Effects of Cinnamaldehyde on PGE₂ Release and TRPV4 Expression in Mouse Cerebral Microvascular Endothelial Cells Induced by Interleukin-1 β

Yue-Ying MA,^a Hai-Ru HUO,*^a Cang-Hai LI,^b Bao-Sheng ZHAO,^a Lan-Fang LI,^a Feng SUI,^a Shu-Ying GUO,^a and Ting-Liang JIANG^a

^a Tang Center for Herbal Medicine Research, Institute of Chinese Materia Medica, China Academy of Chinese Medical Science; and ^b Medical Science Experimental Center, China Academy of Chinese Medical Sciences; Beijing 100700, China. Received April 17, 2007; accepted December 5, 2007; published online December 13, 2007

Cinnamaldehyde is a principle compound isolated from Guizhi-Tang (GZT), which is a famous traditional Chinese medical formula used to treat influenza, common cold and other pyretic conditions. Transient receptor potential vanilloid subtype 4 (TRPV4) is expressed in the anterior hypothalamus and may act as thermosensor. The purpose of the present study was to investigate the effects of cinnamaldehyde on the production of prostaglandin E₂ (PGE₂) and the expression of TRPV4 in mouse cerebral microvascular endothelial cell strain (b.End3). In the research work, the b.End3 cells were cultured in DMEM medium containing interleukin-1 β (IL-1 β) in the presence or absence of ruthenium red (RR), a kind of known TRPV4 inhibitor, or different concentrations of cinnamaldehyde. The results suggested that IL-1 β significantly increase production of PGE₂ and cinnamaldehyde evidently decrease IL-1 β -induced PGE₂ production, while RR showed no inhibitory effect on PGE₂ production. Moreover, it was identified that TRPV4 was expressed at the mRNA and protein levels in b.End3 cells. IL-1 β could up-regulate the expression of TRPV4, RR and cinnamaldehyde could down-regulate the high expression of mRNA and protein of TRPV4 by IL-1 β induced in b.End3 cells. In conclusion, cinnamaldehyde decreased the production of PGE₂ and the expression of TRPV4 in b.End3 cells induced by IL-1 β .

Key words cinnamaldehyde; cerebral microvascular endothelial cells; interleukin-1 β ; prostaglandin E₂; transient receptor potential vanilloid subtype 4

Chinese formula has over one thousand year history. Guizhi-Tang (GZT) is one of the most famous formulae in the classical works of Shang-han-lun, which has been widely used to treat influenza, common cold and other pyretic conditions. Cinnamaldehyde was one of principle compounds isolated from Guizhi-Tang, which was previously found that it relieved fever and pain,¹⁾ inhibited the bacillus tuberculosis and influenza virus effectively, and could regulate the immunological function.²⁾ Our recent research also showed that it could reduce the amount of cyclooxygenase (COX)-1, COX-2 and prostaglandin E₂ (PGE₂), this perhaps the mechanism of its relieving-fever effect.³⁾

PGE₂, a COX-derived metabolite of arachidonic acid, is a well-defined mediator of fever.⁴⁾ Increased mRNA expression and activity of COX or PGE₂ production have been found in fever.⁵⁾ Interleukin-1 β (IL-1 β) is a main 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.⁶⁾

Transient receptor potential vanilloid subtype 4 (TRPV4) is a broadly expressed Ca²⁺-permeable cation channel in the vanilloid subfamily of transient receptor potential (TRP) channels. TRPV4 is expressed in the anterior hypothalamus and currents have similar temperature sensitivity to the pre-optic anterior hypothalamus (POAH) cell currents.⁷⁾ TRPV4 gates in response to a large variety of stimuli, including cell swelling,⁸⁾ warm temperatures (>27 °C), the synthetic phorbol ester 4- α -phorbol 12,13-didecanoate (4 α -PDD), and the endogenous lipid arachidonic acid (AA).⁹⁾ Temperature sensitivity that spans normal body temperature suggests that TRPV4 respond to small changes in body temperature around 37 °C, which suggested that TRPV4 may act as a ther-

mosensor. However, the role of TRPV4 in the thermoregulation remains obscure.

