1. Real-time PCR assay was carried out with ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems), using SYBR Green PCR Master Mix (Applied Biosystems), in order to observe the level of mRNA. The basic protocol for real-time PCR was an initial incubation at 95°C for 5min, followed by 45 cycles of 94°C for 30s, 56°C for 30s, 72°C for 1min 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.

2.7. Assay of COX activity

RCMEC from the above culture were scraped and spun down at 1500×g in a microfuge for 10min at 4°C and washed once with saline. Then cells were suspended in cold buffer (0.1 M Tris–HCl, pH 7.8, containing 1 mM EDTA) and sonicated in an ice bath for 3×4s by an ultrasonic sonicator. The crude homogenate was centrifuged at 10,000×g for 10min in a tabletop microfuge and the supernatant was collected. The protein content was measured with a protein assay kit, then the COX activity was determined by ELISA as indicated in kit instructions. The kit includes isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity.

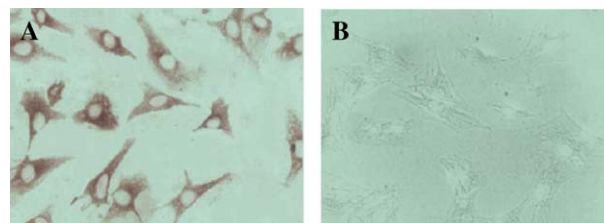


Fig. 1. Characterization of RCMEC. (A) RCMEC at passage 3 cultured on gelatin-coated chamber slides stained positively with antibody to von Willebrand factor. (B) Negative control slide remained unstained (400×).

2.8. Statistical analysis

All the data are given as the mean ± 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. Data were correlated by nonparametric Spearman's rank method. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of the RCMEC cultures

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 days. Primary endothelium culture displayed typical “cobble stone” morphology. Based on morphology, >95% of the population were endothelial cells. Positive immunostaining for von Willebrand factor, a marker for all endothelial cells, was found to be present diffusely in the cytoplasm of cultured RCMEC (Fig. 1A). Negative control slides remained unstained (Fig. 1B).

3.2. Dose- and time-dependent effects of IL-1β on COX mRNA expression, COX activity and PGE2 production

To determine the time-dependent effect of IL-1β, RCMEC were incubated with IL-1β (30 ng/ml) for different times. Real-time RT-PCR reactions were performed to amplify COX-1 and COX-2, and the standard curve was run for each target gene (for example, COX-1 in Fig. 2A and B). Melting curve analysis confirmed that there was no primer dimer in the PCR products (Fig. 2C). For each of the primer sets, non-specific amplification was visualized after electrophoresis and ethidium bromide

Table 1
Sequences of primers used for real-time RT-PCR

Name	Oligo	Primer sequence	Predicted size (bp)	Gene bank accession
GAPDH	Forward primer	5'-TGAACGGGAAGCTCACTGG-3'	260	NM_017008
	Reverse primer	5'-GAGCTTCACAAAGTTGTCAATTGAG-3'		
COX-1	Forward Primer	5'-GGCGTTGCTCATCCATCTACTC-3'	116	S67721
	Reverse primer	5'-AGCATCTGTGAGCAGTACCGG-3'		
COX-2	Forward primer	5'-TTTGTTGAGTCATTACACAGACAGAT-3'	169	S67722
	Reverse primer	5'-ACGATGTGTAAGGTTTCAGGGAGAAG-3'		

