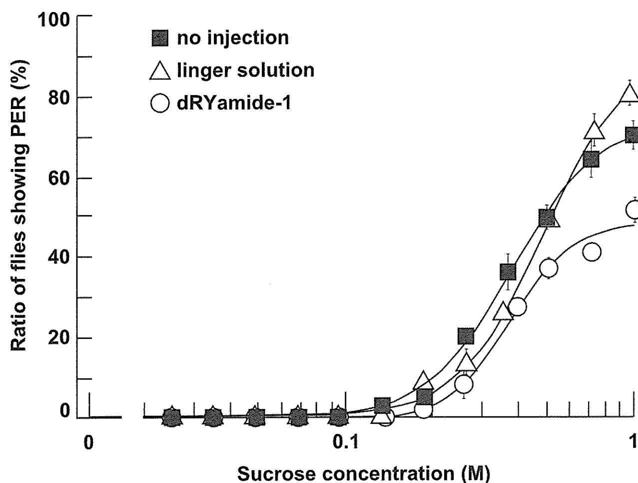

Fig. 4. Effect of dRYamide-1 on PER of the blowfly. Sigmoidal curves show the sucrose concentration–PER relationships in comparison among three fly groups; no injection (closed square); injection with linger solution (open triangle); injection of dRYamide-1 (open circle). Each symbol on the line graph represents the mean ± S.E.M. of data from four sets for each experiment.

electrophysiological response to sucrose in the sugar receptor neurons of the labellar contact chemosensilla [Hiraguchi et al., in preparation]. Probably, dRYamides modulate feeding behavior or appetite not at the peripheral but at the CNS level.

Drosophila neuropeptide F (dNPF) and short neuropeptide F (sNPF) have been indentified in numerous invertebrate species [7]. Both dNPF and sNPF are considered ancestrally related to the vertebrate family of neuropeptide Y (NPY). These peptides have a C-terminal RXRFamide motif. However, in vertebrates, the C-terminus RYamide is conserved for peptides of the NPY family (NPY, PYY, and PP). dRYamide-1 and dRYamide-2 have a C-terminal RYamide, whereas dNPF and sNPF have a C-terminal RFamide. In the pharmacological characterization using CHO cells expressing the receptor, NPY family peptides were potent at activating the recombinant CG5811 (dRYamide receptor). In addition, RFamide peptides affected CG5811. Moreover, NPFF induced robust increases in $[Ca^{2+}]_i$ in CHO-CG5811, and this effect was greater than that of NPY and other RFamide family peptides. It remains unclear why NPFF show higher potency for activating CG5811. The sixth phenylalanine from C-terminal may play a role in its efficacy. Vertebrate NPY and NPFF affected CG5811, whereas dRYamides did not affect the vertebrate NPY receptor (Y1–Y6) and the NPFF receptor (NPFF1R and NPFF2R) (data not shown). In the immunohistochemical study, dRYamides were suspected to be brain-gut peptides in insects. In vertebrates, a number of brain–gut peptides regulate feeding behavior, digestion absorption, metabolism, and growth [1–3]. NPY family peptides are typical brain–gut peptides. NPY is a prominent stimulator of appetitive behavior. In contrast, PYY and PP injection decrease food intake and body weight in rats and mice [19]. The discrepancy between NPY family peptides for feeding behavior can be understood by the difference in the subtype or distribution of the receptor [20]. Both peptides and receptor of NPFs share structural similarity with the vertebrate NPY and receptor family. In addition, NPFs are brain–gut peptides and stimulators of feeding behavior and growth in *Drosophila* [9–12]. Therefore, NPFs are considered homologous of the vertebrate NPY family peptides. Although it is still unclear whether dRYamides are homologs of vertebrate NPY family peptides, similar to NPFs, our results indicate that dRYamides are novel bioactive peptides that affect the receptor, similar to NPY receptor, and control feeding behavior. Further studies are required to elucidate the type of peptide family or physiological function of dRYamides, including the relation to NPFs.

Acknowledgments

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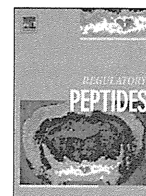

Sustained-release adrenomedullin ointment accelerates wound healing of pressure ulcers

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ABSTRACT

Pressure ulcers are one of the most common complications in elderly, incontinent or paralyzed patients. For the healing of pressure ulcers, the development of granulation tissue and reepithelialization is required. Adrenomedullin (AM), an endogenous vasodilator peptide, is reported to stimulate the proliferation and migration of various cells including endothelial cells, fibroblasts and keratinocytes. Therefore, we hypothesized that AM might accelerate the healing process of pressure ulcers in which these cells were involved. We developed a sustained-release ointment containing human recombinant AM, and applied it in a mouse model of pressure ulcer twice a day for 14 days. Human AM was efficiently absorbed in wound area, but its blood concentration was negligible. AM ointment significantly reduced the wound area on day 5 to 7 after injury. In addition, AM ointment accelerated the formation of granulation tissue and angiogenesis as well as lymphangiogenesis after 7 days of treatment. Immunological analysis revealed that Ki-67-positive proliferating cells in granulation tissue expressed AM receptors. In summary, sustained-release AM significantly improved wound healing of pressure ulcers through acceleration of granulation and induction of angiogenesis and lymphangiogenesis. Therefore, sustained-release AM ointment may be a novel therapeutic agent for pressure ulcers.

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1. Introduction

Pressure ulcers are one of the most common complications in elderly, incontinent or paralyzed patients, resulting from neurological disease, cardiovascular disease and surgical procedures, and susceptibility to pressure ulcers occurs due to unrelieved pressure, shear force or friction [1]. Treatment of pressure ulcers includes pressure reduction, cleaning and surgical intervention; however, long-term therapy is necessary for most patients [1,2]. In the healing process of pressure ulcers, the development of granulation tissue and reepithelialization are critical.

Adrenomedullin (AM) is an endogenous vasodilator peptide [3] that has been shown to have proliferative, migrative and anti-apoptotic effects on various cells including vascular endothelial cells [4,5], smooth muscle cells [6], fibroblasts [7], and keratinocytes [8]. Furthermore, AM is produced in these cells including endothelial cells [9], fibroblasts [10], and keratinocytes [8,11] in response to proinflammatory cytokines. These cells are reported to possess its receptor complexes, calcitonin receptor-like receptor (CRLR)/receptor activity-modifying protein (RAMP)-1,-2, and -3 [12,13], indicating that AM stimulates the proliferation of these cells in an autocrine and/or paracrine manner [8,14]. Considering that the healing process of pressure ulcers involves granulation tissue, with invasion of the wound space in association with proliferation and migration of endothelial cells and fibroblasts, AM may contribute to the healing process of pressure ulcers.

