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ORIGINAL ARTICLE

Cinnamaldehyde up-regulates the mRNA expression level of TRPV1 receptor potential ion channel protein and its function in primary rat DRG neurons *in vitro*

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Cinnamaldehyde (**1**) is a pharmacologically active ingredient isolated from cassia twig (*Ramulus Cinnamomi*), which is commonly used in herbal remedies to treat fever-related diseases. Both TRPV1 and TRPM8 ion channel proteins are abundantly expressed in sensory neurons, and are assumed to act as a thermosensor, with the former mediating the feeling of warmth and the latter the feeling of cold in the body. Both of them have recently been reported to be involved in thermoregulation. The purpose of this paper is to further uncover the antipyretic mechanisms of **1** by investigating its effects on the mRNA expression levels and functions of both TRPV1 and TRPM8. The results showed that **1** could up-regulate the mRNA expression levels of TRPV1 at both 37 and 39°C, and its calcium-mediating function was significantly increased at 39°C, all of which could not be blocked by pretreatment of the neuronal cells with ruthenium red, a general transient receptor potential (TRP) blocker, indicating that the action of **1** was achieved through a non-TRPA1 channel pathway. In conclusion, the findings in our *in vitro* studies might account for part of the peripheral molecular mechanisms for the antipyretic action of **1**.

Keywords: cinnamaldehyde; TRPV1; TRPM8; DRG; capsaicin; menthol

1. Introduction

Guizhi-Tang (decoction of cassia twig) is one of the most effective herbal formulae in the classical works of *Shang-han-lun* (*Treatise on Exogenous Febrile Disease*), and has been widely used to treat influenza, common cold, and other pyretic conditions. Cinnamaldehyde (**1**) is one of the principal ingredients isolated from *Guizhi-Tang*, which was previously found to relieve fever and pain [1], as well as to inhibit the growth of various organisms [2].

Recently, it has been found to inhibit cyclooxygenase (COX)-2 activity with an IC_{50} value of 245 μM *in vitro* and to induce endothelium-dependent and independent vasorelaxant action on isolated rat aorta

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[3,4]. Moreover, **1** has been reported to have anti-oxidant effects, evoke significant spontaneous pain, induce heat and mechanical hyperalgesia, and cause cold hypoalgesia by application to the skin in study participants [5]. In addition, a previous study in our laboratory showed that **1** reduced the TRPV4 expression in b.End3 cells, which indicated this might be the antipyretic mechanism of **1** through a central temperature regulation center [6]. However, the putative peripheral antipyretic and hyperalgesic mechanisms induced by **1** as well as the correlations between them remain obscure.

Accumulating evidence indicates that ion channels of the transient receptor potential (TRP) family are evolutionarily conserved participants in the peripheral mechanisms by which we sense hot and cold temperatures, and that these channels may also contribute, directly or indirectly, to thermoregulation [7]. Among them, several lines of evidence have shown that the capsaicin-sensitive vanilloid receptor (TRPV1), which can be activated by temperature over 42°C or capsaicin, in the detection of noxious stimuli [8], as well as TRPM8, a non-selective, calcium-permeable cation channel of the TRP family, which can be activated by cooling and menthol, might be involved in the thermoregulation through peripheral-sensing mechanism [9].

Thus, in the present study, we investigated the effects of **1** on the mRNA expression levels of both TRPV1 and TRPM8 as well as the functions of these channel proteins as mediators of calcium entrance into the cytoplasm of primary dorsal root ganglion (DRG) neurons *in vitro* in order to further explore the antipyretic mechanisms of **1**. In view of the fact that **1** is a known TRPA1 channel activator, its influence on the expression and functions of the TRPV1 and TRPM8 might be related to the mediation of the TRPA1 channel. So, in this experiment, ruthenium red (RR), a commonly used non-selective TRPA1 blocker, was undertaken to verify this hypothesis.

2. Results and discussion

2.1 Cytotoxic effect of cinnamaldehyde on the primary DRG neurons

To test whether cinnamaldehyde (**1**) treatment has cytotoxic effects, viability assays of the primary DRG neurons were performed to determine the optimal concentrations for **1**. The primary DRG neurons were incubated for 24 h with various concentrations of **1**, followed by viability assay. The results demonstrated that viabilities of the cells for 40, 80, 160, 300, and 400 μM of **1** were 101.86 ± 10.90, 93.99 ± 9.16, 98.36 ± 8.90, 91.80 ± 9.47, and 90.52 ± 9.33 (% control), respectively. No significant differences were found for any group compared with the control group ($p > 0.05$ vs. control), indicating that **1** below 400 μM had no cytotoxic effects on the cells. As a consequence of these studies, all subsequent incubations with **1** were performed below 400 μM.

