Note

UCP1 and TRPM8 Expression in the Brown Fat Did Not Affect the Restriction of Menthol-Induced Hyperthermia by Estradiol in Ovariectomized Rats

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Summary Estradiol (E2) modulates the central and peripheral thermoregulatory responses to cold. Menthol is an agonist of transient receptor potential melastatin type 8 (TRPM8), which is a peripheral cold receptor. E2 suppresses menthol-induced elevation of body temperature (Tb) in ovariectomized rats, but the mechanism is unknown. The aim of the present study is to investigate the effect of E2 on uncoupling protein 1 (UCP1), a thermogenic gene, and TRPM8 mRNA levels in ovariectomized rats applied menthol. A silastic tube was implanted in ovariectomized rats with and without E2 underneath the dorsal skin (E2(+)) and E2(−) groups), and data loggers for Tb measurement into peritoneal cavity. After application of 10% l-menthol or vehicle to the skin of the whole trunk of rats, Tb was measured for 2 h. The interscapular brown adipose tissue (BAT) and spinal ganglia of cervical, thoracic, and lumbar parts were obtained for RT-qPCR assay. In the menthol application, Tb in the E2(+) group was lower than that in the E2(−) group. The UCP1 mRNA in the BAT, TRPM8 mRNA in the BAT and spinal ganglia in all areas did not differ between the E2(+) and E2(−) groups. In conclusion, the UCP1 and TRPM8 expression in the brown fat did not affect the restriction of the menthol-induced hyperthermia by estradiol in ovariectomized rats.

Key Words menthol, UCP1, TRPM8, brown adipose tissue, estradiol

The lifestyle diseases such as obesity and metabolic syndrome become a social problem. Recently, brown adipose tissue (BAT), an important organ for non-shivering thermogenesis to maintain body temperature (Tb) and metabolism in the cold (1), received plenty of attention in a new approach of anti-obesity. The subclavian BAT in the adults activated by cold stimulation induced thermogenesis (2). The thermogenesis induced by the BAT activation by the medical agents and dietary components may contribute to decrease the obesity.

The female hormone estradiol (E2) helps maintain Tb of female rats in the cold. In the previous study, the central administration of E2 to the medial preoptic area in the hypothalamus increased Tb by decreasing the tail skin temperature of ovariectomized rats in the cold (3). The results indicated the central action of E2 affected autonomic thermoregulation in the cold. Then, we focused on the effect of E2 on thermoregulatory responses via a peripheral cold-sensitive receptor, the transient receptor potential melastatin type 8 (TRPM8). E2 suppressed hyperthermia induced by menthol, a TRPM8 agonist and ingredients in mint, in ovariectomized rats. However, E2 did not affect the tail skin temperature and neural activity, according to the assessment by c-Fos immunohistochemistry of the medial preoptic area, which is the thermoregulatory center; and the median preoptic area, paraventricular nucleus, posterior nucleus, and dorsomedial hypothalamus, which are involved in thermogenesis after menthol application (4). The results suggested that the heat dissipation from the tail and the neural activity of the nuclei did not influence the inhibition of menthol-induced hyperthermia by E2. Thus, we focused on a cold-sensitive receptor, TRPM8 and BAT thermogenesis in the female rats applied menthol to investigate the inhibition of menthol-induced hyperthermia by E2.

Based on our previous study (4), we speculated that the effect of E2 on TRPM8 expression in peripheral organs was involved in the inhibition of menthol-induced hyperthermia by E2. TRPM8 mRNA expression in ovariectomized rats administered with E2 (5 μg/kg/d, 4 wk) subcutaneously had a tendency to decrease (5); however, the study did not perform menthol application and did not assess TRPM8 mRNA expression in sensory neurons on the skin. In addition, the administration method of E2 was different from that in our previous study (4). Thus, in the present study, we investigated TRPM8 mRNA expression in the spinal ganglia, which are areas that produce TRPM8 mRNA in sensory neurons, in the ovariectomized rats administrated E2 with menthol application in the same manner as our previous study (4).