Here, we showed the therapeutic potential of AM for the treatment of pressure ulcers. However, one of the greatest disadvantages associated with the use of recombinant AM is its rapid clearance after systemic administration [15]. Therefore, we developed a sustained-release AM ointment to overcome this problem related to the administration of AM in a pressure ulcer model. Our long-lasting drug delivery system allowed AM to be locally applied to a wound in the skin.

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Thus, the purposes of this study were 1) to investigate whether AM has therapeutic potential for the treatment of pressure ulcers, and 2) to investigate the underlying mechanisms of AM in the process of wound healing.

2. Materials and methods

2.1. Preparation of AM

Recombinant human AM was obtained from Shionogi & Co., Ltd. AM was dissolved in saline with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore). The chemical nature and content of AM in vials were verified by high-performance liquid chromatography [3] and radioimmunoassay [16]. All vials were stored frozen at −80 °C from the time of dispensing until the time of preparation for administration.

2.2. Analysis of AM release from ointment in vitro

Rehydrated recombinant human AM (500 μg/ml) was mixed in three kinds of ointment base (at 40 μg/g); white petrolatum (Cosmescience), polyethylene glycol (1:1 mixture of Macrogol 400 and 4000, Nikko Pharmaceutical), and anionic hydrogel (Hiviswako, Wako Pure Chemical Industries). Each ointment (1 g) was placed in a tube, 10 ml distilled water was added over the ointment, and 0.5 ml of supernatant was collected 3, 6, 12 and 24 h later. The concentration of AM in the supernatant was measured with an ELISA kit (Phoenix Pharmaceuticals) according to the manufacturer's instructions.

2.3. AM concentration in wound tissue and plasma

Human and mouse AM concentrations in plasma and wound tissue were measured with a radioimmunoassay kit (Shionogi and Phoenix Pharmaceuticals, respectively), as reported previously [17]. Human and mouse AM radioimmunoassay kits have no cross-reactivity. Briefly, each tissue was boiled in water to inactivate intrinsic proteases. After cooling, acetic acid was added and the mixture was homogenized. The supernatant of the extract, obtained after centrifugation, was lyophilized. For assay, the lyophilized material was dissolved in radioimmunoassay buffer, and the clear solution was subjected to radioimmunoassay. Plasma samples were analyzed without modification. The radioactivity was measured by a gamma counter (ARC-1000M, Aloka). All assay procedures were performed in duplicate.

2.4. Pressure ulcer model

We used 5-week-old male ICR mice (Japan SLC). A pressure ulcer model was produced by repeated induction of ischemia/reperfusion of the skin. Briefly, we anesthetized mice with isofluran (Escain, Mylan Inc), and removed the hair on the back using Epirat depilatory cream (Kanebo), and compressed the skin with a circular punch (Fujiwara Sangyo) for 4 h. After 20 h of reperfusion, we compressed the skin again for 4 h, followed by 20 h of reperfusion. The necrotic tissue was cut off with scissors the next day. All protocols were performed in accordance with the guidelines of the Animal Care and Ethics Committee of the Japanese National Cardiovascular Center Research Institute.

2.5. Study protocol

We randomly allocated ICR mice to three groups: mice with a pressure ulcer (without ointment group; n = 15), mice with a pressure ulcer to which hydrogel alone (50 mg, ointment only group; n = 15) was applied, and mice with a pressure ulcer treated with hydrogel containing AM (2 μg/50 mg, AM ointment group, n = 15). The wound area in each group was covered with a transparent dressing (Tegaderm, 3 M) immediately after application of the ointment. We applied the ointment twice a day and measured the wound area every day for 14 days [18].

2.6. Histological analysis

The wound tissue (n = 6 in each group) was excised on day 7, fixed in 4% formalin, embedded in paraffin, and processed for histological and immunohistological analysis. Tissue sections were stained with hematoxylin and eosin (H–E). To detect proliferative cells, vascular endothelial cells, and lymphatic endothelial cells, we performed immunohistochemical staining of Ki-67 (Dako Cytomation), von Willebrand factor (vWF, Chemicon), and LYVE-1 (R&D Systems).We also conducted CRLR (V-20, Santa Cruz Biotechnology) immunostaining of tissue sections. The images were obtained blindly using a computer-navigated microscope (BIOREVO, KEYENCE). The independent observer chose ten randomly selected areas within wound granulation tissue and the average number of cells positive for Ki-67, vWF, or LYVE-1 were calculated (magnifications: Ki-67: ×400, vWF and LYVE-1: ×200).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA were isolated from wound granulation tissue using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was reverse-transcribed into cDNA using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. PCR was carried out as follows: an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C. The specific primer pairs were: CRLR, 5′-TGTAATAACAGCACGCATGAG-3′ and 5′-GTTATTGGCCACTGCCGTGA-3′; RAMP-1, 5′-CACCATCTCTTCATGGTCACTG-3′ and 5′-CAATCGTGTGCGCCACGTGC-3′; RAMP-2, 5′-TGGATCTCGGCTTGGTGTGAC-3′ and 5′-GCAAGGTAGGACATGTGTTTCG-3′; RAMP-3, 5′-TTGTGGTGAGTGTGCCCAGG-3′ and 5′-CCCATGATGTTGGTCTCCATC-3′ [19]. A set of GAPDH primers was used as an internal control.

2.8. Statistical analysis

Numerical values are expressed as mean ± S.E.M. Continuous variables were determined in four groups in this study. Therefore, for multiple comparisons of more than two groups, we performed one-way analysis of variance (ANOVA). If the result of ANOVA was significant, we used Newman–Keuls' procedure as a post hoc test. For repeated measurements such as chronological analysis, we performed two-way repeated ANOVA with Newman–Keuls' test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Controlled release of AM from ointment

We prepared three types of ointment base: anionic hydrogel, polyethylene glycol and white petrolatum, to determine the optimal base for the development of a sustained-release preparation of AM. AM in anionic hydrogel was gradually released over 24 h, whereas AM in polyethylene glycol was released rapidly, and AM in white petrolatum was hardly released (Fig. 1a). Thus, anionic hydrogel was considered a promising drug delivery base to examine the therapeutic effect of AM. To observe the absorption of AM in wound tissue and into the blood, we measured the concentration of human AM in wound tissue and plasma after local administration of human AM ointment on pressure ulcers in mice. The concentration of human AM in wound tissue was markedly elevated in AM ointment group (512.2 ± 66.7 fmol/g) (Fig. 1c). Although we could detect human AM

