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Research Article

The cold-hot Properties of Aconitum Carmichaelii and Coptis Chinensis on the Temperature Sensor-Transient Receptor potential Vanilloid 4 Channel in Rat Tubular Epithelial Cells

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Abstract

Herbal medicine with hot or cold property is effective to treat the renal diseases with apparent cold or hot symptom. However, the mechanism is not clear. This study was designed to evaluate the effects of Chinese herbs with cold or hot properties on the temperature sensor-transient receptor potential vanilloid 4 (TRPV4) channel. Rat tubular epithelial cells with stable TRPV4 expression were established. One herb with hot properties-Aconitum carmichaelii (Chinese: Fuzi), and one with cold properties-Coptis chinensis (Chinese: Huanglian) were selected. Cell viability, cellular ATP and calcium levels were determined after treatment under different conditions. Compared with the control group, Fuzi significantly increased, while Huanglian decreased, cellular concentrations of ATP. As evidenced by increased intracellular calcium concentration, Fuzi and Huanglian respectively increased TRPV4 activity. These effects are consistent with the function of these herbs as TRPV4 agonists. Fuzi and Huanglian may regulate the activity of TRPV4 and the levels of energy metabolites. TRPV4 is a suitable temperature sensor for the study of cold or hot property of Chinese herbs.

Keywords: cold-hot property; fuzi; huanglian; trpv4; energy metabolite

Introduction

Hot and Cold medications are based on four properties of Chinese medicinal herbs, including cold, hot, warm and cool. These are also called "four xing" in traditional Chinese Medicine (TCM). The "four xing" theory is the central focus of property theory in recent TCM research. According to the rule of TCM- "hot medication in cold syndrome and cold medication in hot syndrome," the addition or subtraction of cold or hot medicine was widely used to treat cold or hot symptoms. For example, the Qufeng Tongluo prescription is typical for chronic kidney disease treatment [1]. It is effective in eliminating the syndromes in patients without clear cold or hot syndromes. However, for patients with clear cold or hot syndromes, the prescription requires the use of adjuvant drugs with a clear hot or cold property to exert their optimal effects [2].

The investigations of Chinese herbals are mostly focusing on the biological actions of active components or the extraction [3]. However, based on TCM theory, Chinese herbals should function dependent on the entire constituent. A series of studies have reported the correlation of chemical components with the cold-hot property of TCM [4-6]. Using finger-print chromatogram tests on 20 Chinese herbs with cold-hot properties, the component-water soluble carbohydrate was supposed to be one of the substances that determined the hot-cold property [6]. Another study proposed that hot properties are positively correlated with manganese levels in herbs, whereas cold properties are related to iron levels [4]. Chemical elements were thought to possess hot-cold properties according to electron donation or acceptance [5]. Pharmacological studies suggested that herbs with cold properties can inhibit synthesis of catecholamines, which would decrease the excitation of the central nervous system and inhibit adrenocortical and metabolic functions [7]. In contrast, herbs with hot properties increased synthesis of catecholamines and functioned oppositely [7]. Chinese herbs may function through two distinct pathways. First, TCM exerts functions by their active compounds, which is now the main focus in TCM research. Second, TCM with the physical characteristics such as "four xing" and "five tastes" may also have the potential to affect tissue microenvironment or cell growth through modulating temperature and pH.

Transient receptor potential channels (TRPCs) are one family of cationic ion channels located on cell membranes [8]. Transient receptor potential vanilloid 4 (TRPV4) is extensively expressed in a variety of tissues and organs, including lung, kidney, muscles and blood vessels [9]. HEK293 cells with TRPV4 overexpression can sense the warm or hot temperature when the temperature is higher than 27°C [10]. The high temperature directly activates TRPV4 as indicated by the inward flow of Ca^{2+} current [10]. In addition to the functional alteration, expression of TRPV4 is also regulated by temperature [11]. Therefore, TRPV4 should be a suitable temperature sensor for the study of cold or hot properties of TCM.