The uncoupling protein 1 (UCP1) in the BAT is a thermogenic gene. Recently, a new role for TRPM8 in
the BAT thermogenesis was introduced (6). TRPM8 activation in the BAT might increase UCP1 upregulation in mice. Chronic dietary menthol application (0.5%, 7 mo) increased $T_b$ and UCP1 protein expression in the BAT of mice; such responses were not observed in TRPM8$^{-/-}$ mice (7). However, the effect of $E_2$ on the BAT thermogenesis via TRPM8 and UCP1 expression in female rodents has not been clarified. Thus, we investigated TRPM8 and UCP1 mRNA expression of the BAT in the female rats applied menthol.

The aim of the present study was to validate the hypothesis that $E_2$ suppressed TRPM8 expression in the spinal ganglia and UCP1 expression in the BAT in ovarioctomized rats applied menthol. The $T_b$, TRPM8 mRNA expression in the spinal ganglia, TRPM8 and UCP1 mRNA expression in the BAT, and plasma catecholamine concentrations related to systemic sympathetic nerve activity were assessed. We compared the results between the ovarioctomized rats with $E_2$ and without $E_2$ to avoid the effect of the concentration of female hormones fluctuates in the female rats.

Materials and Methods

Animals. Virgin female Wistar rats ($n=36$; body weight 165±0.9 g; age 9 wk; Japan SLC, Inc., Hamamatsu, Japan) were used. They were individually housed in cages (37×21×19 cm) at ambient temperature of 26±1°C in 12:12-h light-dark cycle (light on at 07:00 h) and allowed free access to food and water. The Institutional Animal Care and Use Committee of Nara Women's University (Nara, Japan) approved all experimental protocols (Approval number: 19-06).

Surgery. The rat was inserted a temperature sensor in the spinal ganglia and BAT using the RNeasy Mini Kit and RNeasy Lipid Tissues Mini Kit (QIAGEN), respectively, according to the manufacturer’s protocol. The total RNA concentration in the eluent was determined based on the ratio of the absorbance at 260 and 280 nm (NanoDrop ND-2000 Spectrophotometer, Thermo Scientific, Wilmington, DE). Then, cDNA was synthesized using the PrimeScript™ RT Master Mix (TAKARABIO, Otsu, Japan). RT-qPCR was conducted using an RT-PCR kit (QB Green® Premix Ex Taq™, TAKARABIO). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the reference as in a previous report that determined the TRPM8 and UCP1 mRNA levels (7). The oligonucleotide sequences of the primers used are shown in Table 1. Amplification was performed with a StepOne Software v2.1 system (Applied Biosystems, Foster City, CA). The denaturation protocol was 95°C for 30 s, 95°C for 5 s and 64°C for 30 s by 40 cycles, 95°C for 1 min, 95°C for 1.5 s. The mRNA quantity was calculated using the comparative Ct method under the assumption that the primer efficiencies were relatively similar.

Assessment of plasma catecholamine levels. The blood samples obtained in our previous study performed same experimental protocol were used (4). The plasma level of catecholamine was determined a catecholamine kit (CA test TOSOH; Tosoh Corporation, Tokyo, Japan) at SRL, Inc. (Tokyo, Japan).

### Table 1. Primer sequences used for real-time RT-qPCR assays.

| Gene     | Forward primer(5’-3’)                                    | Reverse primer(5’-3’)         |
|----------|----------------------------------------------------------|-------------------------------|
| TRPM8    | GCAGTGGTACATGAAACCGAGT                                    | TGAAGAGTGAACCCGAAATAC         |
| UCP1     | TACCCACGTGCAATGACCA                                       | GCACACAAACATGAGCTTCC          |
| GAPDH    | AAATCCCATCACCATCCTTCA                                      | AATGAGCCCCAGCCTTCTC           |

