Effects of Carvacrol and Volatile Fraction of Winter Savory (Satureja montana L.) on Body Temperature in Humans Who Experience Cold Sensitivity

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Ingestion of winter savory extract has been reported to inhibit the decrease in peripheral body temperature in people who experience cold sensitivity. However, the active ingredients underlying this effect are not known. We sought to elucidate the effects of the volatile components of winter savory on body temperature. Carvacrol (the main volatile component and a transient receptor potential channel ankyrin 1 (TRPA1) agonist in winter savory) increased core body temperature, but did not inhibit the decrease in peripheral body temperature after ingestion. In addition, the volatile fraction of winter savory (which contained various components in addition to carvacrol) induced not only an increase in core body temperature, but also inhibited the decrease in peripheral body temperature. These results suggest that the volatile components in winter savory may be effective for alleviating cold sensitivity, and that carvacrol is one of the active ingredients in winter savory.

Keywords: winter savory, carvacrol, volatile, TRPA1, body temperature, humans, cold sensitivity

Introduction

People who experience cold sensitivity (hie-sho in Japanese) have been reported to feel chilly, particularly in the waist and extremities such as the arm, wrist, fingers, ankle and toes (Terasawa, 1987; Shibahara and Itoh, 1999). This symptom typically accompanies shoulder stiffness, headache, swelling, sleeplessness, dizziness, hot flashes, peripheral numbness or tinnitus (Imai et al., 2007). With regard to the causes of feeling cold, disturbance in peripheral circulation (induced by contraction of the peripheral vessels) has been proposed. (Takatori et al., 1990; Ushiroyama, 2005). Taking into account a survey that > 50% of females aged 18 – 60 years experienced cold sensitivity (Yamato and Aomine, 2002; Takao et al., 2005; Imai et al., 2007), studying methods for alleviating cold sensitivity seems worthwhile.

Recently, we reported that ingestion of winter savory (Satureja montana L.) leaf extract, which has been used as a food condiment and herbal tea, inhibits decreases in peripheral body temperature (PBT) in people who experience cold sensitivity (Masuda et al., 2011). However, the active ingredients underlying this effect are unknown.

Carvacrol is the main component in the volatiles of winter savory (Piccaglia and Marotti, 1993; Skočibušić and Bezić, 2004; Pieto et al., 2007; Grosso et al., 2010). It is used as a pungent or spicy flavoring (Burdock, 2005). Based on an in vitro study (Xu et al., 2006; Lee et al., 2006), carvacrol has been reported to act as a transient receptor potential channel ankyrin 1 (TRPA1) agonist. TRPA1 is also activated by various pungent or spicy volatile components, such as allyl isothiocyanate (Jordt et al., 2004), cinnamaldehyde, eugenol, methyl salicylate (Bandell et al., 2004) and diallyl disulfide (Bautista et al., 2005; Macpherson et al., 2005). TRPA1 is a non-selective calcium-permeable channel and is considered to be involved in the noxious cold sensation and neural transmission related to pungency. Recent studies have suggested that TRPA1 activation is involved in nociception and thermoception, and in thermoregulation and energy metabolism (Iwasaki et al., 2008; Masamoto et al., 2009). Therefore, ingestion of carvacrol or other spicy/pungent...
vapors in winter savory could induce thermogenesis followed by an increase in PBT in people who experience cold sensitivity.

The aim of the present study was to elucidate the effects of the volatile components of winter savory on body temperature (BT). First, we examined whether carvacrol ingestion affected core body temperature (CBT) and body surface temperature (BST) in subjects who experienced cold sensitivity (Exp. 1). Next, we examined the effects of the volatile fraction of winter savory (WSV) on these two temperatures (Exp. 2). To elucidate the relationship between the increase in CBT and TRPA1, we confirmed the ability of carvacrol and WSV to activate human TRPA1 (hTRPA1).

Materials and Methods

This study was conducted with the approval of the Ethics Committee at the University of Shiga Prefecture in accordance with the principles of the Helsinki declaration. Informed consent was obtained from all subjects after a full explanation of the contents.