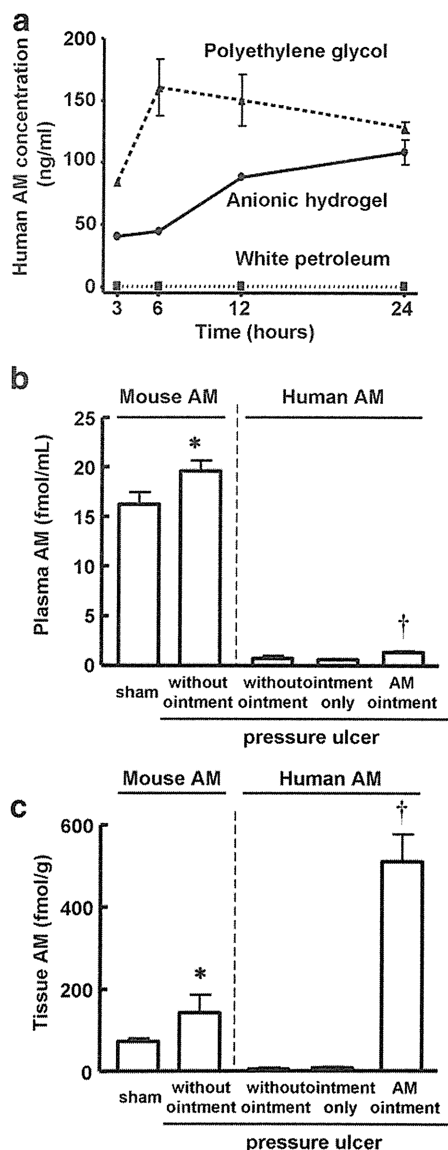

Fig. 1. AM release and absorption from ointment in vitro and in vivo. (a). Comparison of AM release from polyethylene glycol, white petroleum, and anionic hydrogel in vitro. AM in anionic hydrogel was gradually released over 24 h, while AM in polyethylene glycol was released rapidly, and AM in white petrolatum was hardly released. (b) Plasma concentration of mouse and human AM 1 h after treatment with AM ointment (anionic hydrogel). (c) The concentration of mouse and human AM in wound tissue. Mice received no surgical procedure were denoted as sham. The concentration of human AM in wound tissue was significantly elevated in the AM ointment group compared with without ointment and ointment only groups, but its concentration in plasma was negligible. N = 5 in each group.

immunoreactivity in plasma of AM ointment group, the concentration of human AM was significantly low (1.3 ± 0.12 fmol/ml of plasma) compared to that of mouse AM (19.6 ± 1.0 fmol/ml of plasma) (Fig. 1b). In addition, no significant change in blood pressure or heart rate was observed by human AM treatment (data not shown).

3.2. Effect of AM-containing ointment on pressure ulcer

To examine the therapeutic effect of AM ointment on the healing process of pressure ulcers, we applied AM ointment (2 μg AM in 50 mg hydrogel) twice a day, and measured the wound area for two weeks. AM significantly accelerated wound healing on days 5 to 7 after injury, compared to that in without ointment and ointment only groups (Fig. 2a,b). H–E staining of wound tissue on day 7 showed thicker granulation tissue in AM ointment group compared to that in without ointment and ointment only groups, and the newly formed granulation tissue in AM ointment group contained a number of blood vessels compared to those in without ointment and ointment only groups (Fig. 2c, 3c,d).

3.3. Effect of AM-containing ointment on cell proliferation, angiogenesis and lymphangiogenesis

Immunohistochemical analysis of the granulation tissue on day 7 demonstrated that AM ointment significantly increased the number of Ki-67-positive proliferating cells (AM ointment group: 541 ± 46.4/mm^2, p < 0.001 vs. without ointment group: 257.8 ± 24.5/mm^2 and ointment only group: 262.4 ± 24.2/mm^2) (Fig. 3a, b). AM also increased the number of von Willebrand factor (vWF)-positive vessels (AM ointment: 197.4 ± 10.8/mm^2, p < 0.01 vs without ointment: 150.2 ± 3.3/mm^2 and ointment only: 142.2 ± 10.4/mm^2) (Fig. 3c,d), and LYVE-1-positive lymphatic vessels (AM ointment: 38.4 ± 3.8/mm^2, p < 0.001 vs without ointment: 9.6 ± 5.6/mm^2 and ointment only: 5.4 ± 3.0/mm^2) (Fig. 3e,f).

3.4. Expression of CRLR, RAMP-1, -2 and -3 in wound tissue

Immunofluorescent analysis of granulation tissue on day 7 demonstrated that Ki-67-positive cells also expressed CRLR (Fig. 4a). To examine the expression of functional AM receptors in granulation tissue, we performed RT-PCR for CRLR, RAMP-1, -2, and -3 in 3 samples from each group. RT-PCR revealed that these AM receptors were expressed in all granulation tissues (Fig. 4b). RAMP-1 and -3 mRNA expression in granulation tissue was increased by the treatment with AM ointment (Fig. 4b).

4. Discussion

In this study, we showed that 1) anionic hydrogel is a promising ointment base for sustained release of AM, 2) AM containing ointment accelerated wound healing in a pressure ulcer model, and 3) AM administration induced angiogenesis and lymphangiogenesis in wound tissue.

AM is an endogenous vasodilator peptide, and continuous infusion is required because it has an extremely short duration of action [20] but continuous administration of AM may cause hypotension. In the present study, anionic hydrogel could slowly release AM, and AM absorbed in the wound area did not cause any change in blood pressure or heart rate. The hydrogel used in this study is a high-molecular-weight carboxyvinylic derivative, which is extensively used in the manufacture of pharmaceutical gels, and is highly suited for use in controlled-release systems for not only chemicals but also peptides including insulin [21].

The wound healing process is a complex cascade that relies on several mechanisms including a hypoxic phase, inflammatory phase, tissue formation phase, and remodeling phase [22,23], each of which involve distinct cell types [24]. In the tissue formation phase, angiogenesis, granulation, and reepithelialization occur, and endothelial cells [25], fibroblasts [7,8], and keratinocytes [8] are mainly involved [22,23]. It has been demonstrated that AM stimulates proliferation of all these cells and enhances their DNA synthesis and proliferative activity via its receptors [13,26–28]. AM ointment increased granulation tissue formation and accelerated skin wound healing from day 5 to day 7 after injury, suggesting the acceleration of granulation and reepithelialization. In addition, angiogenesis, which is necessary to sustain the newly formed granulation tissue, was induced by AM on day 7 after injury. We also confirmed that Ki-67-positve proliferating cells in would tissue expressed high level of AM receptors. Therefore, sustained release of AM would enhance the proliferation of endothelial cells, fibroblasts, and keratinocytes in wound tissue, leading to acceleration of wound healing in the tissue formation phase [8,14].