Primers were obtained from TAKARABIO (Otsu, Japan).
Statistics. Data are presented as mean±standard error. The baseline value of $T_b$, change in $T_b$ ($\Delta T_b$) from the baseline level was calculated. Values for change in $T_b$ were averaged every 30 min. Differences in the $\Delta T_b$, TRPM8 and UCP1 mRNA levels, and plasma catecholamine concentration between E2(−) and E2(+) groups (§), Vehicle/E2(−) and Vehicle/E2(+) groups (†), Vehicle/E2(−) and Menthol/E2(−) groups (‡), and Vehicle/E2(+) and Menthol/E2(+) groups (§), p<0.05.

Results

Change in body temperature ($\Delta T_b$)

Figure 1 showed the $\Delta T_b$. The baseline of $T_b$ was not different among the groups (Vehicle/E2(−), 37.5±0.1°C; Vehicle/E2(+), 37.6±0.1°C; Menthol/E2(−), 37.4±0.1°C; Menthol/E2(+), 37.5±0.1°C). Two-way ANOVA indicated a significant main effect of time [F(2,59)=78.11, p<0.05] and a significant interaction between time and group on $\Delta T_b$ [F(6,59)=29.59, p<0.05]. The $\Delta T_b$ in Vehicle/E2(−) group was lower than that in Vehicle/E2(+) group at 90–120 min [p<0.05]. The $\Delta T_b$ in Menthol/E2(−) group was greater than that in Menthol/E2(+) group at 60 min [p<0.05]. The $\Delta T_b$ in Menthol/E2(−) and Menthol/E2(+) groups was lower than that in Vehicle/E2(−) and Vehicle/E2(+) groups at 30–120 min [p<0.05].
TRPM8 mRNA in spinal ganglia

Figure 2 showed TRPM8 mRNA expression (A–C). No significant difference was observed in the TRPM8 mRNA expression between any of the groups.

UCP1 and TRPM8 mRNA in the BAT

Figure 3 showed the UCP1 (A) and TRPM8 mRNA (B) expression in the BAT. No significant difference was observed in the UCP1 and TRPM8 mRNA expression in the BAT among the groups.

Plasma catecholamine concentration

Figure 4 showed the plasma levels of adrenaline (A), noradrenaline (B) and dopamine (C) concentrations at 27˚C. No significant differences in plasma levels were observed between groups.

Discussion

The present study revealed that the UCP1 and TRPM8 expression in the brown fat and TRPM8 expression in the DRG did not affect the restriction of the menthol-induced hyperthermia by estradiol in ovariectomized rats.

The ΔTb values were consistent with those in the previous study (4). First, we planned to investigate the TRPM8 protein that expressed after several hours after menthol stimulation; however, we dropped the idea because we could not obtain the appropriate antibodies. The unpublished immunohistological data of Prof. Miyata showed that all commercial antibodies (5, 9) showed the positive responses in the TRPM8−/− mice tissues. Thus, we tried to assess the TRPM8 mRNA in the tissues. E2 did not affect TRPM8 mRNA expression in the spinal ganglia of ovariectomized rats treated with menthol, which means that TRPM8 expression did not affect the inhibitory action of E2 on menthol-induced hyperthermia. E2 induced a decreasing trend in TRPM8 mRNA expression, but did not affect the protein level in the lumbar skin of ovariectomized rats (5). TRPM8 mRNA and protein in the lumbar skin of ovariectomized rats were lower than those in sham rats after exposure to cold (4˚C, 20 min) (9). These results did not correspond with our results, which can be attributed to the fact that previous studies did not apply menthol and investigate TRPM8 mRNA in sensory neurons. Menthol application did not increase TRPM8 mRNA in the spinal ganglia of ovariectomized rats with and without E2. Oxaliplatin, a drug that induces cold hyperalgesia, increased TRPM8 mRNA in the fourth to sixth lumbar parts of the spinal ganglia in male rats (10). Menthol administration (3 mM) to the bladder increased TRPM8 mRNA and protein in the bladder and spinal ganglia of female rats (11). These results did not agree with our results, which could be attributed to the short dermal administration of menthol to the back of female rats in the present study. Our results described the effects of E2 on TRPM8 mRNA in the spinal ganglia of ovariectomized rats after menthol stimulation. After the development of the appropriate TRPM8 antibodies, it is important to investigate the TRPM8 protein in the tissues after menthol application in future studies.