Subjects for measurement of BT and blood flow Changes in the time-course of human BT were measured after the ingestion of carvacrol (Exp. 1) or WSV (Exp. 2). Subjects were selected using a questionnaire focusing on the symptoms of cold sensitivity as reported by Takumi et al. (2010). To avoid fluctuations in basal BT, each subject participated in experiments within 2 weeks after menstruation. Taking this condition into account, the test was carried out in eight Japanese female volunteers in Exp. 1 and Exp. 2. Subject characteristics (values are mean ± SD) in Exp. 1 were: age, 18 ± 22 years; height, 156.1 ± 5.6 cm; body weight, 47.8 ± 2.1 kg; and body mass index (BMI), 19.6 ± 0.9. In Exp. 2, values were: age, 18 ± 20 years; height, 151.3 ± 2.5 cm; body weight, 47.0 ± 1.2 kg; and BMI, 20.5 ± 0.5.

Test protocols for measurement of BT and blood flow

Exp. 1 and Exp. 2 were carried out from late November to late December and from late May to late July, respectively. To reduce the influence of seasonal changes in BT and to accentuate the differences in BST between each sample, room temperature was determined: in Exp. 1 it was 23 ± 0.5°C and in Exp. 2, 25 ± 0.5°C. Room humidity was maintained at approximately 50% in both experiments. Subjects sat for > 30 min in the same temperature-controlled room with a humidity of approximately 50% before sample ingestion.

This study was conducted using a randomized double-blind, placebo-controlled, single-ingestion crossover design. Each measurement was taken between 09:00 and 13:00 to avoid the influence of diurnal variations in BT. Subjects were forbidden from eating and drinking anything other than water after waking on the experiment day. Ingestion of alcohol or irritant foods such as spices on the day before testing was prohibited. Each subject wore the same clothing (sweatsuit and socks) for each measurement in order to exclude the influence of clothing on BT.

The time-course of changes in temperature of the tympanic membrane was measured using an ear thermometer (M30, Terumo Corporation, Tokyo, Japan). This temperature was measured by the subjects themselves every 10 min from 5 min before to 55 min after each sample ingestion.

The time-course of changes in BST at the forehead, neck, wrist, middle finger and ankle was measured using two thermometers (BTH-601, Bio Research Center Co., Ltd., Nagoya, Japan; AM-8000K, Anritsu Meter Co., Ltd. Tokyo, Japan). Thermoprobes were fixed on each body surface by surgical tape. Each temperature was measured every minute from 10 min before to 59 min after sample ingestion.

Blood flow on the tip of the ring finger was measured using an ALF 21D Laser Doppler Flowmeter (Advance Co., Ltd., Tokyo, Japan). One sample was ingested on the first day and the other sample was ingested on the second day. The order of ingestion was randomized. To exclude the effects of the first sample, we arranged a washout period of > 1 day. The washout period was determined after consideration of the absorption, metabolism and urinary excretion of carvacrol in rats (Aussgulen et al., 1987). All subjects were in good health throughout the study period, and there were no complaints of discomfort after ingestion of any sample.

Sample preparation

WSV essential oil was prepared as follows. Briefly, steam was introduced to dried winter savory leaves (200 g, production area: Albania) in a steam distillation apparatus. The condensate was obtained by cooling the evaporated steam. The essence of WSV (1.6 g, yield against winter savory leaves: 0.8%) was then separated from the condensate. The clinical study was conducted by ingestion of each capsule containing the sample (carvacrol in corn oil, WSV in corn oil, or corn oil) with 37°C water (50 mL). The carvacrol dose in Exp. 1 was determined to be 0.44 mg (0.004 kcal, food-additive grade; Sigma Aldrich, St. Louis, MO). This dose corresponded to the content in winter savory extract (600 mg), which is a powder prepared from dried winter savory leaves (2.4 g, about one tablespoon) taken as herbal tea in hot water. WSV contains various other than carvacrol, so the WSV dose in Exp. 2 was determined to be 0.53 mg (which contained one-half of carvacrol in Exp. 1 (0.22 mg)). Carvacrol and WSV were dissolved in corn oil (100 mg, 0.9 kcal, J-Oil Millus, Tokyo, Japan) and packed into hard capsules made of gelatin (Matsuya Corporation., Osaka, Japan). Corn oil (100 mg, 0.9 kcal), a placebo, was packed into hard cap-
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Measurement of carvacrol in WSV
Quantitative analyses of carvacrol was carried out using the high-performance liquid chromatography (HPLC) system described in a previous report (Masuda et al., 2011).