2.2 Effect of the temperature on the viability of the primary DRG neurons

Viability assays of the primary DRG neurons were performed to make sure that the temperature range and duration we selected for the subsequent experiments did not induce neurotoxicity. The primary DRG neurons were incubated for 12 h at 37 or 39°C, followed by viability assays. The results demonstrated that the viability of the cells at 39°C was 98.50 ± 10.41 (% of control) and no significant difference was shown compared with the control group at 37°C ($p > 0.05$ vs. control), indicating that no cytotoxic action was exerted by the higher temperature after the 12 h incubation. As a result of these studies, all subsequent incubations with cinnamaldehyde were performed under those temperature conditions.

2.3 Effects of cinnamaldehyde on the levels of TRPV1 and TRPM8 mRNA expression under the temperature condition of 37°C

Detection of the expression of TRPV1 and TRPM8 mRNA was performed using real-time PCR. Melting curve analysis confirmed that there was no primer dimer in PCR products. For each of the primer sets, the specificity and efficacy of cDNA's amplification for both the target genes and the internal control were visualized and evaluated. The results indicated that no non-specific amplification was visualized and the amplification efficacies of the cDNAs were relatively close and suitable for analysis by the method of $2^{-\Delta\Delta CT}$ (Figures 1 and 2).

In the present study, it is demonstrated that the TRPV1 mRNA expression is significantly up-regulated after the action of 60 or 30 μM cinnamaldehyde (**1**) for both RR-free (170.59 ± 35.29, 126.43 ± 31.37 (% of control); $p < 0.01$, $p < 0.05$ vs. control, respectively) and plus RR (161.54 ± 31.73, 126.92 ± 32.69 (% of control); $p < 0.01$, $p < 0.05$ vs. control, respectively) conditions for 12 h at 37°C (Figure 3), while the effect of **1** on the TRPM8 mRNA expression is not significantly different (88.12 ± 18.75, 90.10 ± 19.80, 95.05 ± 22.92 (% of control); $p > 0.05$ for any groups vs. control). No significant difference was shown between the RR-free and plus RR groups of a similar concentration of **1** ($p > 0.05$ for any groups) and the significant effects of **1** on the TRPV1 mRNA expression were not blocked by pre-incubation of the neurons with RR, indicating that **1** might exert its action on TRPV1 by additional cellular mechanisms other than the TRPA1 channel (Figure 3).

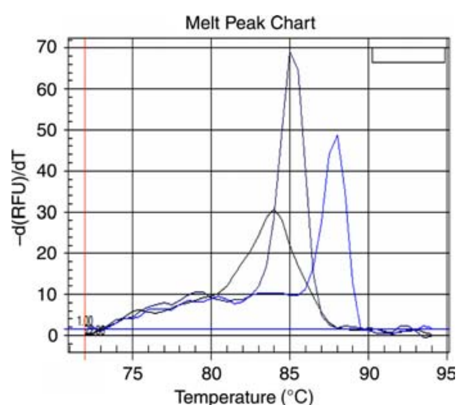

Figure 1. Melting curves of the amplification products from TRPV1, TRPM8, and GAPDH. RFU, relative fluorescence unit.

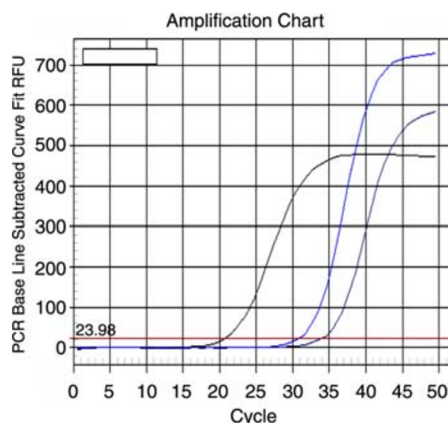

Figure 2. Amplification curves for the cDNAs of TRPV1, TRPM8, and GAPDH.

