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Central and peripheral des-acyl ghrelin regulates body temperature in rats

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ABSTRACT

In the present study using rats, we demonstrated that central and peripheral administration of des-acyl ghrelin induced a decrease in the surface temperature of the back, and an increase in the surface temperature of the tail, although the effect of peripheral administration was less marked than that of central administration. Furthermore, these effects of centrally administered des-acyl ghrelin could not be prevented by pretreatment with [D-Lys3]-GHRP-6 GH secretagogue receptor 1a (GHS-R1a) antagonists. Moreover, these actions of des-acyl ghrelin on body temperature were inhibited by the parasympathetic nerve blocker methylscopolamine but not by the sympathetic nerve blocker timolol. Using immunohistochemistry, we confirmed that des-acyl ghrelin induced an increase of cFos expression in the median preoptic nucleus (MnPO). Additionally, we found that des-acyl ghrelin dilated the aorta and tail artery in vitro. These results indicate that centrally administered des-acyl ghrelin regulates body temperature via the parasympathetic nervous system by activating neurons in the MnPO through interactions with a specific receptor distinct from the GHS-R1a, and that peripherally administered des-acyl ghrelin acts on the central nervous system by passing through the blood–brain barrier, whereas it exerts a direct action on the peripheral vascular system.

1. Introduction

Ghrelin was originally isolated from rat stomach extracts [1] as an endogenous ligand for the GHS-R. Ghrelin, produced predominantly by endocrine cells of the gastric oxyntic glands [2,3], exists as two major molecular forms: ghrelin and des-acyl ghrelin [4]. Ghrelin is composed of 28 amino acids and is octanoylated at Ser3, an unusual post-translational modification that is catalyzed by the enzyme ghrelin *O*-acyltransferase (GOAT) [5,6]. Des-acyl ghrelin, which lacks the Ser3 residue octanoylation, is unable to release GH or bind to the classic GHS-R1a receptor [4]. These characteristics indicate that octanoic acid plays an important role in physiological activity via GHS-R1a, and des-acyl ghrelin has been considered an inactive form of ghrelin. However, it has recently been reported that des-acyl ghrelin has a variety of functions, such as inhibition of cell death in cardiomyocytes and endothelial cells, induction of adipose tissue production, and enhancement of human osteoblastic cell proliferation through a pathway that does not involve the GHS-R1a [7–9]. These findings suggest the presence of an unidentified receptor specific for des-acyl ghrelin. Thus, although ghrelin and des-acyl ghrelin have similar structures, their actions and the mechanisms involved differ at several levels.

Since its discovery in 1999, it has been clarified that ghrelin acts on energy-metabolic systems by promoting growth hormone secretion and food intake [10–12]. Furthermore, centrally or peripherally administered ghrelin increases the respiratory quotient, indicating that it has an inhibitory action on fat oxidation [13]. Intracerebroventricular (i.c.v.) injection of ghrelin in rats transiently decreases the body core temperature [14], and decreases temperature, sympathetic activity and noradrenalin release in brown adipose tissue (BAT) [15,16]. These findings suggest that ghrelin induces positive energy balance not only by increasing energy intake but also by decreasing energy expenditure.

On the other hand, compared to ghrelin, the effects of des-acyl ghrelin on energy intake are less well characterized [17–20]. Also, the action of des-acyl ghrelin on energy expenditure has not yet been investigated. In the present study, therefore, we examined the effects of central and peripheral injection of des-acyl ghrelin on body temperature, and investigated in detail the mechanisms responsible for this effect.