Evaluation of hTRPA1 activation by measurement of the intracellular concentration of calcium ions (Ca\(^{2+}\)) HEK T-REx cells that stably maintained hTRPA1 gene expression were obtained as reported previously (Okumura et al., 2010). The intracellular concentrations of Ca\(^{2+}\) ([Ca\(^{2+}\)]) were measured as reported previously (Okumura et al., 2010). Concentration response curves for TRPA1 were obtained using allyl isothiocyanate (100 μM), WSV (10 μg/mL), and carvacrol (10 μM). Antagonistic inhibition was studied by adding HC030031 (1,2,3,6-tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7-acetamide; 30 μM; ChemBridge Corporation, San Diego, CA), a TRPA1 antagonist, to WSV (10 μg/mL) or carvacrol (10 μM). Each sample was prepared in dimethyl sulfoxide (DMSO) and added to the loading solution to a final DMSO concentration of 0.1% or 0.2%. Ionomycin (5 μM) was added to each well to elicit the maximum fluorescence intensity. The results for the samples are expressed as the percentage response to ionomycin (5 μM). Non-linear least-squares curve fitting and parameter estimation were carried out using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA).

Statistical analyses
Data are means ± SEM. The effects of time, treatment and time × treatment were evaluated by two-way repeated measures ANOVA. For comparison between the two groups at certain times, a post-hoc paired t-test was used. Statistical values were calculated using SPSS 13.0J for Windows (IBM Japan, Tokyo, Japan). p < 0.05 was considered significant.

Results
Changes in BT and blood flow after carvacrol ingestion in Exp. 1
Tympanic temperature after carvacrol ingestion was higher than that after placebo ingestion (p < 0.05 at 5 min to 55 min by two-way repeated measures ANOVA) (Fig. 1a).

BST of forehead temperature became elevated after carvacrol ingestion and tended to be higher than after placebo capsules in the same manner. No odor was detected outside each capsule.

Statistical analyses
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Fig. 1. Time-course of changes in BT after carvacrol ingestion in Exp. 1.
Values are differences from the mean temperature at 5 min (a) and 0 min (b – f), given as means ± SEM (n = 8). Time × treatment effect, p < 0.05 for 5 min to 55 min at the tympanic membrane (a), p < 0.1 for 0-59 min at the forehead (b), no significant difference at the neck (c), wrist (d), middle finger (e) or ankle (f) (two-way repeated measures ANOVA). * indicates p < 0.05 (paired t-test, carvacrol vs. placebo).
ingestion ($p < 0.1$ at $0 - 59$ min by two-way repeated measures ANOVA) (Fig. 1b). There were no significant differences between the carvacrol-ingestion group and placebo-ingestion group in the BST of neck, wrist, middle finger, and ankle (Figs. 1c - f).

Regarding blood flow, there were no significant differences between the carvacrol-ingestion group and the placebo-ingestion group (Fig. 2).

**Changes in BT and blood flow after WSV ingestion in Exp. 2** The tympanic temperature after WSV ingestion was higher than that after placebo ingestion ($p < 0.05$ at 5 min to 55 min by two-way repeated measures ANOVA) (Fig. 3a).

BSTs of the forehead, neck, wrist, middle finger and ankle due to WSV ingestion were higher than those after placebo ingestion ($p < 0.05$ at $0 - 59$ min by two-way repeated measures ANOVA) (Fig. 3b - f).

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**Fig. 2.** Time-course of changes in blood flow at the ring finger after carvacrol ingestion in Exp. 1. Values are means ± SEM ($n = 8$).

**Fig. 3.** Time-course of changes in BT after WSV ingestion in Exp. 2.

Values are differences from the mean temperature at 5 min (a) and 0 min (b - f), given as means ± SEM ($n = 8$). Time × treatment effect, $p < 0.05$ for 5 min to 55 min at the tympanic membrane (a), $p < 0.05$ for $0 - 59$ min at the forehead (b), neck (c), wrist (d), middle finger (e) and ankle (f) (two-way repeated measures ANOVA). * indicates $p < 0.05$ (paired $t$-test, WSV vs. placebo).
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Changes in the time-course of blood flow measured at the ring finger are shown in Fig. 4. Blood flow in the WSV-ingestion group tended to be greater than that of the placebo-ingestion group, but this difference was not significant.