In the present research, it was investigated that the effect of cinnamaldehyde on IL-1 β -induced PGE₂ release and expression of TRPV4 in mouse cerebral microvascular endothelial cells (b.End3) in order to further clarify the antipyretic mechanism of cinnamaldehyde.

MATERIALS AND METHODS

Materials Cinnamaldehyde was extracted from Guizhi-Tang (the purity of 99.98%). IL-1 β was obtained from Pepro Tech Inc. (Rocky Hill, NJ, U.S.A.). PGE₂ ELISA kit was purchased from Suzhou SuDa Technology Development Co. (Suzhou, China). Ruthenium red (RR) was purchased from Sigma (St. Louis, MO, U.S.A.). Dulbecco's minimum essential medium (DMEM) was obtained from GIBICO. TRIzol and electrophoresis reagents were from Takara Co., RNAsafe, Oligo (dT)15, dNTP and SYBR green I were purchased from Tiangen Biotech Company (Beijing, China). M-MLV Reverse Transcriptase and M-MLV Reverse RT 5 $\times$ Buffer were purchased from Promega (WI, U.S.A.), and primers sequences were synthesized by Invitrogen Inc. (Carlsbad, CA, U.S.A.). Antibody against TRPV4 was purchased from Chemicon (Billerica, MA, U.S.A.). All reagents for cell culture were of tissue culture grade, and for RNA extraction, reagents were of molecular biology grade.

Cell Culture and Treatments The b.End3 cells (a gift from Prof. Jin H. M., Department of Physiology & Pathophysiology, Fudan University Shanghai Medical College) were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/l), strepto-

* To whom correspondence should be addressed. e-mail: huohr@yahoo.com.cn

mycin (100 mg/l) and NaHCO_3 (3.6 g/l). The b.End3 cells were sub-cultured into 24-well cell culture plates and maintained until sub-confluence. RR, a known TRPV4 inhibitor, was used as a tool to block TRPV4 and chosen 0.38 mM as cinnamaldehyde's IC_{50} of PGE_2 stimulated by IL-1 β according to the previous study. Subsequently, the cells were incubated in the serum-free medium containing 30 ng/ml IL-1 β in the presence or absence of RR (10 μM) or different concentrations of cinnamaldehyde. In the current experiment, b.End3 cells were divided into 6 groups: control, RR (RR 10 μM), IL-1 β -induced model (IL-1 β 30 ng/ml-induced), RR 10 μM (IL-1 β 30 ng/ml-induced), cinnamaldehyde 0.38 mM (IL-1 β 30 ng/ml-induced) and cinnamaldehyde 0.19 mM (IL-1 β 30 ng/ml-induced) group.

Assay of PGE_2 After incubation, the medium was collected for measurement of PGE_2 . PGE_2 was determined by ELISA according to the manufacturer's recommendations. The absorbance was then measured at 490 nm by an enzyme immunoassay instrument (Bio-Rad, Hercules, CA, U.S.A.).

RNA Extraction and cDNA Synthesis The total RNA from different experimental conditions was obtained by means of TRIzol method. Subsequently, the amount of RNA was determined by measuring the optical density at 260 nm and RNA was reverse transcribed to cDNA using the Taqman[®] Reverse Transcription Reagents (Applied Biosystems). Reverse transcription was performed at 42 °C for 30 min followed by RT inactivation at 94 °C for 5 min. cDNA was analyzed immediately or stored at -20 °C until it was used. The sequences for the TRP4 sense primer was 5'-AGAAAGGTCGTGGAGAAGC-3' and for the antisense primer was 5'-ATCAGTCAGGCGCTTCTTGT-3' with the expected product length of 174 base pairs. Real-time polymerase chain reaction (PCR) assay was carried out with IQ5-Type Real-time PCR meter (Bio-Rad, U.S.A.). All samples were run in triplicate, the relative expression values were normalized to the expression value of GAPDH. PCR mix was prepared according to the manufacturer's instructions and thermal cycler conditions were as follows: 1×5 min 94 °C, 55 cycles denaturation (30 s, 94 °C), annealing (30 s, 60 °C) and extension (30 s, 72 °C). Relative expression of the TRPV4 genes was calculated to use the formula $2^{-\Delta\Delta\text{CT}}$.