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2. Materials and methods

2.1. Animals and i.c.v. injection of des-acyl ghrelin and ghrelin

Male Wistar rats (Charles River Japan, Inc., Yokohama, Japan), weighing 300–350 g, were housed in individual Plexiglas cages in an animal room maintained under a constant light–dark cycle (lights on from 7:00 to 19:00 h) and temperature (22 ± 1 °C) for at least one week. Food and water were provided ad libitum. i.c.v. cannulae were implanted into the lateral cerebral ventricles using a method that has been described previously [21], and after surgery all rats were housed individually in Plexiglas cages. During a 6-day postoperative recovery period, the rats became accustomed to the handling procedure. Des-acyl ghrelin (Peptide Institute, Inc., Osaka, Japan) and ghrelin (Peptide Institute, Inc., Osaka, Japan) was dissolved in saline, and 10 μl of the solution was injected into each free-moving rat through a 27-gauge injection cannula connected to a 50-μl Hamilton syringe. All procedures were performed in accordance with the Japan Physiological Society's guidelines for animal care, and this study was approved by the experimental animal committee of Miyazaki University (authorization number: 2006-052-6).

2.2. Measurement of body temperature

We removed food and water from each cage at 18:00 h and switched off ambient illumination at 19:00 h. Subsequently, during the dark period, we started infrared thermographic imaging of the back (back surface temperature) and tail (caudal surface temperature) of the rats at 19:30 h, and saved images taken at 1 min intervals during the following 30 min (FLIR SC620, FLIR Systems, Danderyd, Sweden). Thereafter, des-acyl ghrelin was administered by i.c.v. (0.01, 0.1, 0.5 nmol/10 μl) or intraperitoneal (i.p.; 0.01, 0.03, 0.1 mg/kg body weight) injection, and measurements were conducted for the following 60 min. The camera was newly calibrated and fixed in a standardized position 1.30 m vertically above each rat. The FLIR SC620 has a thermal resolution of <0.04 °C, an accuracy of ±2%, and a picture resolution of 640 × 480 pixels. The average value during the 10 min before administration was assumed to be 0, and the values obtained thereafter were indicated as increases or decreases. Moreover, i.c.v. injection of ghrelin (0.5 nmol/10 μl) was also performed under the same conditions.

2.3. Influence of [D-Lys3]-GHRP-6 on the effects of des-acyl ghrelin on body temperature

Rats were subjected to implantation of a stainless steel cannula into the lateral left ventricle using the method described above. The GHS-R1a antagonist [D-Lys3]-GHRP-6 (Sigma–Aldrich Co., St. Louis, USA; 10 nmol/5 μl) was dissolved in saline and i.c.v. injected at 19:30 h, and then 30 min later, saline, ghrelin (0.5 nmol/5 μl) and des-acyl ghrelin (0.5 nmol/5 μl) were injected into the lateral ventricle using a 50-μl Hamilton syringe. After injection, measurements were performed every 1 min for 60 min.

2.4. Influence of an autonomic nerve blocker on the effects of des-acyl ghrelin on body temperature

Rats were subjected to implantation of a stainless steel cannula into the lateral left ventricle using the method described above. The muscarinic receptor antagonist methylscopolamine (Sigma–Aldrich Co., St. Louis, USA; 0.5 mg/kg body weight) or the adrenergic receptor antagonist timolol (Sigma–Aldrich Co., 0.5 mg/kg body weight) was dissolved in saline and i.p. injected at 19:30 h, and then 30 min later, saline and des-acyl ghrelin (0.5 nmol/10 μl) were injected into the lateral ventricle using a 50-μl Hamilton syringe. After injection, measurements were performed every 1 min for 60 min. Moreover, peripheral administration of des-acyl ghrelin (0.03 mg/kg body weight) was also performed under the same conditions.

2.5. Immunofluorescence staining of cFos in the MnPO

Immunohistochemical staining for cFos was performed 90 min after i.c.v. (0.5 nmol/10 μl) and i.p. (0.03 mg/kg body weight) injection of des-acyl ghrelin during the dark period. Frozen brain sections were cut with a cryostat at a thickness of 10 μm. The cFos staining was performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the method described. Briefly, after blocking the sections with diluted normal goat serum, they were incubated at 4 °C for 24 h with anti-cFos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.3% Triton X-100/phosphate-buffered saline (PBS). After being washed for 30 min with 0.1% tween 20/PBS, the sections were incubated for 2 h with a biotinylated second antibody and for an additional 2 h with avidin–biotin–peroxidase complex. The sections were then stained with 0.02% 3,3′-diaminobenzidine and 0.05% hydrogen peroxide in Tris buffer.