Aconitum carmichaelii (Chinese: Fuzi) and *Coptis chinensis* (Chinese: Huanglian) are characterized by their hot and cold properties, respectively. In the present study, we chose these two representative Chinese herbs with opposing cold and hot properties to examine their effects on energy metabolism and TRPV4 function in renal tubular epithelial cells. This study will provide experimental evidence for the property theory of TCM.

Materials and Methods

Cell culture and establishment of a stable cell line expressing TRPV4

Renal tubular epithelial cells (RTECs; designated as NRK-52E cells) were obtained from BioHermes Bio & Medical Technology (Beijing, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high-glucose containing 10% fetal bovine serum in an incubator with 5% CO_2 at 37°C. Cells at 90% confluence were digested with trypsin and passaged at a 1:2 ratio.

Adult male Sprague-Dawley rats (3-4 months old) were obtained from the Animal Center of Xi'an Jiaotong University. Total RNA was isolated from the renal distal convoluted tubule (Invitrogen; Carlsbad, CA, USA). The cDNA of trpv4 was obtained through reverse transcription of total mRNA (Takara; Los Angeles, CA, USA). pLenti virus encoding trpv4 was constructed and transfected into human embryonic kidney 293T cells (HEK 293T) (ATCC; Manassas, VA, USA). RTECs were cultured in 6-well plates and transfected with the virus when they reached 50% confluence. RTECs stably expressing TRPV4 ($RTEC^{TRPV4}$) were obtained using antibiotic selection.

Western blot analysis

Stable expression of TRPV4 in RTECs was confirmed using Western blot analysis. Cells at the logarithmic growth phase were washed twice with PBS and 100 μL of cell lysis buffer was added to each well and incubated on ice for 5 min. Protein was isolated after centrifugation. Protein concentrations were measured using the BCA protein assay kit (Thermo; West Palm Beach, FL, USA). Equivalent amounts of proteins were processed for SDS-PAGE and Western blotting. The primary antibodies used were CK18 (1:400), TRPV4 (1:500) and β-actin (1:2000), and were purchased from Proteintech Group, Inc. (Chicago, IL, USA). The gels were scanned by an infrared laser imaging system (Odyssey; Guelph, Ontario, Canada).

Preparation of drug solution and treatment

Fuzi and Huanglian were obtained from Tongrentang Pharmacy (Beijing, China). Fuzi and Huanglian (50 g of each) were dissolved in water with the volume of six-fold of the drug respectively for 15-30 min and then boiled for 1 h. Filtrate was collected and sterilized at 121°C for 30 min. The stock solution was kept at 4°C until use. The following study was divided into eight groups: 1) control group, 2) Fuzi group (25 mg/mL), 3) Huanglian group (25 mg/mL), 4) TRPV4 antagonist group (ruthenium red, RR; 10 μM), 5) TRPV4 agonist group (4α-phorbol 12,13-didecanoate, 4α-PDD; 10 μM), and temperature-controlled groups (group 6: 22°C for 3 h; group 7: 28°C for 2 h; group 8: 38°C for 1 h).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells in the logarithmic growth phase were seeded in a 96-well culture plate. After treatment with the indicated drugs for 24 h, 20 μL of MTT (5 mg/mL) was added to each well. The medium was removed 4 h later and 150 μL of dimethyl sulfoxide was added to each well to dissolve the precipitate. Absorbance was measured at 570 nm using an automated microplate reader (Tecan; San Jose, CA, USA). Five independent experiments were repeated for each group. Cell viability was calculated according to the following formula: cell viability (%) = average absorbance of treated group/average absorbance of control group × 100%.

ATP assay

Cells in the logarithmic growth phase were seeded in a 96-well culture plate. After treatment with the indicated drugs for 24 h, the cells were lysed on ice. ATP levels were determined with a ATP assay kit according to the manufacturer's instructions (Beyotime; Nantong, China). The ATP standard sample was diluted to 0.1, 1 and 10 μM in the lysis buffer provided by the kit. Luminescence was detected by a luminometer (Promega, Madison, WI, USA). The concentration of ATP in the samples was based on a standard curve.