Activation of hTRPA1 by carvacrol and WSV using hTRPA1-expressing cells  Activation of hTRPA1 by carvacrol using hTRPA1-expressing cells is shown in Fig. 5. Carvacrol increased the \([\text{Ca}^{2+}]\) in hTRPA1-expressing HEK T-REx cells. Addition of the TRPA1 antagonist HC030031 to carvacrol significantly inhibited the increase in \([\text{Ca}^{2+}]\) (\(p < 0.005\)), while slight increases in \([\text{Ca}^{2+}]\) were seen in HEK T-REx cells not expressing hTRPA1. Therefore, we confirmed that carvacrol activates hTRPA1.

WSV increased \([\text{Ca}^{2+}]\) in hTRPA1-expressing HEK T-REx cells (Fig. 6a). Addition of the TRPA1 antagonist HC030031 to WSV significantly inhibited the increase in \([\text{Ca}^{2+}]\) (\(p < 0.005\)), while slight increases in \([\text{Ca}^{2+}]\) were seen in HEK T-REx cells not expressing hTRPA1. The maximum response value by WSV (10 μg/mL) was similar to that of allyl isothiocyanate (100 μM) (Fig. 6b). The half-maximal effective concentration (EC50) value of WSV (1.78 μg/mL) was obtained from the concentration–response curve (Fig. 6b). These results suggested that WSV could activate hTRPA1.

Contribution ratio of carvacrol in WSV versus WSV for hTRPA1 activation  To estimate the contribution ratio of hTRPA1 activation by carvacrol in WSV versus that by WSV, we determined the concentration-response curve of carvacrol in WSV (which was calculated from the carvacrol content in WSV (42%)) (Fig. 7). Thus, the EC50 value of carvacrol in WSV (4.98 μM) was similar to that of carvacrol (4.01 μM).

Discussion  Experiment 1 was carried out from late November to late December; Exp. 2 was carried out from late May to late July. Metabolic and body-temperature responses to cold air differ according to seasonal changes (van Ooijen et al., 2004). Therefore, one must determine the room temperature to observe the changes in BT, which corresponds to seasonal changes. In our experiments, the PBT decreased gradually to room temperature. In an experiment at 25 ± 0.5°C from late October to early December, there were no significant differences between winter savory extract and placebo in the decrease in PBT (data not shown). In an experiment at 22 ± 0.5°C from late October to early December, ingestion of winter savory extract inhibited the decrease in PBT when compared with placebo (data not shown). Based on these results, the room temperature in Exp. 1 was set at 23 ± 0.5°C.

We observed that ingestion of carvacrol (the main volatile component in winter savory) increased the tympanic temperature in Exp. 1 (Fig. 1a). Tympanic temperature reflects CBT (Childs et al., 1999). Masamoto et al. (2009) reported that TRPA1 agonists, such as allylisothiocyanate and cinnamaldehyde, enhanced thermogenesis. Furthermore, Iwasaki et al. (2008) reported that these TRPA1 agonists induced adrenaline secretion via the central nervous system by TRPA1 activation. Carvacrol was confirmed to be able to activate hTRPA1 using hTRPA1-expressing cells (Fig. 5). Therefore, hTRPA1 activation by carvacrol may induce thermogenesis.
followed by an increase in CBT.

Ingestion of WSV (which contains various components besides carvacrol in winter savory) was found to increase CBT in Exp. 2 (Fig. 3a). Our in vitro study using hTRPA1-expressing cells revealed that WSV could activate hTRPA1 (Fig. 6a and b). The ability of WSV to activate hTRPA1 of WSV may be dependent on that of carvacrol (Fig. 7). Therefore, the increase in CBT after WSV ingestion may be induced by hTRPA1 activation by carvacrol.