2.6. Measurement of perfusion pressure

After the rats had been killed, tail arteries and the aorta were rapidly removed and suspended from two L-shaped stainless steel wires in an organ bath (LABO Support Co., Ltd., Osaka, Japan) filled with Tyrode solution and maintained at 37 °C. The bath solution was continuously bubbled with 95% O_2 and 5% CO_2. Isometric tension was recorded using a PowerLab/8SP computerized data acquisition system (AD Instruments, Castle Hill, NSW, and Australia). When the contraction reached a plateau upon treatment with phenylephrine, des-acyl ghrelin (0.2, 2, 20 μM) was added to the bath cumulatively. Additionally, to study endothelium-dependent vasodilation via the NO-cGMP pathway, rings with intact endothelium that had been pretreated with N^G-nitro-L-arginine methyl ester (L-NAME: 100 μM) for 30 min were contracted by treatment with phenylephrine. When the contraction reached a plateau, des-acyl ghrelin (2 μM) was added to the bath employing the same schedule as that described above.

2.7. Statistical analysis

All results are presented as mean ± S.E.M. Effects of des-acyl ghrelin and ghrelin on body temperature were analyzed using repeated-measures ANOVA. Furthermore, AUC values were analyzed by Student's *t* test. Perfusion pressure was analyzed using Tukey's multiple comparison test. Values were considered to differ significantly at $p < 0.05$.

3. Results

3.1. Effects of central and peripheral administration of des-acyl ghrelin on body temperature

To evaluate the effects of des-acyl ghrelin on thermogenesis, we performed infrared thermographic imaging of the back (back surface temperature) and tail (caudal surface temperature) after i.c.v. injection of des-acyl ghrelin. The back surface temperature after i.c.v. injection of des-acyl ghrelin was significantly decreased at a concentration of 0.1 and 0.5 nmol compared with the saline group for at least 1 h (Fig. 1B). Moreover, i.c.v. injection of 0.1, 0.5 nmol des-acyl ghrelin acutely increased the caudal surface