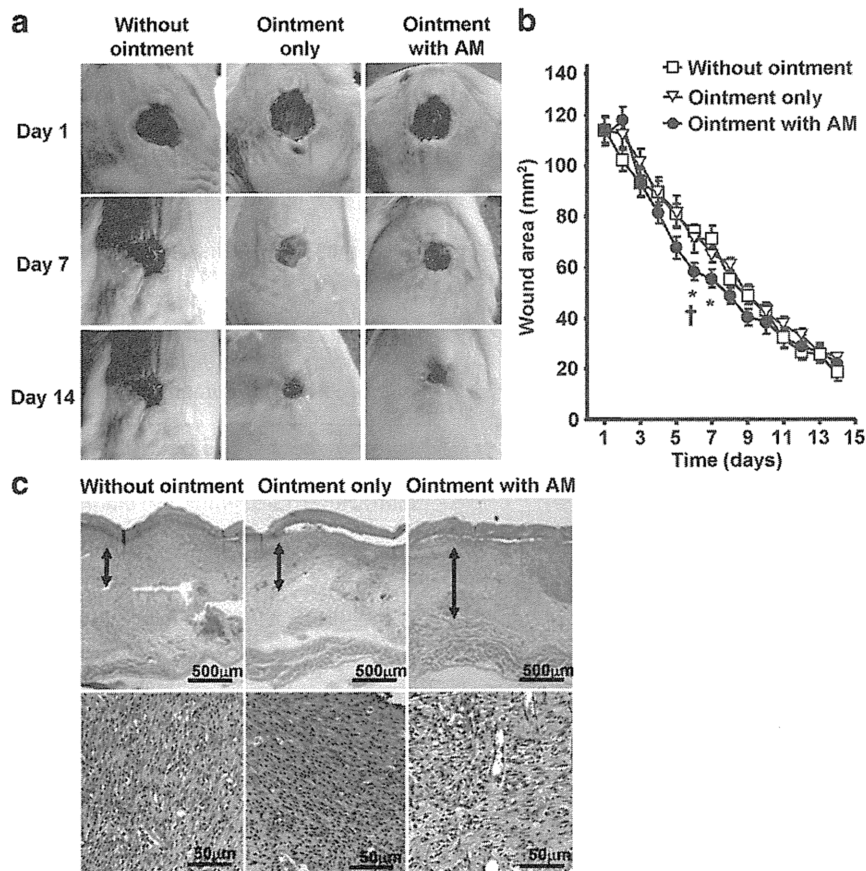

Fig. 2. Effect of AM ointment on pressure ulcer. (a) Gross appearance of wounds at indicated time points. (b) Time course of wound area. AM significantly accelerated wound healing in the early phase (days 5 to 7), compared to that in the without ointment and ointment only groups. N = 15 in each group. (c) Photomicrographs of granulation tissue of wound area stained with hematoxylin and eosin on day 7. Thicker granulation tissue was observed in the AM ointment group compared with that in the without ointment and ointment only groups, and the newly formed granulation tissue in the AM ointment group contained a number of large blood vessels. N = 6 in each group. Scale bars in upper panel of c, 500 μm; lower panel of c, 50 μm. *$p<0.05$ versus without ointment. †$p<0.05$ versus ointment only.

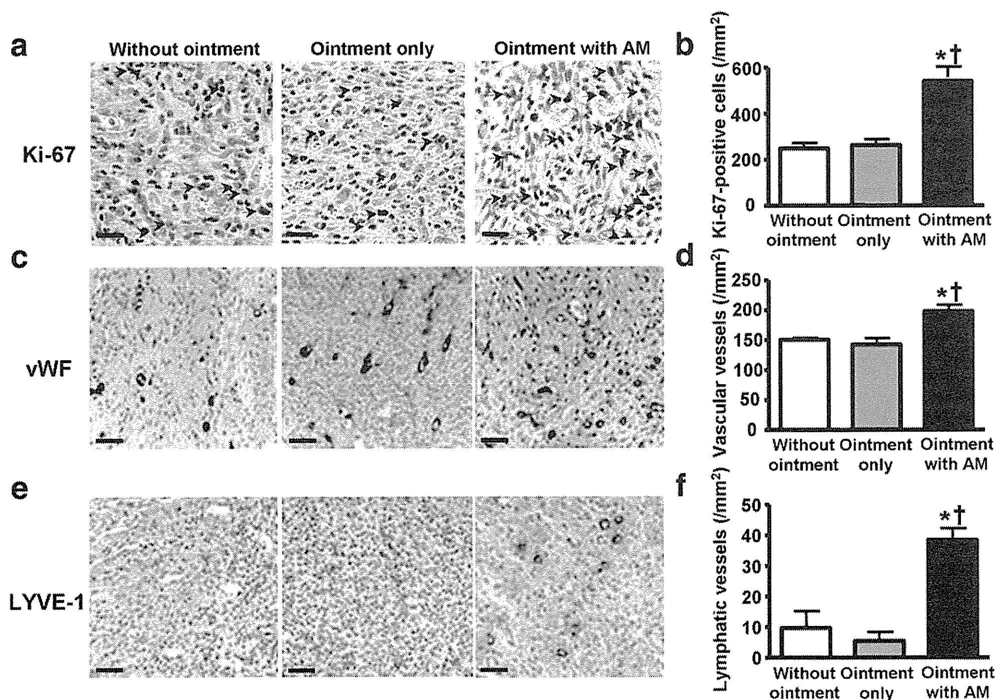

Fig. 3. Effects of AM ointment on cell proliferation, angiogenesis and lymphangiogenesis. (a,c,e) Representative microphotographs of wound tissue stained for Ki-67 (a), von Willebrand factor (vWF, c), and LYVE-1 (e) on day 7. (b,d,f) Semi-quantitative analysis of Ki-67 (b), vWF (d) and LYVE-1 (f)-positive cells. AM ointment significantly accelerated cell proliferation, angiogenesis, and lymphangiogenesis in wound tissue compared with that in the without ointment and ointment only groups. N = 6 in each group. Scale bars in a, 100 μm; in c and e, 50 μm. *$p<0.05$ versus without ointment. †$p<0.05$ versus ointment only.