Ingestion of carvacrol and WSV (which were confirmed to have the ability to activate hTRPA1 in hTRPA1-expressing cells) increased CBT. Based on the reports on thermogenesis by TRPA1 agonists in rats and mice (Iwasaki et al., 2008; Masamoto et al., 2009), ingestion of TRPA1 agonists in winter savory may induce thermogenesis in humans. Therefore, it is expected that ingestion of other TRPA1 agonists would also be effective for increasing CBT followed by alleviation of cold sensitivity.

In Exp. 2, although the amount of carvacrol was one-half of that used in Exp. 1, CBT increased (Fig. 3a). Therefore, carvacrol dose in Exp. 2 (0.22 mg) was sufficient to increase CBT. However, we did not confirm the dose-dependent increase in CBT. Hence, further study is needed to elucidate the adequate dose of carvacrol to increase CBT.

Carvacrol ingestion did not induce an increase in BSTs (Fig. 1b − f) or blood flow in the ring finger in Exp. 1 (Fig. 2). However, BSTs after WSV ingestion were higher than those after placebo ingestion (Fig. 3b − f), and blood flow after WSV ingestion tended to be higher when compared with that after placebo ingestion in Exp. 2 (Fig. 4). The volatiles in winter savory reportedly contain various components other than carvacrol (Piccaglia and Marotti, 1993; Skočibušić and Bezić, 2004; Pieto et al., 2007; Grosso et al., 2010). Among the factors related to cutaneous vasodilation, the following are considered to be important: (i) the decrease in vasoconstriction in the sympathetic noradrenergic system; and (ii) activation in the non-adrenergic vasodilation system (Charkoudian, 2010). Intravenous injection of volatile components such as allyl isothiocyanate and cinnamaldehyde in mice have been reported to induce peripheral vasodilation and increase peripheral blood flow via the autonomic neuronal pathways (Pozgai et al., 2010). In addition, based on an in vitro study, various monoterpenes have been reported to have a vasodilation effect (Santos et al., 2011). Therefore, the volatiles in winter savory may have similar components and similar effects. Further study is necessary to elucidate the active principles in WSV that affect the BST.

Okuda et al. (1993) reported that the agent that induces thermogenesis followed by peripheral vasodilation is an effective agent for alleviating cold sensitivity. In the present study, carvacrol ingestion increased tympanic temperature (Fig. 1a) but not PBT (Fig. 1d − f). We postulate that weak thermogenesis increases PBT. Therefore, the ingestion of greater doses of carvacrol or simultaneous ingestion of other components having a peripheral-vasodilation effect may alleviate cold sensitivity.

Previously, we reported that ingestion of winter savory extract (which contains volatiles and non-volatiles) inhibited the decrease in PBT (wrist, middle finger, middle toe) (Masuda et al., 2011). In the present study, ingestion of WSV increased CBT and alleviated the decrease in PBT (wrist, middle finger and ankle). It is thought that the volatiles in winter savory are important for alleviation of cold sensitivity. However, it is not clear if the volatiles in winter savory are...
the only effective ingredients in winter savory extract (which contains volatiles and non-volatiles). Winter savory extract (which was obtained from winter savory by hot-water extraction and lyophilization) contains various polyphenols other than volatiles (Darbour et al., 1996; Ćetković et al., 2007; Gião et al., 2009; Silva et al., 2009). Among these polyphenols, rosmarinic acid has been found to improve endothelium-dependent vasodilation based on an in vitro study (Ersoy et al., 2008). In addition, other polyphenolic components in winter savory (e.g., naringenin, eriodictyol, luteolin) have been reported to induce vasodilation based on an in vitro study using rat aortae (Sánchez de Rojas et al., 1996a; Sánchez de Rojas et al., 1996b; Sánchez de Rojas et al., 1999). Therefore, the non-volatiles in winter savory, such as polyphenols, might be expected to contribute to the increase in peripheral blood flow. To answer this question, further studies into the effects of non-volatiles in winter savory on human BT are needed.

In conclusion, we clarified that ingestion of carvacrol (the main volatile component and hTRPA1 agonist in winter savory) increased CBT in people who have cold sensitivity. In addition, the volatile components in winter savory (which activate hTRPA1) not only increased CBT, but also inhibited the decrease in PBT. These results suggest that the volatile components in winter savory are effective for alleviating cold sensitivity and that carvacrol is one of the active ingredients in winter savory.

References