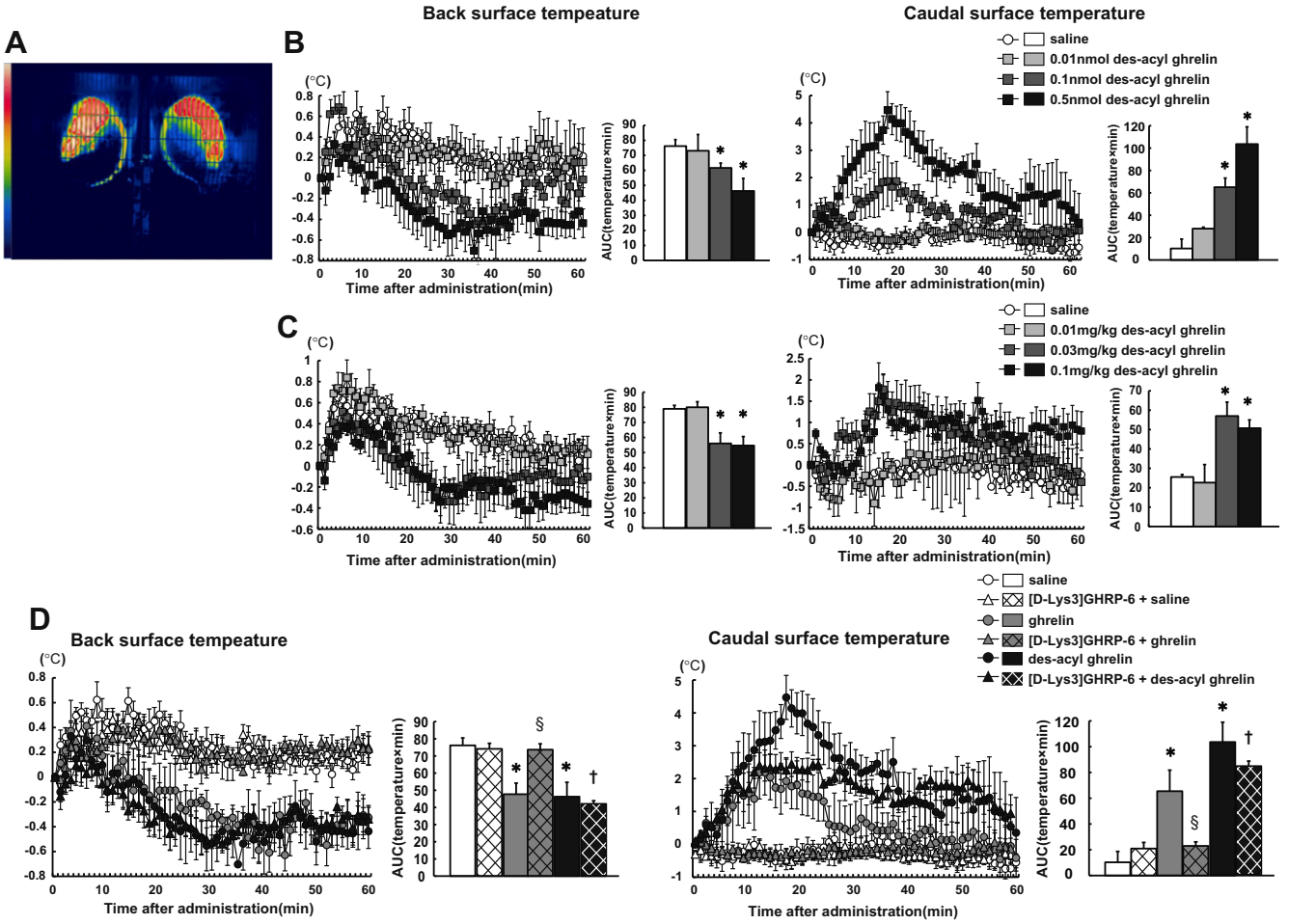

Fig. 1. (A) Infrared images showing surface temperature changes in the rat. (B) Changes in back surface temperature and caudal surface temperature from the baseline after i.c.v. injection of saline ($n = 11$) and des-acyl ghrelin (0.01 nmol: $n = 5$, 0.1 nmol: $n = 5$, 0.5 nmol: $n = 7$). (C) Changes in back surface temperature and caudal surface temperature from the baseline after i.p. injection of saline ($n = 5$) and des-acyl ghrelin (0.01 mg/kg: $n = 5$, 0.03 mg/kg: $n = 7$, 0.1 mg/kg: $n = 5$). (D) Changes in back surface temperature and caudal surface temperature from the baseline after i.c.v. injection of saline, ghrelin, and des-acyl ghrelin with (saline: $n = 5$, ghrelin: $n = 6$, des-acyl ghrelin: $n = 6$) or without (saline: $n = 11$, ghrelin: $n = 7$, des-acyl ghrelin: $n = 7$) [D-Lys3]-GHRP-6 pretreatment. AUC of back surface temperature indicates cumulative change from the baseline during 60 min after injection of saline, ghrelin and des-acyl ghrelin. AUC of caudal surface temperature indicates cumulative change from the baseline during 30 min after injection of saline, ghrelin and des-acyl ghrelin. Each symbol or bar and vertical line represents the mean ± SEM. Significant differences from the saline group ($^{*}P < 0.05$), from the ghrelin group ($^{\S}P < 0.05$), and from the group subjected to combined treatment with [D-Lys3]-GHRP-6 and saline ($^{\dagger}P < 0.05$) are indicated.

temperature (Fig. 1B). i.p. injection of des-acyl ghrelin at a concentration of 0.03 and 0.1 mg/kg body weight also decreased the back surface temperature and increased the caudal surface temperature acutely, although the effect was less marked than that of i.c.v. injection (Fig. 1C).