2.4 Effects of cinnamaldehyde on the levels of TRPV1 and TRPM8 mRNA expression under the temperature condition of 39°C

From the experimental results, it could be identified that the TRPV1 mRNA expression was markedly up-regulated after cinnamaldehyde 60, 30 or 15 μM stimulation for both RR-free (251.49 ± 40.59, 180.20 ± 35.64, 125.74 ± 28.71 (% of control); $p < 0.01$, $p < 0.01$, $p < 0.05$ vs. control, respectively) and plus RR (233.33 ± 36.27, 172.55 ± 30.39, 126.47 ± 27.45 (% of control); $p < 0.01$, $p < 0.01$, $p < 0.05$ vs. control, respectively) conditions for 12 h at 39°C in a dose-dependent

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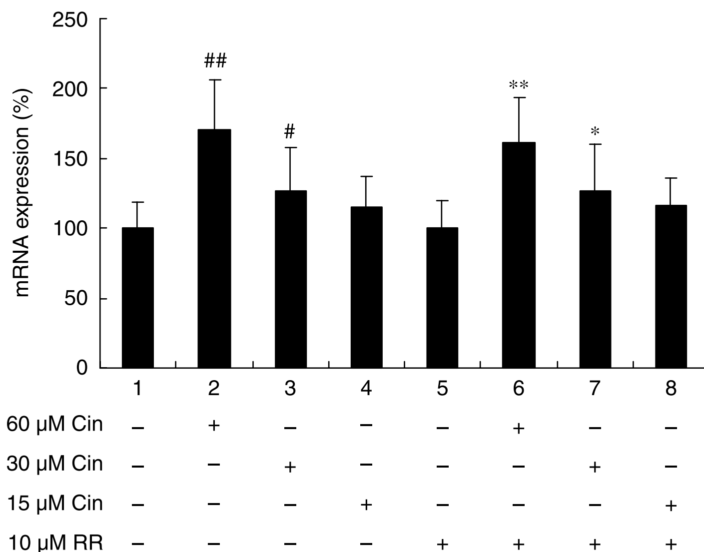

Figure 3. Effects of cinnamaldehyde on the levels of the TRPV1 mRNA expression under the temperature condition of 37°C. TRPV1 mRNA expression levels of TRPV1 in the presence of 60 μM cinnamaldehyde, 30 μM cinnamaldehyde, 15 μM cinnamaldehyde, or 10 μM RR either alone or in different combinations were measured using the method of relative quantitative real-time RT-PCR ($n = 10$). The results represent three independent experiments. $^{##}p < 0.01$, $^{#}p < 0.05$ vs. normal control; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. RR alone. Cin, cinnamaldehyde; RR, ruthenium red.

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manner (Figure 4), while the effect of cinnamaldehyde on the TRPM8 mRNA expression was not significantly different (96.32 ± 19.83, 98.10 ± 19.05, 94.88 ± 20.26 (% of control); $p > 0.05$ for any groups vs. control). As demonstrated under the temperature condition of 37°C, the significant effect of cinnamaldehyde on the TRPV1 mRNA expression was not blocked by pretreatment of the neurons with RR (Figure 4).

2.5 *Effects on the functions of TRPV1 and TRPM8 ion channel proteins in DRG neurons pretreated with cinnamaldehyde under the temperature condition of 37°C*

The experimental results revealed that most of the small-sized DRG neurons (around 70% of the total neurons) exhibited a significant elevation of intracellular Ca^{2+} induced by 5 μM capsaicin in the presence of extracellular 1.25 mM calcium. This is consistent with previous reports [10]. It was demonstrated that the concentration of intracellular calcium increased upon addition of capsaicin, and reached the peak value at about 120 s, and then decreased toward the resting level gradually (Figure 5). The elevated amplitude of intracellular calcium by capsaicin could not be changed markedly by preincubation of the DRG neurons with any of the three doses of cinnamaldehyde (105.32 ± 20.66, 101.60 ± 18.71, 104.08 ± 19.43 (% of control); $p > 0.05$ for any groups vs. control), indicating that cinnamaldehyde might not change the calcium-mediating function of TRPV1 receptor channel protein under this temperature condition.