Western Blot Analysis After treatment, cells were prepared using lysis buffer (50 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1% sodium dodecyl sulphate (SDS); and 100 mM NaCl) and the protein concentration was estimated by the bicinchoninic acid assay. An equal amount of protein for each sample was separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.).

The membrane was blocked with 5% (w/v) nonfat dry milk in phosphate buffered saline (PBS) containing 0.02% Tween 20 (PBST) for 1 h with the room temperature, and followed by incubation at 4 °C overnight with a specific primary antibody. The primary antibodies were polyclonal rabbit anti-TRPV4 (1 : 500 dilution), the nitrocellulose membrane was then washed with PBST. After washing, the membrane was incubated with peroxidase-conjugated goat-anti-rabbit secondary antibody at 1 : 1000 dilution at room temperature for 1 h. The final reaction products were visualized by using enhanced chemiluminescence and recorded on X-ray film. For quantitative analysis the bands were scanned in a Foto/Ana-

lyst Investigator PC system using PC Image 5.0 software (Fotodyne Inc. Hartland, WI, U.S.A.), and the densities of the bands were quantitated by using Image Tool 3.00 to analyze the gray scale of every group.

Immunofluorescence Assay When the cultures reached confluence, the cells were cultured in sterile cover slips and treated under different condition as described above. After the treatment, the cells were fixed with 4% formaldehyde in PBS for 20 min and washed with PBS. The fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 25 min at room temperature (RT), washed with PBS for 3 times, then treated with anti-TRPV4 polyclonal antibody at 1 : 300 dilution overnight at 4 °C, washed with PBS for 3 times. Cells were then incubated at 1 : 100 dilution of fluorescen-conjugated goat anti-rabbit IgG for 1 h at room temperature, and washed with PBS for 3 times. Finally, the cells were sealed with glycerine. The expression of TRPV4 of every group was evaluated as Qwin Standard V3.1.0 data processing system.

Statistical Analysis All the data are given as the mean $\pm$ S.D. Every experiment was repeated at least two times and the data were pooled. The results were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparisons test among groups. Data were correlated by nonparametric Spearman's rank method. Value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Cinnamaldehyde on PGE_2 Production Induced by IL-1 β To determine whether cinnamaldehyde affects the IL-1 β -induced PGE_2 production, b.End3 cells were incubated with RR or cinnamaldehyde for 12 h in the presence of 30 ng/ml IL-1 β . After incubation, PGE_2 productions were detected by ELISA. The results showed that PGE_2 production treated with IL-1 β in b.End3 cells was significantly higher than normal cells ($p < 0.01$ vs. control). The IL-1 β induced increase in PGE_2 production was inhibited by cinnamaldehyde 0.38 mM and 0.19 mM ($p < 0.05$ vs. IL-1 β), while RR had no inhibitory effect ($p > 0.05$ vs. IL-1 β) on the PGE_2 production of IL-1 β inducement in b.End3 cells (Fig. 1).

Effect of Cinnamaldehyde on Expression of TRPV4

Fig. 1. Effect of Cinnamaldehyde on PGE_2 Production Induced by IL-1 β

The b.End3 cells were incubated with indicated concentrations of cinnamaldehyde for 4 h in the presence or absence IL-1 β (30 ng/ml). After incubation, the medium was collected and the amount of PGE_2 was determined by ELISA. Data are means $\pm$ S.D. ($n=6$). # $p < 0.01$ vs. control; ** $p < 0.01$, * $p < 0.05$ vs. IL-1 β . RR, ruthenium red; CA, cinnamaldehyde.

mRNA Induced by IL-1 β To determine the expression of TRPV4 in b.End3 cells, it was performed by real-time PCR to analyze the expression of TRPV4 mRNA. In the course of our studies, it was identified that TRPV4 mRNA was expressed in b.End3 cells. TRP mRNA expression was up-regulated upon IL-1 β stimulation ($p < 0.01$ vs. control), IL-1 β -induced expression of TRPV4 mRNA was down-regulated by RR ($p < 0.01$ vs. IL-1 β) and cinnamaldehyde 0.38 mM ($p < 0.05$ vs. IL-1 β). This indicated that down-regulating the expression of TRPV4 mRNA may be one of the mechanisms of cinnamaldehyde to reduce the anti-inflammation action (Fig. 2).