3.2. Influence of pretreatment with [D-Lys3]-GHRP-6 on the thermoregulatory action of centrally administered des-acyl ghrelin

It has been suggested that des-acyl ghrelin is unable to bind to the GHS-R1a receptor [4]. In addition, several reports have suggested the existence of a specific receptor for des-acyl ghrelin [22,23]. Therefore, we investigated whether GHS-R1a is involved in the thermoregulatory action of des-acyl ghrelin. First, we demonstrated that i.c.v. injection of ghrelin decreased the back surface temperature and increased the caudal surface temperature, as was the case for i.c.v. injection of des-acyl ghrelin, and that these effects were completely inhibited by pretreatment with the GHS-R1a antagonist [D-Lys3]-GHRP-6 (Fig. 2D). On the other hand, pretreatment with [D-Lys3]-GHRP-6 did not attenuate the decrease in the back surface temperature or the increase in the caudal surface temperature induced by centrally administered des-acyl ghrelin (Fig. 2D).

3.3. Influence of pretreatment with an autonomic nerve blocker on the thermoregulatory action of des-acyl ghrelin

It has been suggested that the autonomic nervous system is involved in the action of ghrelin on body temperature. Therefore we investigated the effect of pretreatment with the sympathetic nerve blocker timolol or the parasympathetic nerve blocker methylscopolamine on the thermoregulatory action of des-acyl ghrelin. Pretreatment with timolol did not attenuate the decrease in the back surface temperature and increased the caudal surface temperature elicited by centrally administered des-acyl ghrelin (Fig. 2A). On the other hand, pretreatment with methylscopolamine completely inhibited the effect of des-acyl ghrelin on the back surface temperature and the caudal surface temperature (Fig. 2B). Similar results were also obtained with peripheral administration of des-acyl ghrelin (Fig. 2C and D).

3.4. Des-acyl ghrelin induces cFos expression in the MnPO

Immunohistochemical analysis revealed induction of cFos expression in the MnPO after central and peripheral administration