For the imaging experiment of TRPM8, the basal temperature of the bath solution for which the cells were maintained was 26°C. Higher basal temperature was not necessary for this study because TRPM8 receptors are not significantly activated at temperature >25°C when the cells are near resting membrane

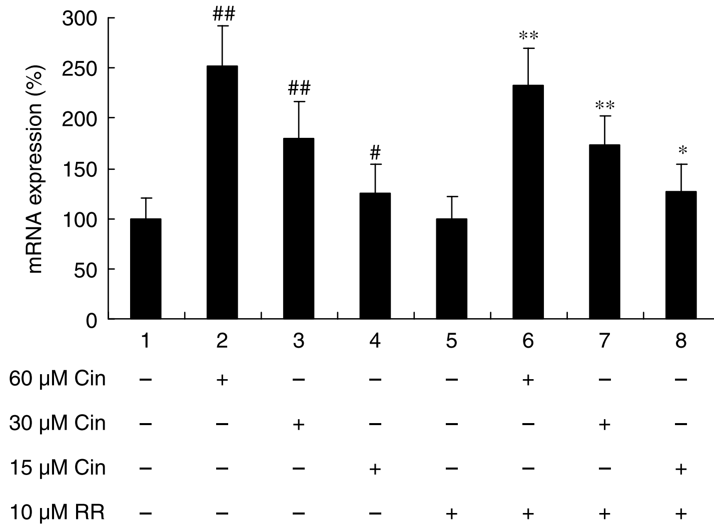

Figure 4. Effects of cinnamaldehyde on the levels of the TRPV1 mRNA expression under the temperature condition of 39°C. TRPV1 mRNA expression levels of TRPV1 in the presence of 60 μM cinnamaldehyde, 30 μM cinnamaldehyde, 15 μM cinnamaldehyde, or 10 μM RR either alone or in different combinations were measured using the method of relative quantitative real-time RT-PCR ($n = 10$). The results represent three independent experiments. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. normal control; $**p < 0.01$, $*p < 0.05$ vs. RR alone. Cin, cinnamaldehyde; RR, ruthenium red.

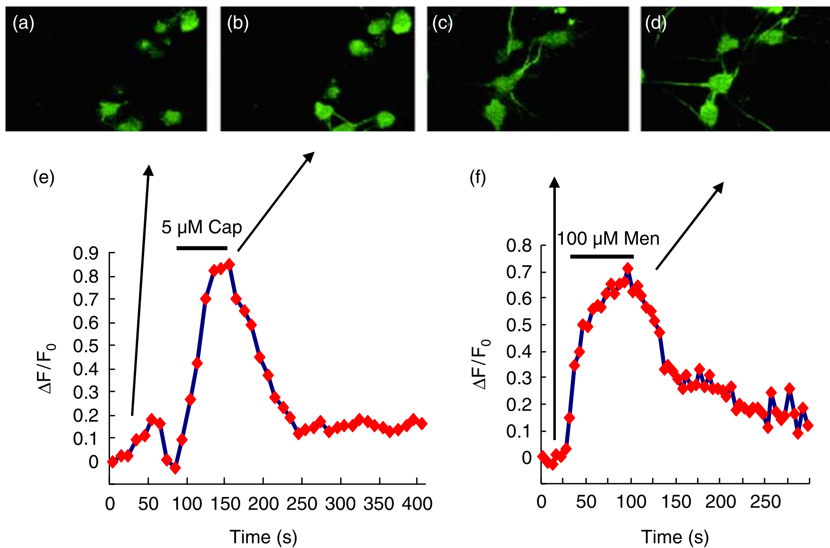

Figure 5. Intracellular changes of fluorescent intensity evoked by TRPV1 and TRPM8 agonists in DRG neurons. (a),(c) Loaded DRG neurons with Ca^{2+} indicator Fluo-4 AM dye. (b) Capsaicin (5 μM)-induced increase of intracellular fluorescent intensity of DRG neurons. (d) Menthol (100 μM)-induced increase of intracellular fluorescent intensity of DRG neurons. (e) Capsaicin (5 μM)-induced intracellular changes of fluorescent intensity in DRG neurons. (f) Menthol (100 μM)-induced intracellular changes of fluorescent intensity in DRG neurons. Cap, capsaicin; Men, menthol.

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potentials [11]. Of the DRG neurons tested, nearly 10% of them demonstrated increased Fluo-4 fluorescence intensity of intracellular Ca^{2+} induced by menthol (Figure 5). It was identified that the elevated amplitude of intracellular calcium by menthol could not be changed markedly by incubation of the DRG neurons with cinnamaldehyde of various doses (98.60 ± 17.84, 97.25 ± 19.13, 102.24 ± 21.03 (% of control); $p > 0.05$ for any groups vs. control), indicating that cinnamaldehyde might not change the calcium-mediating function of TRPM8 receptor channel protein at 37°C.