Measurement of calcium concentration

Intracellular calcium concentration was detected as previously described [12]. Cells in the logarithmic growth phase were seeded in a 96-well culture plates. After treatment with the indicated drugs for 24 h, 100 μM Fura-2/AM (Biotium, US) was added to each well and incubated for another 30 min. Images were acquired under a fluorescence microscope and fluorescence intensity was measured with a microplate reader (Tecan; San Jose, CA, USA).

Statistical Analysis

Data are presented as mean ± SD and analyzed using SPSS 13.0 (SPSS Inc.; Chicago, IL, USA). Differences between means were analyzed by one-way ANOVA. $P < 0.05$ was set as the threshold of significance.

Results

Identification of RTECs and stable expression of TRPV4 in RTECs

Cytokeratin-18 (CK18) is selectively expressed in RTECs [13]. Fibroblasts were used as negative controls. As shown in Fig. 1A, CK-18 was expressed in RTECs, but not in fibroblasts. Expression of TRPV4 in $RTEC^{TRPV4}$ was higher than that in normal RTECs (Fig. 1B).

Cell viability and ATP analysis

Cell viability in the Fuzi, Huanglian, RR, 4α-PDD, 38°C and the temperature-regulated groups were not significantly different from that in the control group (Table 1). Regulation of the temperature at 22°C and 28°C, however, significantly decreased cell viability ($P < 0.01$).

Compared with the control group, ATP levels in the Fuzi, 4α-PDD and 38˚C temperature-regulated groups were significantly increased (Table 1). In contrast, ATP levels in the Huanglian, 22°C and 28°C groups were significantly ($P < 0.05$) decreased compared to those in the control group.

Analysis of intracellular calcium concentration

After application of Fura-2/AM, images were taken under a fluorescence microscope. The cells were tightly arranged in the Fuzi group, and especially in the 38°C group. Cells in the 22°C and 28°C groups were sparsely distributed (Fig. 2). Compared with the control group, intracellular calcium concentration was not affected in the 22°C and RR groups, but was significantly increased in the Fuzi, Huanglian, 4α-PDD, 28°C and 38°C groups ($P < 0.05$) (Figure. 3).

Discussion

The effects of TCM with cold or hot properties on TRP channel function were previously investigated, and focused on Transient receptor potential ankyrin 1 (TRPA1) and the transient receptor potential cation channel subfamily M member 8 (TRPM8) [14]. Drugs with cold properties, including bacailin and emodin, up-regulate TRPM8 and down-regulate TRPA1 expression [14]. In contrast, drugs with hot properties, such as evodiamine and cinnamaldehyde, have the opposite effects on TRPM8 and TRPA1 expression [14]. In addition, cinnamaldehyde has the capacity to inhibit the function of TRPM8 [15]. TRPV4 is widely distributed in distal convoluted

tubule epithelial cells [16]. Whether the cold or hot properties of TCM affects TRPV4 is still unknown. In the present study, we enhanced TRPV4 function by overexpressing TRPV4 in RTECs using lentiviral transfection. A stable RTEC cell line with high TRPV4 expression was established. Based on the sensitive temperature range for TRPV4 [17], we tested a temperature gradient to verify the temperature-dependent effects on TRPV4. A temperature of 22°C for 3 h slightly increased TRPV4 activation, as evidenced by increased intracellular calcium concentration. Temperatures of 28°C for 2 h and 38°C for 1 h both significantly activated TRPV4. These results consistently support the temperature sensitivity of TRPV4 [10]. Moreover, the temperature range of 22°C to 38°C was established in this study as the sensitive temperature range for TRPV4. Under normal conditions, the TRPV4 antagonist did not affect either intracellular calcium concentration or agonist-activated channel activity [12]. These positive controls are consistent with the known effects of this established system [12]. Therefore, the system is beneficial for screening TCM with cold or hot properties [10].