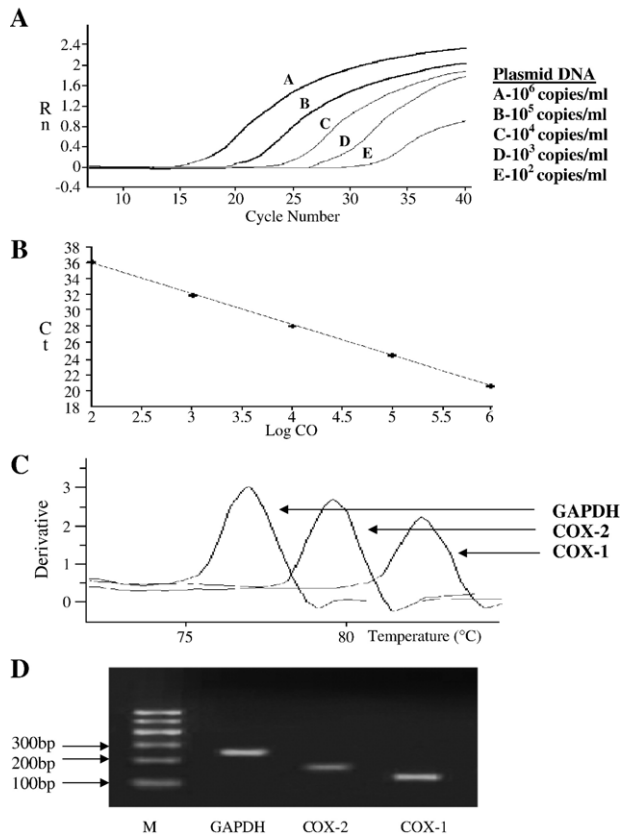


Fig. 2. Real-time quantitative PCR analysis. (A and B) Linear stand curve from plasmid DNA concentrations of COX-1. (C) Melting curves of the amplification products from COX-1, COX-2 and GAPDH. (D) Gel electrophoretic analysis. Gene mRNA-specific DNA bands were identified by analyzing the real-time PCR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

staining of agarose gels (Fig. 2D). 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-1 β . While COX-1 mRNA was present and remained constant during the incubation period (Fig. 3A). ELISA results indicated that IL-1 β also increased the COX-2 activity and PGE₂ production in a time-dependent manner (Fig. 3B and C), while COX-1 activity was not significantly altered during the incubation period (Fig. 3B). The production of COX-2 activity and PGE₂ production were increased significantly by IL-1 β as early as 8 h ($P < 0.05$), reached a maximum by 12 h of incubation ($P < 0.01$) and then declined thereafter.

To determine the concentration-dependent effect of IL-1 β , RCMEC were incubated with various concentrations of IL-1 β for 4 h or 12 h. Real-time RT-PCR results indicated that IL-1 β increased the COX-2 mRNA in a concentration-dependent manner, While COX-1 mRNA remained constant (Fig. 4A). ELISA results indicated that IL-1 β also increased the COX-2 activity and PGE₂ production in a concentration-dependent manner (Fig. 4B and C), while COX-1 activity was not significantly altered during the incubation period (Fig. 4B). Concentrations as low as 10 ng/ml were effective in increasing the COX-2 mRNA expression and PGE₂ production ($P < 0.05$).

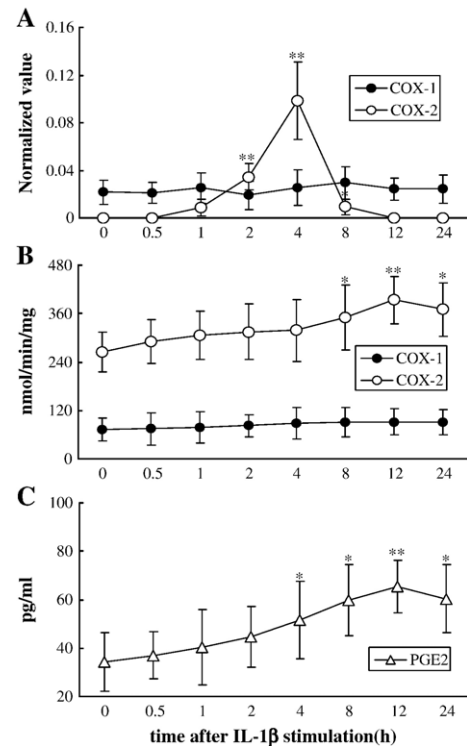


Fig. 3. Time course of mRNA expression and activity of COX, and PGE₂ production in RCMEC stimulated by IL-1 β . RCMEC were incubated with 30 ng/ml IL-1 β for different times. After incubation, the medium was collected and the amount of PGE₂ was determined by ELISA. The cells were harvested, mRNA expression and activity of COX were analyzed by real-time RT-PCR and ELISA, respectively. (A) Normalized COX mRNA expression; (B) COX activity; (C) PGE₂ production. Statistical significance: ** $P < 0.01$, * $P < 0.05$ vs. non-IL-1 β .

3.3. Effect of cinnamaldehyde on IL-1 β -induced expression of COX mRNA

To determine whether cinnamaldehyde affect the IL-1 β -induced expression of COX, the cells were incubated with various concentrations of cinnamaldehyde for 4 h in the presence of 30 ng/ml IL-1 β . Real-time RT-PCR analysis indicated that IL-1 β -induced expression of COX-2 was not significantly altered by cinnamaldehyde, COX-1 mRNA expression also remained constant (Fig. 5A).