2.6 *Effects on the functions of TRPV1 and TRPM8 ion channel proteins in DRG neurons pretreated with cinnamaldehyde under the temperature condition of 39°C*

The elevated amplitude of intracellular calcium evoked by capsaicin could be promoted markedly by the pretreatment of the DRG neurons with 60 or 30 μM cinnamaldehyde (**1**) for both RR-free (126.79 ± 21.43, 119.64 ± 19.64 (% of control); $p < 0.01$, $p < 0.05$ vs. control, respectively) and plus RR (129.02 ± 23.67, 118.75 ± 21.43 (% of control); $p < 0.01$, $p < 0.05$ vs. control, respectively) conditions for 12 h at 39°C. No significant difference between the RR-free and plus RR groups of the similar **1** concentration was shown ($p > 0.05$ for any groups), and the significant increases of the calcium-mediating function of the TRPV1 channel after the action of 60 or 30 μM of **1** could not be masked by the preincubation of the DRG neurons with RR ($p < 0.01$ or $p < 0.05$ vs. RR alone, respectively), indicating that the up-regulated function of TRPV1 induced by **1** was probably not achieved through the TRPA1 pathway (Figure 6). For the imaging experiment of TRPM8, it was shown that the elevated amplitude of intracellular calcium induced by menthol could not be changed markedly by the pretreatment of the DRG neurons with **1** of various doses (96.17 ± 18.20, 93.44 ± 19.84, 98.05 ± 21.46 (% of control); $p > 0.05$ for any groups vs. control), suggesting that **1** might not change the

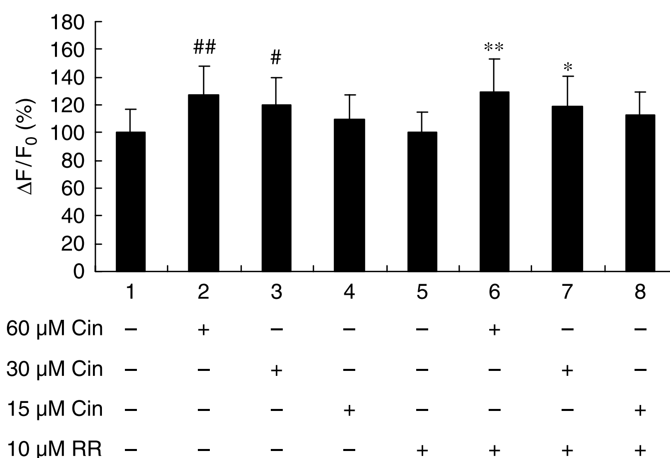

Figure 6. Effects of cinnamaldehyde on the functions of TRPV1 ion channel protein under the temperature condition of 39°C. The functions of TRPV1 in the presence of 60 μM cinnamaldehyde, 30 μM cinnamaldehyde, 15 μM cinnamaldehyde, or 10 μM RR either alone or in different combinations were measured using the method of confocal microscopy ($n = 10$). The results represent three independent experiments. $^{##}p < 0.01$, $^{#}p < 0.05$ vs. normal control; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. RR alone. Cin, cinnamaldehyde; RR, ruthenium red.

calcium-mediating function of TRPV1 receptor channel protein.

2.7 Discussion

Over the past decade, considerable evidence supporting a role for TRP channels in temperature sensation has emerged from a number of mammalian and non-mammalian systems. These data can be most readily explained by the existence of multiple thermally sensitive TRP channels, each contributing to temperature sensation over a different range and in different anatomical locations. TRPV1-4s are activated when the temperature is higher than thresholds while TRPM8 and TRPA1 are activated when the temperature is lower than thresholds [12].

Among these recently cloned and characterized 'thermoTRP' channels, the following facts support the involvement of tonic TRPV1 activation in body thermoregulation: (1) agonists of TRPV1 cause hypothermia in rodents and humans [13]; (2) there is an absence of agonist-induced hypothermia in TRPV1 knock-out mice [14]; (3) antagonists of TRPV1 representing multiple different chemotypes cause hyperthermia in rats [10]; (4) there is a correlation of antagonism of TRPV1 activation by antagonists with hyperthermia in dogs and monkeys [15]; (5) TRPV1 knock-out mice show some impairment in thermoregulation [16]; and (6) there is an absence of antagonist-induced hyperthermia in knock-out mice [17].

TRPM8 has recently been identified as a distant relative of TRPV1 (the receptors show only 20% identity in protein sequence) by a bioinformatics approach [17]. Recently, several lines of evidence have indicated a potential functional role of TRPM8 in thermoregulation as a menthol- and cold-sensitive ion channel in sensory neurons for the detection of cold temperature of $<25°C$. In contrast to TRPV1, agonists of TRPM8 evoke hyperthermia in rodents [9,18]. Additional support for the participation of TRPM8 in physiological cold transduction comes from the observation that its expression is confined largely to a subpopulation of small-diameter peripheral sensory neurons that under normal conditions are distinct from those that express TRPV1 [9,19,20].