Fuzi and Huanglian were chosen in this study because they are two representative drugs with a hot and cold property, respectively. Because of the lack of similar studies of these two traditional Chinese herbs in a cell culture system, we determined the final concentration based on the previous study of herbs in an *E. coli* system [18]. We confirmed that a concentration of 25 mg/mL had the optimal effect in our preliminary study. The cell line overexpressing TRPV4 was used to investigate the effects of hot and cold properties of TCM on cell viability, ATP level and intracellular calcium concentration. Our data showed that neither Fuzi nor Huanglian affected cell viability. Fuzi increased, while Huanglian decreased cellular ATP levels, which confirms the distinct hot or cold property of Fuzi and Huanglian, respectively. These data implicated the possibility of drugs with a hot or cold property regulating the temperature of the microenvironment.

Although Fuzi and Huanglian did not affect cell viability, incubation at 22°C or 28°C, but not 38°C, significantly decreased cell viability. These data suggest that 37°C is the optimal temperature for cell culture and cold or hot properties of TCM do not affect cell growth. In our study, the hot property drug Fuzi or incubation at 38°C increased cellular ATP levels compared with normal control (without drug treatment and incubation at 37°C), whereas the cold property drug Huanglian and incubation at 22°C and 28°C decreased cellular ATP levels. These data suggest that the temperature change of the environment as well as the property of the drugs affect the cellular ATP level. The effects of drugs with a hot property on cellular ATP levels are likely due to: (1) the cellular ATP contained in the drug itself; and (2) released ATP activated by the drugs. It is possible that the cellular ATP in Fuzi itself was released on exposure of the cells, as Fuzi had a similar effect on the cellular ATP level as the 38˚C incubation. Whereas Huanglian decreased the cellular ATP level, the cold property of the drug would absorb the cellular ATP, leading to the decrease in the ATP level. These effects of TCM are similar to those of the low-temperature incubations.

The regulation of cellular ATP represents the cold or hot property of the TCM. Additionally, we found that TRPV4 function is regulated by the cold-hot property of TCM. Inconsistent with the effects on the ATP levels, either the cold property drug or hot property drug activates TRPV4. The effects were consistent with the differential temperature incubations. These data further support the temperature sensitivity of TRPV4. Most importantly, Chinese herbs with hot or cold properties have similar effects, indicating Chinese herbs regulate the temperature of the cellular microenvironment.

Fuzi is a type of TCM with the properties of being acrid, sweet and hot. It can be theoretically used to supply yang, reverse yin deficiency, get rid of cold and relieve pain [2]. Clinically, the drug is applied to treat yang deficiency syndrome and cold arthralgia syndrome [2], which is consistent with the recording in the Divine Farmer's Materia Medica, a Chinese book on agriculture and medicinal plants. After administering a hot property drug, expression of genes related to energy metabolites in patients with cold syndrome were affected, indicating that cold syndrome and hot property drugs can mediate the hot or warm reaction in the body [2]. There was a report that Fuzi significantly enhanced liver ATP and affected energetic metabolites in rats [19]. Yu *et al.* [20] found that Fuzi, to some extent, increases energy metabolism in rats through regulating metabolite-related gene expression. Likewise, herbs that have the potential to get rid of cold stimulate mitochondrial ATP generation, presumably through alteration of the levels of reactive oxygen species [2].

In contrast, Huanglian is characterized as being bitter and cold. It can be used to clear heat, eliminate dampness, purge fire and detoxify [2]. The efficacy of Huanglian is opposite to that of Fuzi as recorded in the Divine Farmer's Materia Medica. Huanglian has the potential to inhibit the growth and energy metabolism of dysentery bacillus [21]. After administering a Huanglian decoction to patients with hot syndrome, the body temperature was balanced by the drug administration. Previously, the compound bisandrographolide A from the plant *Andrographis paniculata* has been used in traditional medicine in many regions of Asia and was reported to activate TRPV4 channels [22]. Interestingly, *Andrographis paniculata* has a similar cold property as Huanglian.

Conclusion

The hot and cold property of TCM has been defined based on clinical experience over a period of a thousand years. Our current study provides experimental evidence for these properties of TCM. Our results suggest that the cold or hot property of TCM can be distinguished by cellular ATP levels after application to cells. Additionally, TRPV4 can be regulated by Chinese herbs with a cold or hot property. However, whether the cold or hot property is due to one specific active component or multiple components still requires further investigation.

Disclosure statement

The authors declare no conflict of interest.

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