3.4. Effect of cinnamaldehyde on IL-1 β -induced COX activity and PGE₂ production

To determine whether cinnamaldehyde affect the IL-1 β -induced COX activity and PGE₂ production, RCMEC were incubated with various concentrations of cinnamaldehyde for 12 h in the presence of 30 ng/ml IL-1 β . After incubation, the COX activity and PGE₂ production were detected by ELISA. As shown in Fig. 5C, cinnamaldehyde decreased IL-1 β -induced PGE₂ production in a dose-dependent manner. Consistent with the observation mentioned in Introduction (Huss et al., 2002), cinnamaldehyde also decreased IL-1 β -induced COX-2 activity in a dose-dependent manner, significant difference was shown as the concentration reached 400 μ M ($P < 0.05$). In contrast,

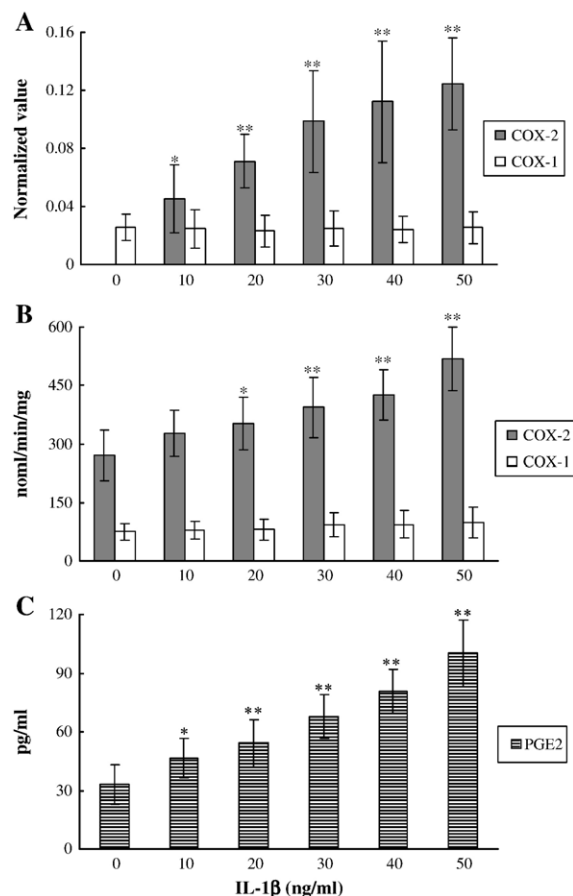


Fig. 4. The dose–response analysis of IL-1 β on mRNA expression and activity of COX, and PGE₂ production in RCMEC. RCMEC were incubated with various concentrations of IL-1 β for 4h or 12h. After incubation, the medium was collected and the amount of PGE₂ was determined by ELISA. The cells were harvested, mRNA expression and activity of COX were analyzed by real-time RT-PCR and ELISA respectively. (A) Normalized COX mRNA expression; (B) COX activity; (C) PGE₂ production. Statistical significance: ** P <0.01, * P <0.05 vs. non-IL-1 β .

COX-1 activity was not significantly altered during the incubation period (Fig. 5B).

3.5. Correlations

According to Spearman's nonparametric rank correlation method, data analysis revealed that cinnamaldehyde-mediated reduction of IL-1 β -induced COX-2 activity correlated with a decrease in PGE₂ production ($r=0.956$, $P<0.05$).

4. Discussion

The systemic response of fever can be the result of pathogenic infection. In particular, the release of lipopolysaccharide (LPS) from Gram-negative bacterial cell wall during most infections triggers a febrile response by stimulating monocytes and macrophages to synthesize and release several pyrogenic cytokines such as IL-1 β , tumor necrosis factor (TNF), and IL-6 (see review by Kluger, 1991). Three routes are considered for the 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 (Blatteis et al., 2000; Romanovsky et al., 2000); (2) via circumventricular organs, such as the organum vasculosum laminae terminalis and the subfornical organ, which lack a blood-barrier (Takahashi et al., 1997); and (3) via interaction with cells located in the blood–brain interface, i.e. endothelial cell (Cao et al., 1996; Tilders et al., 1994). When some of the afferent pathways are activated, PGE₂ will be released into the hypothalamus and binds to EP3 receptors on the cells in the hypothalamic thermoregulatory center (Ushikubi et al., 2000); this is followed by PGE₂-induced neuronal mechanisms involving cyclic AMP and neurotransmitters (Lin et al., 1982) to elevate the temperature set-point, resulting in fever.