For a specific gene, from transcription start to translation, there are several critical processes and steps that are involved which are under refined physiological regulation and strict control. In the present study, we examined the effects of cinnamaldehyde (**1**) on TRPV1 and TRPM8 receptor potential ion channels both at the transcription levels of gene expression and on the functional activities of the post-translational proteins. The levels of the TRPV1 and TRPM8 mRNA expression were detected by real-time quantity PCR with SYBR green dye, and significantly different mRNA expression levels in various groups were observed using the relative quantitative method of $2^{-\Delta\Delta CT}$, which is especially suitable for the detection of target genes with relatively short PCR products of equal amplification efficiency as those in our experiments. For the pair of primer sets, non-specific amplification was not visualized after electrophoresis and ethidium bromide staining of agarose gels; however, amplification efficiencies of TRPV1 and TRPM8 mRNA in the various groups were also assayed. The results showed that real-time PCR conditions used in the study were suitable for the detection of the TRPV1 and TRPM8 mRNA expression in the DRG neuronal cells as well as for the analyses of relative expression levels of various groups.

By using camptothecine (CPT) as an agent to eliminate the non-neuronal cells, we acquired primary DRG cultures with high purity and viability. On this basis, we first studied the effects of **1** on the expression of TRPV1 and TRPM8 mRNA *in vitro*. It was demonstrated that the level of the TRPV1 mRNA expression

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in the neurons of the primary DRG cultures was significantly increased after being incubated with **1** for 12 h under the temperature condition of 37°C. Interestingly, when the temperature at which DRG neurons incubated with **1** is increased to 39°C, a simulated *in vitro* pathological condition for fever, the up-regulated TRPV1 mRNA expression levels in the DRG neurons induced by **1** seemed more significant with all of the three doses markedly higher than the control in a dose-dependent manner. Since TRPV1 and TRPM8 are non-selective cation channels with preference for Ca^{2+} ions, measurement of $[Ca^{2+}]$i evoked by their corresponding agonists can be used to assess the channel's activity. With capsaicin and menthol as agonists of TRPV1 and TRPM8 channels, respectively, as well as Fluo-4-AM as a calcium indicator, the increased amplitude of intracellular $[Ca^{2+}]$i induced by capsaicin or menthol was measured by confocal microscopy.

Of note, there also exists a bimodal modulation of TRPA1 gating by menthol. At low micromolar concentrations, menthol leads to an overall increase of TRPA1 current [21]. That is the reason why in this experiment we set the application concentration of menthol to 100 μM, at which menthol induces a rapid channel block of TRPA1 rather than an agonizing action. It follows that the increased calcium provoked by menthol in the present study could be considered to be achieved through the opening of just TRPM8 channels. The calcium measurement results showed that the intracellular fluorescence intensity induced by capsaicin in the DRG neurons was not significantly different between the three **1** groups of different doses and the control group under the temperature condition of 37°C, which is not consistent with the results for the mRNA expression level under the same temperature condition. This apparently paradoxical phenomenon for the inconsistence of changes between the mRNA expression level and calcium-mediating function of the TRPV1 channel might be explained by our speculation that the up-regulated TRPV1 mRNA expression level could be reversed through one or several post-transcriptional pathways though the specific mechanisms still need to be further researched. In contrast, after being pretreated with **1** for 12 h under the temperature condition of 39°C, the intracellular fluorescence intensity induced by capsaicin in the DRG neurons was significantly higher than that in the control group, which is fundamentally in agreement with the results acquired in the PCR experiments under the same temperature condition. These results seemed to support the notion that a higher body temperature than the normal one such as fever might be the preferred settings for **1** to exert its antipyretic action.

On the premise that the TRPA1 channel could be activated by **1**, concerns have been raised with respect to the possibility that the up-regulated expression and function of TRPV1 by **1** were accomplished through the pathway of TRPA1. Thus, RR, a known non-selective antagonist of TRPA1, was employed in this experiment to detect whether TRPA1 was involved in the **1**-induced gene expression and functional changes for TRPV1. From the experimental results, it could be identified that the significant effect of **1** on the TRPV1 mRNA expression and function was not blocked by the pretreatment of the neurons with RR, indicating that the effect produced by **1** is mediated through the non-TRPA1 channel pathway.