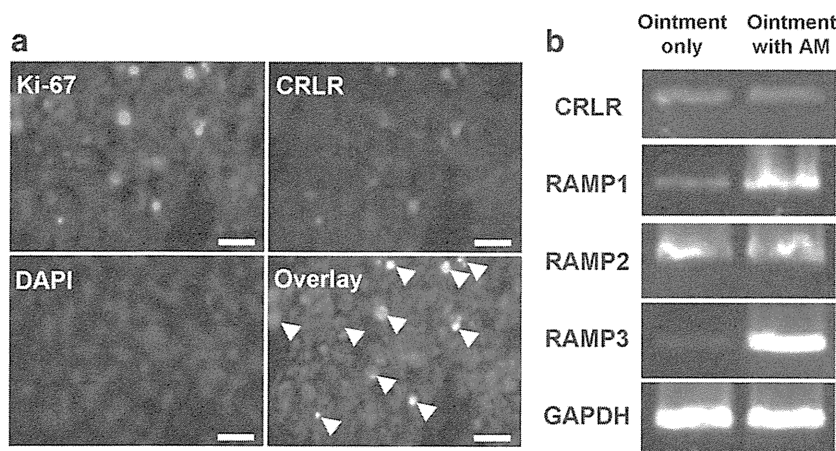

Fig. 4. Expression of CRLR and RAMPs in granulation tissue. (a) Immunostaining of CRLR and Ki-67 in sections was performed 7 days after induction of pressure ulcer. Arrow heads indicate double-stained cells. Proliferating Ki-67-positive cells in granulation tissue were also positive for CRLR. Ki-67: green, CRLR: red, DAPI: blue. Scale bar equals 20 μm. (b) RT-PCR revealed AM receptors including CRLR, RAMP-1, -2, and -3 were expressed in granulation tissue on day 7. The data shown are representative of three experiments. GAPDH was served as internal control.

Previous reports have shown that AM are essential for angiogenesis and vascular integrity [29,30]. Recently, we reported that AM is a major effector of lymphangiogenesis [31]. We demonstrated that AM accelerated proliferation, migration, and network formation of cultured lymphatic endothelial cells, and accelerated lymphangiogenesis in a mouse model of lymphedema [31]. Fritz-Six KL et al. also demonstrated that AM signaling is important for the development of lymphatic vasculature [32]. Because lymphangiogenesis as well as angiogenesis are crucial in the wound-healing process [33], we considered that administration of AM induced both angiogenesis and lymphangiogenesis, resulting in acceleration of wound healing in a mouse model of pressure ulcer. It is well known that blood and lymphatic vessels contribute to transportation of gases, liquids, nutrients, signaling molecules and circulating cells between tissues and organs [34]. Therefore, AM would contribute to acceleration of the healing process of pressure ulcers by not only inducing the formation of granulation but also improving local circulation through angiogenesis and lymphangiogenesis.

In summary, sustained-release AM accelerates wound healing of pressure ulcers through accelerating granulation and induction of angiogenesis and lymphangiogenesis. Therefore, sustained-release AM ointment may be a novel therapeutic agent for pressure ulcers.

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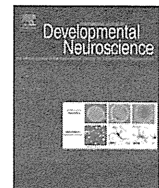

Ghrelin levels are reduced in Rett syndrome patients with eating difficulties

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ABSTRACT

Most patients with Rett syndrome (RTT) have both gastrointestinal problems and somatic growth failure, including microcephaly. Ghrelin is a peptide hormone involved in growth hormone secretion, interdigestive motility, and feeding behavior. Plasma ghrelin assays have previously been described for other neurodevelopmental disorders. To examine the pathophysiology of RTT, we measured plasma levels of ghrelin in patients with RTT. A case–control study examining plasma levels of ghrelin, serum growth hormone, and insulin-like growth factor-1 (IGF-1) was performed on 27 patients with RTT and 53 controls. Plasma levels of total (T)- and octanoyl (O)-ghrelin were significantly lower in patients with RTT than in controls. Plasma levels of T-ghrelin correlated significantly with serum IGF-1 levels and head circumference. Significantly lower levels of plasma T-ghrelin and O-ghrelin were observed in RTT patients with eating difficulties, while lower levels of plasma T-ghrelin were observed in RTT patients with constipation, in comparison to patients without either of these symptoms. Alterations in plasma ghrelin levels may reflect various clinical symptoms and signs in RTT patients, including growth failure, acquired microcephalus, autonomic nerve dysfunction, and feeding difficulties. We describe the role of ghrelin in RTT and suggest this peptide as a novel biological marker in patients with RTT.

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1. Introduction

Rett syndrome (RTT, MIM 312750) is an X-linked neurodevelopmental disorder, caused in the vast majority of cases by mutations in *Methyl-CpG-binding protein 2* (*MeCP2*) (Amir et al., 1999). RTT is characterized by deceleration of head growth, mental retardation, motor disabilities, autistic behavior, epilepsy, periodic breathing, coldness of extremities, eating difficulties, constipation, emotional disturbances, and sleep disruption, followed by somatic growth failure (Oddy et al., 2007; Percy et al., 2010; Schultz et al., 1993). Ghrelin is an acylated peptide hormone produced mainly in gastrointestinal (GI) tissues (Kojima et al., 1999). Ghrelin exerts multiple physiological functions including the stimulation of growth hormone (GH) secretion, the modulation of energy metabolism and circulation, and the regulation of autonomic functions and seizure threshold (Kojima and Kangawa, 2005). The exogenous administration of ghrelin stimulates GH secretion and increases caloric intake and GI motility. Symptoms and signs of RTT, such as growth failure, GI problems, autonomic dysfunction, and neuropsychiatric symptoms, seem to be explained by the disturbance in ghrelin secretion. Therefore, we have speculated that ghrelin plays an important role in patients with RTT. However, plasma ghrelin has not been studied previously in RTT. We compared the plasma levels of ghrelin in RTT patients and healthy controls and assessed the relationship between plasma ghrelin, growth factors, and clinical symptoms and signs.

2. Methods

Clinical diagnosis of RTT was confirmed in 27 female patients according to the recently proposed Rett Syndrome Diagnostic Criteria (Percy et al., 2010). We

Abbreviations: RTT, Rett syndrome; GH, growth hormone; IGF-1, insulin-like growth factor-1; T-ghrelin, total-ghrelin; O-ghrelin, octanoyl-ghrelin; MeCP2, methyl-CpG-binding protein 2; GI, gastrointestinal; DQ, developmental quotient; IQ, intelligence quotient; BMI, body mass index; OFc, occipito-frontal head circumference; EDTA-2Na, disodium dihydrogen ethylenediamine tetraacetate dihydrate; RIA, radioimmunoassay; IRMA, immunoradiometric assay; SD, standard deviations; CNS, central nervous system; SNpc, substantia nigra pars compacta.