The UCP1 was the indicator of the BAT non-shivering thermogenesis in the cold in rodents. The UCP1 mRNA in the BAT increased after cold exposure in male (12) and female (3) rats. We hypothesized that the menthol-induced hyperthermia was caused by the BAT non-shivering thermogenesis, and checked the TRPM8 mRNA in the BAT, because, in vitro, the TRPM8 protein expressed in the BAT, and TRPM8 activation by menthol administration directly increased UCP1 expression in primary cultured BAT (7); however, E2 did not affect TRPM8 and UCP1 expression in the BAT in ovariectomized rats applied menthol. In addition, the UCP1 expression did not differ between vehicle and menthol applications, which suggested that other factors might contribute to the suppression of menthol-induced hyperthermia by E2. Shivering induces thermogenesis and contributes to maintain Tb. The application of 10% menthol induced shivering and increased Tb in male mice (13), which could be inhibited by E2. In future studies, it will be necessary to investigate the effects of E2 on shivering or plasma irisin concentration, which is associated with shivering (14) during menthol application.

Cold stimulation increased the concentration of plasma catecholamine, an indicator of systemic sympathetic nerve activity (15). The hydrogel containing L-menthol (2%) and ethanol (40%), which was applied to the skin, increased plasma noradrenaline and dopamine concentrations in rats (16). The plasma adrenaline and dopamine concentrations in ovariectomized rats were greater than those in sham rats (17). These results indicated that E2 might affect the plasma catecholamine concentration in ovariectomized rats applied menthol. We hypothesized that the suppression of E2 increased sympathetic nerve activity due to menthol.

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Fig. 4. Plasma levels of adrenaline (A), noradrenaline (B) and dopamine (C) concentrations. Values are presented as the mean±standard error (Vehicle/E2(−); n=5, Vehicle/E2(+) n=5, Menthol/E2(−); n=6, Menthol/E2(+); n=5).
application, which decreased systemic thermogenesis; however, there was no difference in the plasma catecholamine concentrations between the groups. It is speculated that small sample size of each group might have affected the large standard error because of the experimental limitations. Thus, catecholamine might not be involved in the suppression of menthol-induced hyperthermia by E2.

In conclusion, the present study revealed that E2 did not affect UCP1 and TRPM8 expression in the BAT in ovariectomized rats applied menthol; thus, it did not influence the suppression of menthol-induced hyperthermia by E2 at least. The mechanism is need to be clarified.

Authorship
Research conception and design: YU and IS; experiments: IS, KA, and CT; statistical analysis of the data: IS and YU; interpretation of the data: YU and IS; writing of the manuscript: YU and IS.

Disclosure of state of COI
No conflicts of interest to be declared.