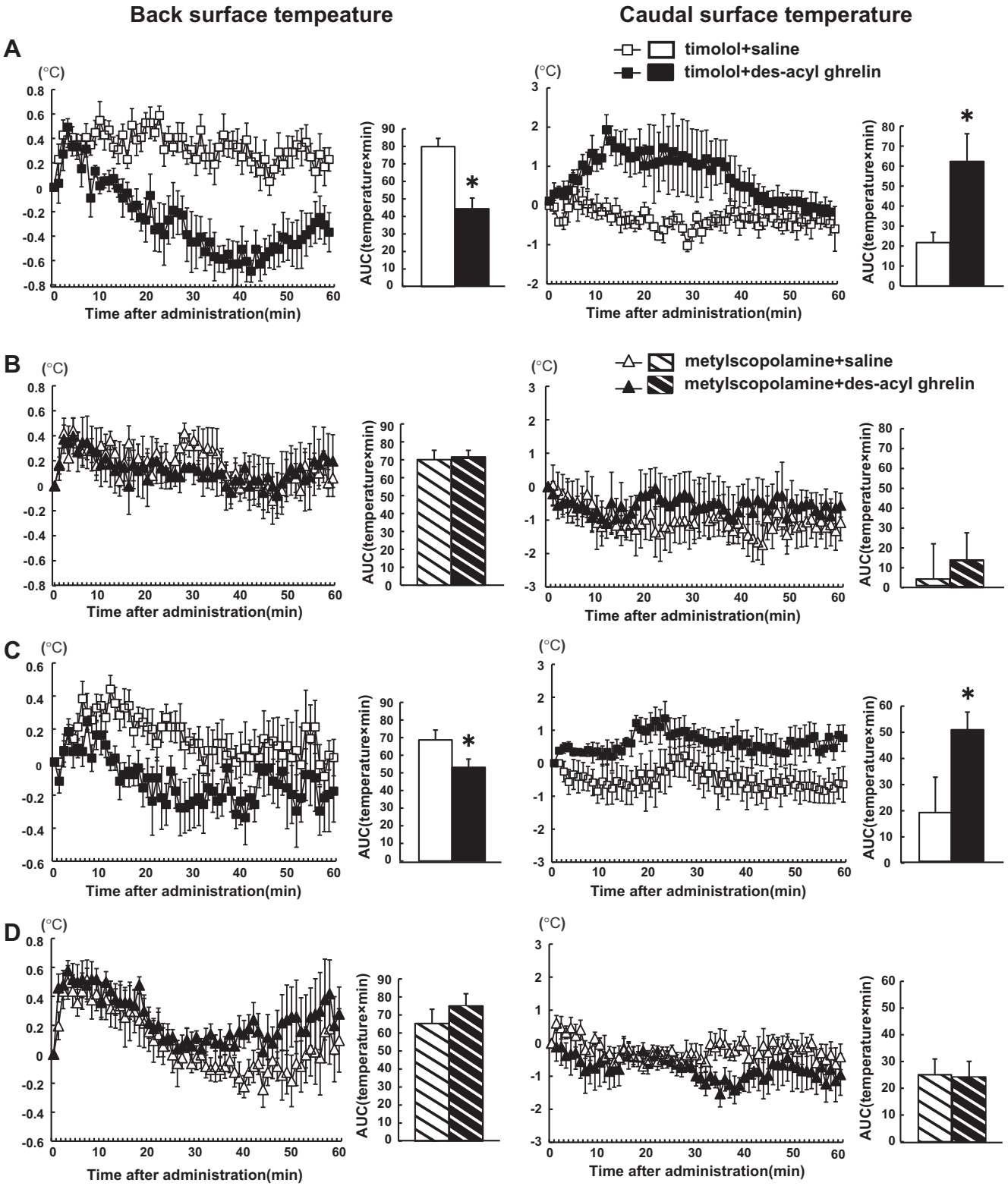

Fig. 2. (A) Changes in back surface temperature and caudal surface temperature from the baseline after i.c.v. injection of saline and des-acyl ghrelin with timolol pretreatment. (B) Changes in back surface temperature and caudal surface temperature from the baseline after i.c.v. injection of saline and des-acyl ghrelin with methylscopolamine pretreatment. (C) Changes in back surface temperature and caudal surface temperature from the baseline after i.p. injection of saline and des-acyl ghrelin with timolol pretreatment. (D) Changes in back surface temperature and caudal surface temperature from the baseline after i.p. injection of saline and des-acyl ghrelin with methylscopolamine pretreatment. AUC of the back surface temperature indicates cumulative change from the baseline during 60 min after injection of saline and des-acyl ghrelin. AUC of the caudal surface temperature indicates cumulative change from the baseline during 30 min after injection of saline and des-acyl ghrelin. Each symbol or bar and vertical line represents the mean ± SEM. Significant differences from the saline group ($^*P < 0.05$) are indicated.