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Table 1
Clinical characteristics of patients with Rett syndrome.

Characteristics	Controls					RTT					p
				Correlations					Correlations		
				T-ghrelin	O-ghrelin				T-ghrelin	O-ghrelin	
Age (years)	16.9	(±10.2)	[N = 53]	0.30*	0.34*	15.8	(±8.3)	[N = 27]	−0.40*	−0.36	0.60
Weight (kg)	39.8	(±16.1)	[N = 53]	000	0.05	27.9	(±11.7)	[N = 27]	−0.59**	−0.48*	000**
BMI (kg/m^2)	18.7	(±3.3)	[N = 53]	−0.13	−0.16	15.4	(±3.5)	[N = 27]	−0.55**	−0.31	000**
BMI-Z	−0.1	(±1.0)	[N = 53]	−0.59**	−0.58**	−2.2	(±2.2)	[N = 27]	−0.21	0.07	0.00*
Height/length (cm)	141.8	(±23.6)	[N = 53]	−0.01	0.11	131.8	(±19.3)	[N = 27]	−0.49**	−0.57**	0.06
Height/length-Z	−0.3	(±0.9)	[N = 53]	−0.36**	−0.22	−2.7	(±0.9)	[N = 27]	0.00	0.06	0.00**
OFC (cm)	52.1	(±3.1)	[N = 27]	−0.74**	−0.56**	50.4	(±2.5)	[N = 27]	−0.66**	−0.49**	0.03*
OFC-Z	1.9	(±1.5)	[N = 27]	−0.61**	−0.53**	0.3	(±1.8)	[N = 27]	−0.66**	−0.48*	0.00**
Total-ghrelin (fmol/ml)	215.5	(±115.0)	[N = 53]	–	0.81**	113.5	(±51.9)	[N = 27]	–	0.67*	0.00*
Octanoyl-ghrelin (fmol/ml)	26.8	(±14.1)	[N = 53]	0.81**	–	16.9	(±8.3)	[N = 27]	0.67**	–	0.00**
O/T ratio	0.13	(±0.05)	[N = 53]	−0.33*	0.20	0.16	(±0.1)	[N = 27]	−0.39*	0.34	0.06

All of the data represent means ± standard deviations. BMI-*Z*, BMI-for-age *Z* score; Height/length-*Z*, height/length-for-age *Z* score; OFC, occipito-frontal head circumference; OFC-Z, OFC-for-age *Z* score; T-ghrelin, total ghrelin; O-ghrelin, octanyl ghrelin. O-ghrelin was significantly lower in patients with RTT than in controls (**$p \leq 0.01$). Weight, BMI, height/length ratio, and OFC-*Z* in RTT was significantly lower in comparison to controls (**$p \leq 0.01$). Age, weight, and OFC-*Z* demonstrated significant inverse correlations with the plasma levels of T-, and O-ghrelin in both RTT patients and controls.

* $p \leq 0.05$.

** $p \leq 0.01$.

measured plasma levels of ghrelin in 27 patients with RTT and 53 age- and gender-matched healthy controls. A genetic analysis of *MeCP2* was performed in all patients with RTT. Of the 27 RTT patients, 9 were in stage III and 18 were in stage IV. All of the RTT patients had a developmental quotient (DQ) or intelligence quotient (IQ) below 20. None of the RTT patients had undergone gastrostomy or had received medications targeting the autonomic nervous system, which might have influenced the plasma ghrelin levels. Eating difficulties were defined as present if dietary intake required more than 30 min, on average, during each mealtime in the 2 weeks prior to this study, as recorded by both caretakers and occupational therapists (Oddy et al., 2007). The clinical data that we collected on RTT patients included weight, height, body mass index (BMI), and occipito-frontal head circumference (OFC). These data were converted into the standard deviation *Z* score established by the US National Center for Health Statistics/World Health Organization (de Oris et al., 2006). Written informed consent was obtained from each patient's or control's parents. The study protocol was approved by the Ethical Committee of the Kurume University School of Medicine.

2.1. Extraction and derivation

The extraction of plasma ghrelin from blood was performed by a method described previously (Hosoda et al., 2000). In brief, samples were obtained by venipuncture between 0800 and 1000 after an overnight fast. Blood samples were put into chilled polypropylene tubes containing EDTA-2Na and aprotinin (700 kIU/mL), and immediately centrifuged to obtain plasma samples. The separated plasma samples were acidified by the addition of 1.0 N HCl (10% of sample volume), and then stored at −80 °C. The plasma samples were semi-purified before the ghrelin radioimmunoassay (RIA) using Sep-Pak C18 cartridges. Two ghrelin-specific RIAs were used; one recognizes the N-terminal portion of octanoyl-modified active ghrelin (O-ghrelin), while the other recognizes the C-terminal portion of ghrelin irrespective of its octanoyl-modification. The plasma levels of ghrelin were determined by a validated in-house RIA as previously described (Hosoda et al., 2000). O-ghrelin is post-transationally octanoylated at Ser3 (Hosoda et al., 2000; Kojima et al., 1999). Plasma levels of total ghrelin (T-ghrelin) were estimated as the sum of non-octanoyl and octanoyl ghrelin levels. Both antibodies used in our ghrelin-specific RIAs exhibited complete cross-reactivity with human n-octanoyl ghrelin (Hosoda et al., 2000). The intra- and inter-assay coefficients of variations were all less than 7.0%. The detection limits of plasma T- and O-ghrelin were 40 and 5 fmol/mL, respectively. All assays were performed in duplicate.

2.2. Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) assays

Serum concentrations of GH and IGF-1 were measured in duplicate by immunoradiometric assays according to the manufacturer's protocol (Active Growth Hormone IRMA DSL-1900 and Active Non-Extraction IGF-1 IRMA DSL-2800, respectively, Diagnostics System Laboratories, Webster, TX, USA) or by radioimmunoassay kit (SRL Inc., Tokyo, Japan). Each assay was calibrated with manufacturer-supplied standards.

2.3. Statistical methods

The concentrations of plasma ghrelin and serum GH and IGF-1 were compared both between and within groups by *t*-tests, and Pearson's correlation coefficients were used to measure monotonic associations between variables. Data are summarized as means ± standard deviations (SD). For statistical analysis, we used analysis of variance. The reference curves for plasma ghrelin concentrations were constructed by additive models incorporating the interaction terms of age and age × disease group as the nonlinear parameters. The approximate significance of smooth terms was obtained by *F*-tests. If a difference deviated by more than 2 SDs from the mean, the data were excluded as outliers. $p \leq 0.05$ was considered significant.