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REFERENCES
1) Gordon CJ. 1993. Temperature Regulation in Laboratory Rodents. the Press Syndicate of University of Cambridge, Cambridge.
2) Yoneshiro T, Aita S, Matsushita M, Kameya T, Nakada K, Kawai Y, Saito M. 2011. Brown adipose tissue, whole-body energy expenditure, and thermogenesis in healthy adult men. Obesity (Silver Spring) 19: 13–16.
3) Uchida Y, Kano M, Yasuhara S, Kobayashi A, Tokizawa K, Nagashima K. 2010. Estrogen modulates central and peripheral responses to cold in female rats. J Physiol Sci 60: 151–160.
4) Uchida Y, Atsumi K, Hirano S, Koyanagi N. 2018. Estradiol administration suppresses body temperature elevation induced by application of menthol to ovariectomized rats. J Therm Biol 78: 281–289.
5) Kubo T, Tsuji S, Amano T, Yoshino F, Niwa Y, Kasahara K, Yoshida S, Mukaisho KI, Sugihara H, Tanaka S, Kimura F, Takahashi K, Murakami T. 2017. Effects of beta-estradiol on cold-sensitive receptor channel TRPM8 in ovariectomized rats. Exp Anim 66: 337–343.
6) Uchida K, Sun W, Yamazaki J, Tominaia M. 2018. Role of thermo-sensitive transient receptor potential channels in brown adipose tissue. Biol Pharm Bull 41: 1135–1144.
7) Ma S, Yu H, Zhao Z, Luo Z, Chen J, Ni Y, Jin R, Ma L, Wang P, Zhu Z, Li L, Zhong J, Liu D, Nilius B, Zhu Z. 2012. Activation of the cold-sensing TRPM8 channel triggers UCP1-dependent thermogenesis and prevents obesity. J Mol Cell Biol 4: 88–96.
8) Shima N, Yamaguchi Y, Yuki K. 2003. Distribution of estrogen receptor beta mRNA-containing cells in ovariectomized and estrogen-treated female rat brain. Anat Sci Int 78: 85–97.
9) Noguchi W, Ishizuka O, Imamura T, Kurizaki Y, Yamagishi T, Yokoyama H, Lei Z, Silwal SG, Nishizawa O, Andersson KE. 2013. The relationship between alpha1-adrenergic receptors and TRPM8 channels in detrusor overactivity induced by cold stress in ovariectomized rats. J Urol 189: 1975–1981.
10) Kawashiri T, Egashira N, Kurobe K, Tsutsumi K, Yamashita Y, Ushio S, Yano T, Oishi R. 2012. L type Ca(2+) channel blockers prevent oxalipatin-induced cold hyperalgesia and TRPM8 overexpression in rats. Mol Pain 8: 7.
11) Jun JH, Kang HJ, Jin MH, Lee HY, Im YJ, Jung HJ, Han SW. 2012. Function of the cold receptor (TRPM8) associated with voiding dysfunction in bladder outlet obstruction in rats. Int Neurourol J 16: 69–76.
12) Ricquier D, Bouillaud F, Toumelin P, Mory G, Bazin R, Arch J, Penicaud L. 1986. Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. Evidence for a rapid beta-adreno-receptor-mediated and transcriptionally regulated step during activation of thermogenesis. J Biol Chem 261: 13905–13910.
13) Tajino K, Matsumura K, Kosada K, Shibakusa T, Inoue K, Fushiki T, Hosokawa K, Kobayashi S. 2007. Application of menthol to the skin of whole trunk in mice induces autonomic and behavioral heat-gain responses. Am J Physiol Regul Integr Comp Physiol 293: R2128–R2135.
14) Lee P, Linderman JD, Smith B, Brychta RJ, Wang J, Idelsohn C, Perron RM, Werner CD, Phan GQ, Kammula US, Kebebew E, Pacak K, Chen KY, Celi FS. 2014. Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. Cell Metab 19: 302–309.
15) Nomura T, Kawano F, Kang MS, Lee JH, Han EY, Kim CK, Sato Y, Ohira Y. 2002. Effects of long-term cold exposure on contractile muscles of rats. Jpn J Physiol 52: 85–93.
16) Sudo J, Iwase H, Terui J, Kakuno K, Soyama M, Takayama K, Nagai T. 1998. Transdermal absorption of L-dopa from hydrogel in rats. Eur J Pharm Sci 7: 67–71.
17) Cao X, Zhou C, Chong J, Fu L, Zhang L, Sun D, Hou H, Zhang Y, Li D, Sun H. 2015. Estrogen resisted stress-induced cardiomyopathy through increasing the activity of beta(2)-AR-galphas signal pathway in female rats. Int J Cardiol 187: 377–386.