As reported in the literature, the TRPV1 expressed in thermal sensory neurons innervating the body surface takes part in body thermoregulation and pain perception through transducing the changes in thermal energy into an electrical signal by opening its channels to temperature of >42°C [22,23]. However, this threshold temperature would be

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lowered by some endogenous or exogenous inflammatory mediators to some extent in acute or chronic inflammatory states [23]. Thus, combined with the results found in our experiments, we have the reason to postulate that the up-regulation of the TRPV1 expression and function might compose an important molecular basis for promoting the sensitivity of the TRPV1 channel to higher temperatures as well as for hypersensitivity to pain. As a result, some temperature-lowering mechanisms might be initiated through integration of temperature information collected from TRPV1 channels by the central nervous system, which, in turn, contributes to the relief of fever under pathological conditions.

In conclusion, our data indicate that the expression levels and functions of TRPV1 in the DRG neurons could be up-regulated by **1** through the non-TRPA1 channel pathway, which may be one of the main molecular antipyretic mechanisms of **1** as well as an important reason for hyperalgesia evoked by **1**. These results also suggest that *Guizhi-Tang*'s antipyretic action, especially the peripheral, might be partially attributed to **1**. It is important to note, however, that the analyses in the present study were all based on *in vitro* results. Thus, other *in vivo* studies using more precise techniques and strategies are still necessary to further clarify the pathophysiological role of TRPV1 in thermoregulation as well as to elucidate the involvement of **1**-induced up-regulation of TRPV1 in thermoregulation in acute or chronic inflammatory states. In addition, **1**, a putatively selective agonist of TRPA1, whether a similar influence of it on TRPV1 could occur to TRPA1 or whether some objective correlations exist between these actions still remains to be further researched in the future study.

3. Experimental

3.1 Materials

Cinnamaldehyde (>99%) was extracted from *Guizhi-Tang*. CPT was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Laminin, poly-L-lysine, nerve growth factor (NGF), RR, menthol (≥99%), and capsaicin (≥99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). An RNA extraction kit, RNAsafe, Oligo (dT) 15, dNTP, and SYBR green were obtained from Tiangen Biotech Company (Beijing, China). Superscript III® reverse transcriptase was purchased from Invitrogen (Carlsbad, CA, USA). Primers were designed and synthesized by Shanghai Yingjun Invitrogen, Inc. (Shanghai, China). Fluo-4-AM and Pluronic F-127 were purchased from Molecular Probes, Inc. (Eugene, OR, USA). All reagents for cell culture were of tissue culture grade, and for RNA extraction, reagents were of molecular biology grade. All other materials purchased from Sigma-Aldrich were of analytical grade unless otherwise indicated.

3.2 Primary cultures of DRG cells

Routine cultures of rat DRG were obtained from newborn Sprague–Dawley (SD) rats bred on the campus of Peking Union Medical College. All protocols were approved by the Animal Care and Use Committee of Peking University Health Science Center, and we followed the Guidelines of Animal Use and Protection adopted from the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All possible efforts were made to minimize unnecessary suffering of animals. Briefly, newborn SD rats were killed by decapitation and submerged in 95% ethanol for approximately 2 min. DRG were aseptically removed and

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meticulously cleaned of rootlets and connective tissue. The tissue was disaggregated by incubating in L15 nutrient mixture (pH corrected to 7.4) containing 0.1% collagenase for 15 min at 37°C with light trituration every 5 min, and then transferred to 0.25% trypsin for 30 min at 37°C with the same procedures. Following digestion, horse serum was added to 10% and the cell suspension centrifuged at low speed for 8 min. The cell pellet was resuspended in neurobasal medium consisting 1% fetal bovine serum, 1% B-27, 10 ng/ml NGF, 2500 mg/ml glucose, and 2 g/l $NaHCO_3$. Cells were seeded onto surfaces precoated with poly-L-lysine (50 g/ml 70,000–150,000 Da) and laminin (10 mg/ml). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air; the medium was replaced routinely every 2 days. CPT dissolved in DMSO (10 mM stock) was included on day 3 of the cultures to a final concentration of 20 μM for a total period of 48 h.

3.3 *Treatment of primary cultures of DRG neurons*

The primary DRG neurons were cultured into six-well cell culture plates or coverslips specifically for confocal microscopy. After the non-neuronal cells were eliminated using CPT, the medium was then replaced by a serum- and NGF-free culture medium and recultured for another 24 h prior to stimulation of experimental temperature conditions and/or cinnamaldehyde of various doses. For the assay of real-time PCR and confocal experiments, the cells were incubated with various concentrations of cinnamaldehyde for 12 h at 37 or 39°C in a humidified atmosphere of 5% CO_2/95% air.