3. Results

3.1. Comparisons between Rett syndrome patients and controls

Plasma levels of T-ghrelin were significantly lower in patients with RTT (113.5 ± 51.9) than in controls (215.5 ± 115.0), at 52.7% of the control values ($p \leq 0.01$). Plasma levels of O-ghrelin were significantly lower (16.9 ± 8.3) than in controls (26.8 ± 14.1), at 63.1% of the control values ($p \leq 0.01$).

3.2. The clinical background variables of Rett syndrome patients and the concentrations of plasma ghrelin

Neither T- nor O-ghrelin levels varied significantly with type of *MeCP2* mutations. Weight, BMI, height, and OFC-Z in RTT patients were significantly lower than in controls ($p \leq 0.01$). Age, weight, and OFC-*Z* showed significant inverse correlations with plasma levels of T- and O-ghrelin in both RTT patients and controls (Table 1). Moreover OFC-*Z* showed significantly positive correlations with serum levels of IGF-1. Plasma levels of T-ghrelin correlated significantly with IGF-1 levels (179.4 ± 107.7) in patients with RTT ($p \leq 0.05$). In controls, plasma levels of T- and O-ghrelin tended to decrease until around 10 years of age, then increased during adolescence until they plateaued at around 25 years of age; these patterns were different in patients with RTT (Fig. 1A and B). RTT patients suffered from the following symptoms: periodic breathing (17/27), sleeping disruption (18/27), cold hands/feet (22/27), epilepsy (23/27), eating difficulties (7/27), and constipation (14/27). Plasma levels of T-ghrelin were significantly lower in RTT patients with eating difficulties and constipation than in patients without each symptom ($p \leq 0.01$ and $p \leq 0.05$, respectively). Plasma levels of O-ghrelin were also significantly lower in RTT patients with eating difficulties than in those without ($p \leq 0.05$) (Table 2).

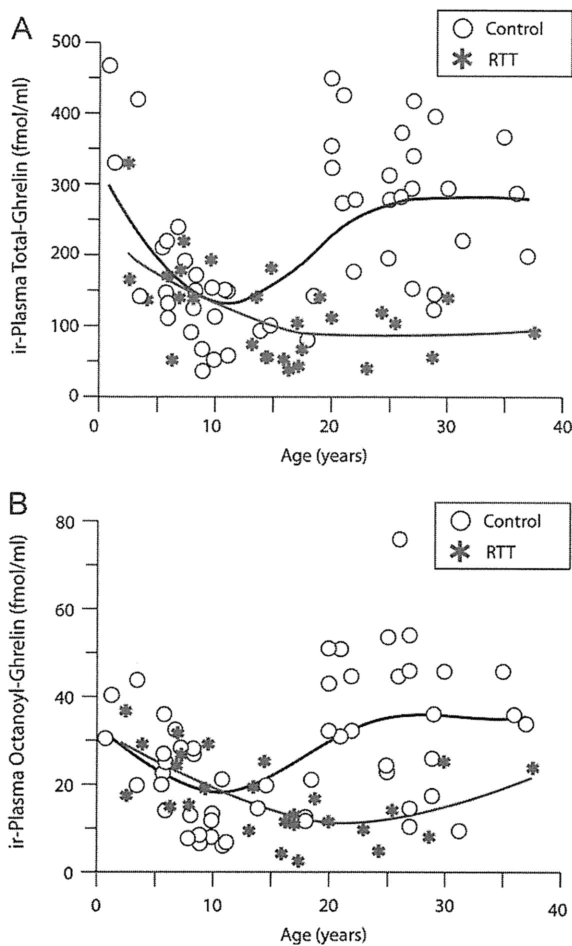

Fig. 1. Reference curves for plasma ghrelin levels were constructed using generalized additive models. The following additive models were fitted: plasma ghrelin concentrations = *s*(age) + disease group + *s*(age × disease group) where *s*( ) is a smoothing function. The test for the age × disease group interaction for concentrations of both plasma T-ghrelin (A) and O-ghrelin (B) was statistically significant with *p* values of 0.004 and 0.01, respectively: T-ghrelin = total ghrelin; O-ghrelin = octanoyl ghrelin.

4. Discussion

4.1. Age and ghrelin

In general, plasma ghrelin levels are high during infancy, then decrease until puberty, at which time they increase in an age-dependent manner (Chanoine, 2005). In this study, patients with RTT showed a different pattern of ghrelin level variability compared to both the previous report (Chanoine, 2005) and to our controls. Our results showed that the ghrelin levels in RTT continued to decrease after 10 years of age (Fig. 1A and B).

4.2. Ghrelin and signs and symptoms of Rett syndrome

Plasma ghrelin inversely correlated with weight, BMI, and height in controls, whereas these relationships were not observed in RTT (Table 1). Mice with Mecp2-null mutations are significantly smaller than wild-type mice (Guy et al., 2001), and numerous genes in the hypothalamus were found to be deregulated in both Mecp2-null and MECP2-Tg male mice (Ben-Shachar et al., 2009). Interestingly, ghrelin-producing cells exist even in the internuclear space of the hypothalamus, and ghrelin exerts an orexigenic effect via a feeding-related neuropeptide (Ferrini et al., 2009). Ghrelin is also known to promote proliferation of nerve cells and neural stem cells, and it maintains normal plasma IGF-1 levels via GHS-R, thus promoting physical growth (Sun et al., 2004). Previous reports have pointed out an association between autism and plasma and cerebrospinal fluid IGF-1 and OFC (Huppke et al., 2001; Riikonen, 2008). Interestingly, OFC inversely correlated with plasma ghrelin concentrations. This implies that ghrelin may be involved in neurogenesis or neuroprotective effects, either directly or via induction of IGF-1. On the other hand, about two-thirds of circulating ghrelin is derived from the stomach (Kojima and Kangawa, 2005). Although plasma levels of T- and O-ghrelin were lower in RTT than in controls, the ratio of O-ghrelin to T-ghrelin did not differ significantly between the two groups. This suggests that ghrelin production and substrate modification was properly performed in the stomach. The pathophysiology of RTT is still unknown. We demonstrated that plasma ghrelin levels were markedly decreased in patients with RTT. Moreover, they were lower in RTT patients with eating difficulties and constipation. A recent report showed that acetylcholine regulates ghrelin secretion as an interactive modulator of the autonomic nerve system (Hosoda and Kangawa, 2008). These results may reflect dysfunction in the integration of the central nervous system (CNS) and autonomic and peripheral nervous systems in RTT. Therefore, ghrelin may be the biological marker of CNS, autonomic nervous system dysfunction, and growth failure in RTT. Previous neuropathological studies have demonstrated decreased melanin content of the zona compacta nigra and a reduction in the number of basal forebrain cholinergic neurons in the CNS (Armstrong, 1992). Patients with RTT also were reported to have Parkinson-like symptoms, and Mecp2-null mice (Mecp2$^{null/y}$) showed dysfunction of the dopaminergic system (Samaco et al., 2009). Jiang et al. (2008) reported that ghrelin's receptor, GHSR-1a, is highly expressed in the substantia nigra pars compacta (SNpc), and ghrelin inhibited MPTP-induced dopaminergic neuronal loss in the SNpc in a mouse model of Parkinson's disease.