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

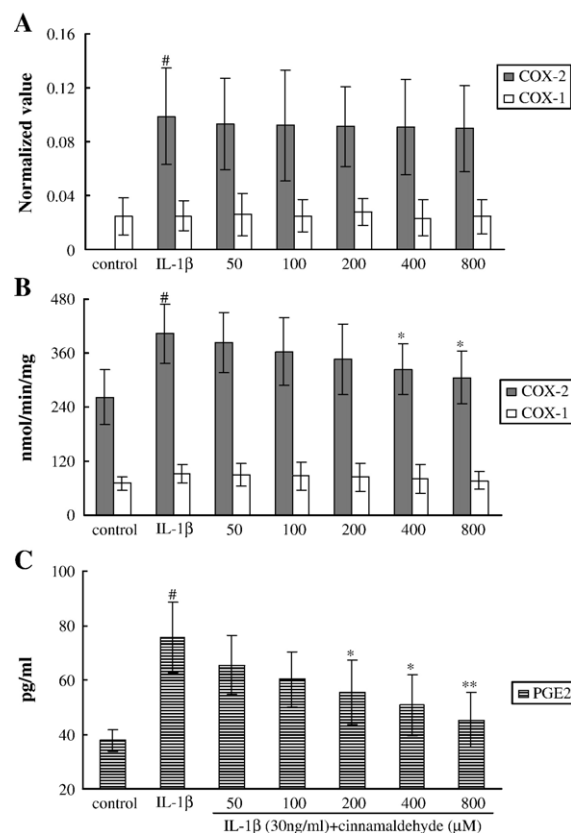


Fig. 5. Effect of different doses of cinnamaldehyde on mRNA expression and activity of COX activity and PGE₂ production induced by IL-1 β . RCMEC were incubated with indicated concentrations of cinnamaldehyde for 4h or 12h in the presence or absence of 30 ng/ml IL-1 β . After incubation, the medium was collected and the amount of PGE₂ was determined by ELISA. The cells were harvested, mRNA expression and activity of COX were analyzed by real-time RT-PCR and ELISA respectively. (A) Normalized COX mRNA expression; (B) COX activity; (C) PGE₂ production. Statistical significance: # P <0.01 vs. control; ** P <0.01, * P <0.05 vs. IL-1 β .

involved in prostanoid-mediated physiological functions. A second isoform, COX-2, has been identified and has been demonstrated to be highly expressed under experimental conditions (Inoue et al., 2002; Whittle, 2004). For this paper, we isolated and cultured functionally active RCMEC, then the confluent of endothelial cells was incubated in M199 medium in the presence or absence of IL-1 β . Cerebral microvascular endothelial cells, a site of the blood–brain barrier in vivo, regulate a number of physiological and pathophysiological processes in the brain. IL-1 β has been reported to induce COX expression, activity and prostaglandin biosynthesis in many types of endothelial cells, including human umbilical vein endothelial cells (Caughey et al., 2001; Uracz et al., 2002). In addition, intraperitoneal injection of IL-1 β in rats can induce these enzymes in brain endothelial cells in vivo. However, study on COX mRNA expression, activity and prostaglandin biosynthesis in cultured brain endothelial cells with IL-1 β -inducement has not been reported in the literature. The COX activity and PGE₂ production were then detected by ELISA. The levels of expression of COX-1 and COX-2 were measured by real-time quantity PCR with SYBR Green dye. 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 RCMEC. As previously observed (Albert et al., 1994; Dubois et al., 1998; Raz et al., 1988), we also found that IL-1 β induced mRNA expression and activity of COX-2 and PGE₂ production in a dose- and time-dependent manner in RCMEC, while mRNA expression and activity of COX-1 were not significantly altered. These findings suggest IL-1 β is a potent inducer of COX-2 and prostaglandin biosynthesis in cultured RCMEC and strongly support the opinion that PGE₂ may gain access to the brain via interaction with endothelial cell.

In this study, we first examined the effect of cinnamaldehyde on central mediator of fever in cultured RCMEC. The results showed that the molecular basis of the effect of cinnamaldehyde was manifested at the posttranscriptional level. IL-1 β -induced COX-2 activity was significantly decreased by cinnamaldehyde in cultured RCMEC. Cinnamaldehyde also significantly reduced IL-1 β -induced PGE₂ production. The correlation analysis indicated that there is a positive correlation between reduced COX-2 activity and decreased PGE₂. These suggest that decreased production of PGE₂ by cinnamaldehyde is partly caused by down-regulation of COX-2 activity. Huss et al. reported that cinnamaldehyde inhibits COX-2 activity. In their strategy, a rapid semi-homogeneous COX-2 enzymatic assay using scintillation proximity assay was developed to identify inhibition of COX-2-catalyzed PGE₂ biosynthesis. In our study, using a cell-based assay, the strategy employed involves examining the effect of cinnamaldehyde on mRNA expression and activity of COX. Based on the strategy, we first find that cinnamaldehyde reduces the IL-1 β -induced COX-2 activity, but does not reduce IL-1 β -induced COX-2 mRNA, and as a result PGE₂ production is suppressed in cultured RCMEC.

In conclusion, cinnamaldehyde inhibits IL-1 β -induced PGE₂ production through the inhibition of COX-2 activity in cultured RCMEC. These results suggest that Guizhi-Tang's antipyretic property might be partly ascribable to cinnamaldehyde. Other active constituents of Guizhi-Tang remain to be studied in the future.

Acknowledgments

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