Fig. 2. Effect of Cinnamaldehyde on TRPV4 mRNA Expression Induced by IL-1 β

The b.End3 cells were incubated with indicated concentrations of cinnamaldehyde for 4 h in the presence or absence IL-1 β (30 ng/ml). After incubation, the cells were harvested, TRPV4 mRNA expression was analyzed by real-time RT-PCR. Data are means $\pm$ S.D. ($n=6$). ## $p < 0.01$ vs. control; ** $p < 0.01$, * $p < 0.05$ vs. IL-1 β . RR, ruthenium red; CA, cinnamaldehyde.

Fig. 3. Effect of Cinnamaldehyde on TRPV4 Protein Expression Induced by IL-1 β

The b.End3 cells were incubated with indicated concentrations of cinnamaldehyde for 4 h in the presence or absence IL-1 β (30 ng/ml). After incubation, the cells were harvested, TRPV4 protein expression was analyzed by Western blotting and immunofluorescence assay, respectively. Top: Performed by Western blot; Bottom: Performed by immunofluorescence assay. Data are means $\pm$ S.D. ($n=6$). ## $p < 0.01$ vs. control; ** $p < 0.01$, * $p < 0.05$ vs. IL-1 β . RR, ruthenium red; CA, cinnamaldehyde.

Fig. 4. Effect of Cinnamaldehyde on TRPV4 Protein Expression Induced by IL-1 β

The b.End3 cells were incubated with indicated concentrations of cinnamaldehyde for 4 h in the presence or absence IL-1 β (30 ng/ml). After incubation, the cells were harvested, TRPV4 protein expression was analyzed by Western blotting and immunofluorescence assay, respectively. Top: Performed by Western blot; Bottom: performed by immunofluorescence assay. A: control; B: RR (10 μ M); C: IL-1 β -induced (30 ng/ml); D: RR (10 μ M)+IL-1 β (30 ng/ml); E: CA (0.38 mM)+IL-1 β (30 ng/ml); F: CA (0.19 mM)+IL-1 β (30 ng/ml). RR, ruthenium red; CA, cinnamaldehyde.

Effect of Cinnamaldehyde on Expression of TRPV4 Protein Induced by IL-1 β By using Western blot analysis and immunofluorescence assay, it was verified that the expression of TRPV4 protein in b.End3 cells. When cells were stimulated by IL-1 β , the expression of TRPV4 protein increased significantly ($p < 0.01$, vs. control). RR ($p < 0.01$, vs. IL-1 β) and cinnamaldehyde 0.38 mM ($p < 0.05$, vs. IL-1 β) decreased the high expression of TRPV4 induced by IL-1 β , this result was consistent with that of TRPV4 mRNA expression level (Figs. 3, 4).

DISCUSSION

The elevation of body temperature that occurs during fever is centrally regulated by neurons in the preoptic region of the hypothalamus. Several studies demonstrated the importance of cytokine IL-1 β , which is released mostly by immune cells during infection or injury to signal the brain and initiate neuroendocrine activation, behavioral changes, and induction of fever.¹⁰ It is also well established that the final mediator of fever is PGE₂ which acts on EP₃ type prostanoid receptors in the hypothalamus.¹¹ There are several theories as to how peripheral cytokines can activate the central mechanism of fever; such theories include the existence of carrier-mediated transport of cytokines into the brain,¹² the entrance of cytokines to the brain through areas devoid of blood-brain barrier (BBB),¹³ and the role of neuronal afferents to signal the thermoregulatory center.¹⁴ Although it is possible that all these mechanisms are involved in fever induction to some extent, it is more likely that cytokine-induced cerebral endothelial prostaglandin production is the dominant player in the signal transduction from the periphery to the thermoregulatory center.¹⁵ Studies in the last decades have indicated that brain endothelial cells are the major sources of PGE₂ under various inflammatory states. Together with our previous findings, the results presented in this study strongly supports the opinion that PGE₂ gains access to the brain *via* interaction with endothelial cell.