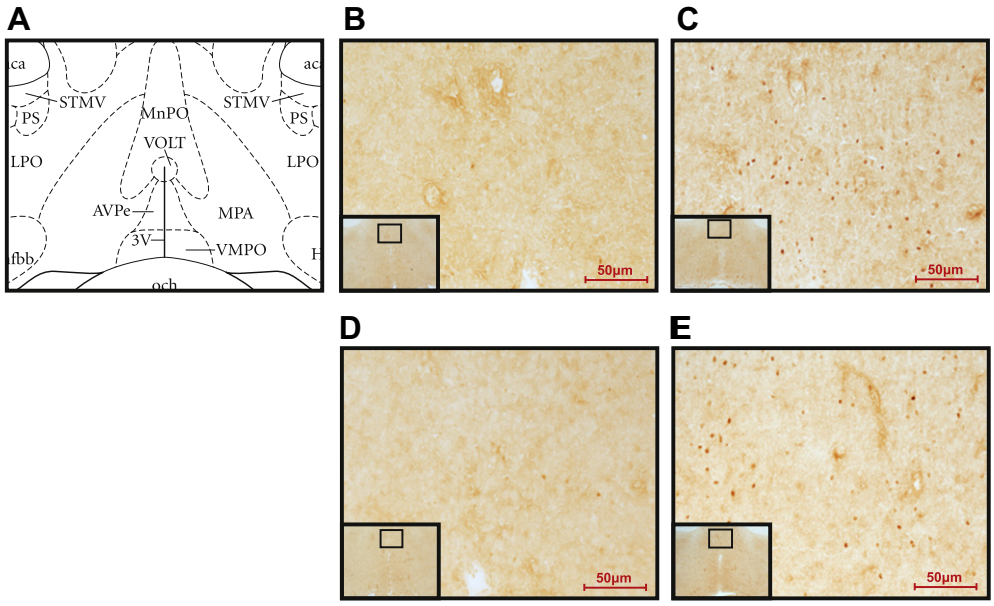

Fig. 3. Immunofluorescence staining of the MnPO for cFos expression after i.c.v. injection of saline (B) and des-acyl ghrelin (C). Immunofluorescence staining of the MnPO for cFos expression after i.p. injection of saline (D) and des-acyl ghrelin (E). (A) Schematic representation of the surrounding MnPO.

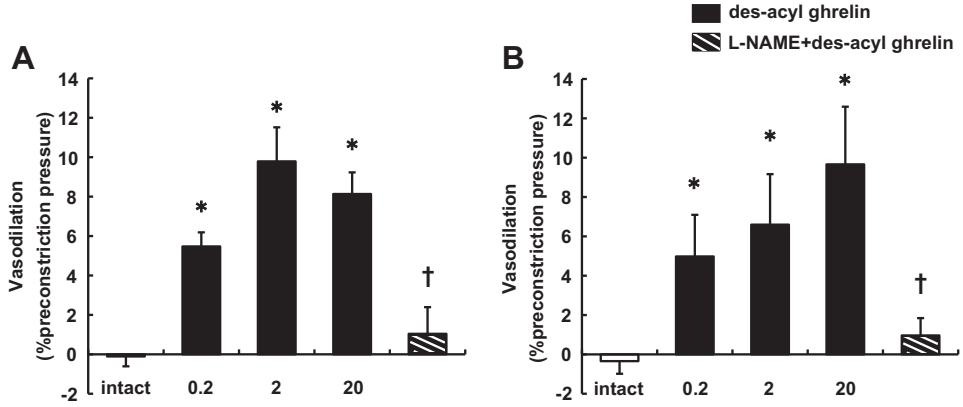

Fig. 4. (A) Vasodilative effect in an intact preparation ($n = 5$), and of des-acyl ghrelin in the presence (2 μM: $n = 7$) or absence (0.2 μM: $n = 5$, 2 μM: $n = 12$, 20 μM $n = 6$) of L-NAME in the tail artery. (B) Vasodilative effect in an intact preparation ($n = 5$), and of des-acyl ghrelin in the presence (2 μM: $n = 5$) or absence (0.2 μM: $n = 5$, 2 μM: $n = 5$, 20 μM $n = 7$) of L-NAME in the aorta. Vasodilation values are indicated as a percentage of the decrease in maximal tension in response to phenylephrine. Each bar represents the mean ± SEM. Significant differences from the intact group ($^{*}P < 0.05$), and from treatment with 2 μM des-acyl ghrelin ($^{\dagger}P < 0.05$), are indicated.

of des-acyl ghrelin, whereas no effect was evident in saline-treated animals (Fig. 3B–E).