3.4 *RNA isolation and reverse transcription*

Total RNA from different samples was extracted with the RNA extract kit according to the manufacturer's protocol. To avoid DNA contamination, RNA was treated with the RNase-Free DNase set (Qiangen, Hilden, Germany). The concentration of RNA was measured spectrophotometrically and samples were stored at −20°C until use. The first-strand cDNA was prepared from 1 μg of total RNA with random hexanucleotide primers using Superscript III® reverse transcriptase. Reverse transcription was performed at 42°C for 30 min followed by inactivation at 94°C for 5 min. The resulting cDNA was further used as a template for PCR amplification immediately or stored at −20°C until use.

3.5 *Quantitative real-time PCR*

For PCR amplifications, the following primer pairs were used: rat TRPV1, forward primer 5′-AGT AAC TGC CAG GAG CTG GA-3′ (position 450–469 in the sequence of rat TRPV1) and reverse primer 5′-GTG TCA TTC TGC CCA TTG TG-3′ (position 576–595 in the sequence of rat TRPV1); rat TRPM8, forward primer 5′-TGC AGG AGA ACA ACG ATC AG-3′ (position 3004–3023 in the sequence of rat TRPM8) and reverse primer 5′-GTA AGC GAA GAC GAC GAA GG-3′ (position 3088–3107 in the sequence of rat TRPM8); and rat GAPDH (internal control), forward primer 5′-CCT TCA TTG ACC TCA ACT ACA TG-3′ (position 180–202 in the sequence of rat GAPDH) and reverse primer 5′-CTT CTC CAT GGT GGT GAA GAC-3′ (position 371–391 in the sequence of rat GAPDH). Real-time PCR was performed according to the protocol of the QIANGEN SYBR Green PCR Kit in an IQ5-type real-time PCR meter (Bio-Rad, Hercules, CA, USA) with GAPDH as the endogenous control. Each amplification program consisted of: one cycle of 94°C for 5 min, followed by 50 cycles of 94°C at 30 s, 55°C for 30 s, and 72°C for 30 s. Melting curves were generated after the last extension step to

verify the specificity and identity of PCR products. Each sample was tested in triplicate. Relative gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

3.6 Ca^{2+} imaging experiments on DRG neurons

DRG neurons were dissociated from neonate SD rats and plated on coverslips precoated with poly-D-lysine and laminin as described above. Ca^{2+} imaging experiments were performed on neurons the second day in culture after the purification processing. The neurons were maintained in normal bath solution. The normal bath solution contains HEPES-buffered (pH 7.4) Hanks' balanced salt solution supplemented with 0.01% bovine serum albumin, 0.8 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM pyruvate, and 1 mM ascorbic acid. To load the Ca^{2+} indicator Fluo-4, the DRG neurons on coverslips were incubated with 5 μM Fluo-4-AM in 0.01% pluronic acid (Molecular Probes) for 30 min at 37°C. After dye loading, the cells were washed three times with normal bath solution and put in a closed chamber on a microscope stage (Olympus-CK). Fluo-4 fluorescence in the cells was detected with a Peltier-cooled charge-coupled device camera (Penta MAX-III System; Roper Scientific, Trenton, NJ, USA) under a 20× objective. Excitation and emission were achieved by a fluorescence filter set at 488 and 510 nm, respectively. Images were taken at one frame per 5 s and digitized using WinView software (Roper Scientific). The increased amplitude of intracellular Ca^{2+} induced by capsaicin or menthol was tested by bath application of 5 μM capsaicin and 100 μM menthol, respectively. Unless otherwise indicated, capsaicin or menthol was applied a new dish in each test and in each experiment, and cells were exposed to capsaicin or menthol only once. For Ca^{2+} imaging data analysis, relative fluorescence intensity $\Delta F/F_0$ was used, and neurons with $\Delta F/F_0$ values of ≥ 0.2 (i.e. equal or above 20% of increase) were assigned as responsive cells [24].

3.7 Statistical analysis

Data are presented as mean ± SD. Each type of experiment was performed on the number of cells indicated (n) in at least three animals. Data were compared using Student's t-tests or one-way ANOVAs with secondary comparisons made by using a Student–Newman–Keuls test. A p-value <0.05 was considered statistically significant, unless otherwise indicated.

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