The TRPV4 channel, a member of the TRP family of channels, was identified originally as a channel activated by hypotonic cell swelling,¹⁶ but in the later reports showed that it can be activated also by the phorbol derivatives, temperatures $>27^{\circ}\text{C}$, acidic pH and citrate and several endogenous substances. TRPV4 has been cloned from the rat, mouse, human, and chicken¹⁷ and was widely expressed in mammalian tissues, including lung, heart, kidney, sensory neurons, sympathetic nerves, brain, skin, gut, salivary gland, sweat glands, inner ear, endothelium, and fat tissue.¹⁸ The ability of this unique channel to respond to a broad variety of signals has evoked hypotheses about its possible involvement in processes ranging from sensory detection and thermoregulation to regulation of vascular tone and signaling in the brain. In addition to peripheral temperature sensing, TRPV4 might also play a role in regulating thermogenesis. TRPV4 is expressed in the preoptic and anterior hypothalamus, the control center of thermogenesis that contains specialized warm- and cool-sensitive neurons, which are also activated by hyposmolality.^{19,20} In addition, recent findings from Vriens *et al.*²¹ indicate that the endocannabinoid anandamide and its hydrolysis product arachidonic acid (AA) are potent endogenous agonists for TRPV4 channels acting through the cy-

tochrome P450 epoxygenase-dependent formation of 5',6'-epoxyeicosatrienoic acid (5',6'-EET). Their data suggested that cell swelling is coupled to TRPV4 activation by means of a sequence of events consisting of (i) swelling-induced activation of phospholipase A₂ (PLA₂), (ii) PLA₂-dependent release of AA from membrane phospholipids, (iii) cytochrome P450 epoxygenase dependent metabolism of AA to 5',6'-EET, and (iv) activation of TRPV4 by 5',6'-EET. Furthermore, Marrelli *et al.*²² also demonstrated that endothelial Ca²⁺ was significantly reduced following PLA₂ inhibition. Although many studies have implied the involvement of TRPV4 channel in temperature sensation, the role of this channel in the thermoregulation has not been evaluated.

Cinnamaldehyde is a main compound isolated from Guizhi-Tang, which is a famous traditional Chinese medical formula used to treat influenza, common cold and other pyretic conditions. Cinnamaldehyde has been reported to inhibit the growth of *Clostridium botulinum*,²³ *Staphylococcus aureus*,²⁴ and *Salmonella enterica* serovar typhimurium.²⁵ Recently, cinnamaldehyde has been found to inhibit cyclooxygenase (COX)-2 activity with IC₅₀ value of 245 μM *in vitro*²⁶ and shown to be effective in inducing apoptotic cell in a number of human cancer cells through activation of the proapoptotic Bcl-2 family proteins and mitogen-activated protein kinases (MAPKs) pathway.²⁷ A previous study from our laboratory demonstrated that cinnamaldehyde could reduce activities of PLA₂ and COX-2.

In the present study, b.End3 cells were incubated in DMEM medium containing interleukin IL-1 β in the presence or absence of cinnamaldehyde. After incubation, the medium was collected and the levels of PGE₂ were measured by ELISA. The cells were harvested, the expression of TRPV4 mRNA and protein were analyzed by real-time PCR, western blot and immunofluorescence assay, respectively. The results suggested that cinnamaldehyde significantly decreased the release of PGE₂, while RR had no significant difference compared with model group. IL-1 β increase the expression of TRPV4 and RR decreased both the basal and the IL-1 β -induced expression of TRPV4. cinnamaldehyde decreased the expression of mRNA and protein of TRPV4, but the effect of cinnamaldehyde was weaker than RR's. The results of the present study suggested that cinnamaldehyde decrease TRPV4 expression might by means of directly inhibited TRPV4 and indirectly inhibited pathways of PLA₂, AA and 5',6'-EET.

Generally speaking, it was demonstrated that TRPV4 is functionally expressed in b.End3 cells. Cinnamaldehyde reduced IL-1 β -induced PGE₂ production and the expression of TRPV4, which suggested that Guizhi-Tang's antipyretic property might be partly ascribable to cinnamaldehyde.

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