3.5. Vasodilation of the tail artery and aorta by des-acyl ghrelin

We next examined whether des-acyl ghrelin directly induced vasodilation of the tail artery and aorta pre-contracted with phenylephrine. As shown in Fig. 4, des-acyl ghrelin at a concentration of 0.2, 2 and 20 μM caused vasodilation of the pre-contracted tail artery (Fig. 4A). To explore the mechanisms responsible for the vasodilative effects of des-acyl ghrelin on the tail artery, we examined the effects of pretreatment with the nitric oxide synthase (NOS) inhibitor, L-NAME. The vasodilation induced by des-acyl ghrelin (2 μM) was significantly inhibited by pretreatment with L-NAME (Fig. 4A). In addition, we carried out the same experiments using the aorta, and similar results were obtained (Fig. 4B).

4. Discussion

In the present study, we demonstrated that central administration of des-acyl ghrelin decreased the back surface temperature and increased the caudal surface temperature. Furthermore, we investigated the functional relationship between des-acyl ghrelin and GHS-R1a using the GHS-R1a antagonist [D-Lys3] GHRP-6. Ghrelin was found to decrease the surface temperature of the back and increased the surface temperature of the tail; however, pretreatment with [D-Lys3] GHRP-6 completely inhibited these actions of ghrelin on thermoregulation. On the other hand, the action of des-acyl ghrelin on thermoregulation was not inhibited by pretreatment with [D-Lys3] GHRP-6. These results suggested that ghrelin and des-acyl ghrelin exerted similar actions through different receptors. Further studies will need to focus on the biological significance of this difference.

Moreover, these thermoregulatory effects of des-acyl ghrelin were inhibited by pretreatment with the parasympathetic blocker methylscopolamine, but not by the sympathetic blocker timolol. In addition, we confirmed that cFos expression was induced in the MnPO by administration of des-acyl ghrelin. Previous research using rats has shown that the MnPO is a key structure involved in regulation of body temperature, receiving and integrating skin and core temperature information, and then sending efferent signals to thermoregulatory effector organs [24,25]. Additionally, it has been well demonstrated that the tail of the rat is a major thermoregulatory organ, and that its blood circulation is controlled by the central nervous system [26,27]. These facts suggest that des-acyl ghrelin acts on a receptor that is specific for it and independent of the GHS-R1a, and activates MnPO neurons and the parasympathetic nervous system, causing inhibition of thermogenesis and facilitating the dissipation of heat from tail vessels.

On the other hand, peripheral administration of des-acyl ghrelin decreased the surface temperature of the back and induced an increase in the surface temperature of the tail, these actions being apparently weaker than those resulting from central administration. Similarly to the case of central administration, the effects of peripherally administered des-acyl ghrelin on body temperature were inhibited only by pretreatment with methylscopolamine, and not with timolol. Moreover, cFos expression in the MnPO was induced by peripheral as well as by central administration. Because it has been reported that des-acyl ghrelin crosses the blood–brain barrier in a blood to brain direction [28], these result indicate that peripherally administered des-acyl ghrelin passes through the blood–brain barrier and exerts a central effect.

Furthermore, we examined the direct effects of des-acyl ghrelin on blood vessels, and found that it caused vasodilation of the tail artery and aorta via an endothelium/NO-dependent mechanism.

In conclusion, the present study has demonstrated that des-acyl ghrelin regulates body temperature through two pathways, one involving central activation of the hypothalamic thermoregulatory center and autonomic nerve activity, and the other involving direct control of cardiovascular tissues.

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