Table 2
Plasma levels of T- and O-ghrelin in RTT patients by symptom.

Symptoms	Total ghrelin						*p*	Octanoyl ghrelin						*p*
	+			–				+			–			
Periodic breathing	104.9	(±57.6)	[N = 17]	128.2	(±39.0)	[N = 10]	0.27	17.7	(±8.9)	[N = 17]	15.6	(±7.3)	[N = 10]	0.54
Sleep disruption	111.8	(±57.7)	[N = 18]	116.9	(±40.9)	[N = 9]	0.82	17.6	(±8.6)	[N = 18]	15.6	(±7.8)	[N = 9]	0.55
Cold hands/feet	116.6	(±51.1)	[N = 22]	102.0	(±60.3)	[N = 5]	0.59	16.7	(±8.4)	[N = 22]	17.9	(±8.6)	[N = 5]	0.78
Epliepsy	107.9	(±53.6)	[N = 23]	146.1	(±25.2)	[N = 4]	0.18	17.1	(±8.5)	[N = 23]	16.1	(±8.3)	[N = 4]	0.82
Eating difficulties	56.5	(±20.9)	[N = 7]	133.5	(±44.0)	[N = 20]	0.00**	11.5	(±7.5)	[N = 7]	18.8	(±7.8)	[N = 20]	0.04*
Constipation	93.5	(±58.2)	[N = 14]	135.1	(±34.7)	[N = 13]	0.03	15.4	(±8.9)	[N = 14]	18.6	(±7.5)	[N = 13]	0.33

All of the data represent means ± standard deviations. T-ghrelin, total ghrelin; O-ghrelin, octanoyl ghrelin. Significantly lower levels of plasma T-ghrelin were observed in patients with eating difficulties (**$p \le 0.01$) or constipation (*$p \le 0.05$).
* $p \le 0.05$.
** $p \le 0.01$.

5. Conclusions

Intravenously administered forms of ghrelin have recently become clinically available and have been safely used for the treatment of patients with functional dyspepsia, eating disorders, and overly low BMI. Ghrelin increases daily food intake, enhances the sensation of hunger, and augments growth hormone release (Akamizu et al., 2008). Therefore, our study demonstrated that ghrelin is potentially an effective treatment for various symptoms and signs in patients with RTT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijdevneu.2011.07.003.

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ORIGINAL ARTICLE

Effects of amino acids infused into the vein on ghrelin-induced GH, insulin and glucagon secretion in lactating cows

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ABSTRACT

To investigate the effects of amino acids on ghrelin-induced growth hormone (GH), insulin and glucagon secretion in lactating dairy cattle, six Holstein cows were randomly assigned to two infusion treatments in a cross-over design. Mixture solution of amino acids (AMI) or saline (CON) was continuously infused into the left side jugular vein via catheter for 4 h. At 2 h after the start of infusion, synthetic bovine ghrelin was single injected into the right side jugular vein through the catheter. Ghrelin injection immediately increased plasma GH, glucose and non-esterified fatty acids ($P < 0.05$) with no difference between both treatments. Additionally, plasma insulin and glucagon concentrations were increased by ghrelin injection in both treatments. The peak value of plasma insulin concentration was greater in AMI compared with CON ($P < 0.05$). Plasma glucagon concentration showed no difference in the peak value reached at 5 min between both treatments, and then the plasma levels in AMI compared with CON showed sustained higher values ($P < 0.05$). After plasma glucose concentration reached the peak, the decline was greater in AMI compared with CON ($P < 0.05$). These results showed that the increased plasma amino acids may enhance ghrelin action which in turn enhances insulin and glucagon secretions in lactating cows.

Key words: *amino acid, ghrelin, glucagon, insulin, lactating cow.*

INTRODUCTION

Ghrelin is a hormone mainly secreted by the abomasum in ruminants (Hayashida *et al.* 2001). Ghrelin can stimulate growth hormone (GH) secretion (Kojima *et al.* 1999; Takaya *et al.* 2000; Itoh *et al.* 2005) and appetite (Nakazato *et al.* 2001), the ghrelin concentration in plasma is related to the feeding and nutritional status of ruminants (Sugino *et al.* 2004). In lactating cows, negative energy balance increases periprandial ghrelin concentrations (Bradford & Allen 2008), and the importance of ghrelin for GH secretion might be augmented during early lactating periods (Itoh *et al.* 2005). Itoh *et al.* (2006) further reported that ghrelin injection increased plasma pancreatic hormones and glucose levels during the lactating period. Recently, Roche *et al.* (2008) reported that long-term infusion of ghrelin increased milk production several days into postpartum. Thus, ghrelin could play an important role as a GH-releasing and catabolic hormone for milk production in lactating cows.

Several published reports are available on the relationship between available nutrients and ghrelin secretion. A high-protein diet increased ghrelin plasma levels in rats (Vallejo-Cremades *et al.* 2004), and oral low dose of essential amino acid mixture increased ghrelin secretion in fasting humans (Knerr *et al.* 2003). Additionally, high plasma levels of α-amino nitrogen (AAN) could stimulate ghrelin secretion in sheep (Sugino *et al.* 2010). These previous results suggest that amino acids may up-regulate ghrelin secretion,

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however, the direct effects of amino acids on ghrelin action remain unclear.

The objectives of the present study were to investigate the effects of amino acid infusion into the jugular vein on the circulating levels of ghrelin and on the role of ghrelin in GH secretion, pancreatic hormone secretion and nutrient (glucose and non-esterified fatty acids (NEFA)) metabolism in lactating dairy cows.

MATERIALS AND METHODS

The procedures used in the present study were carried out in accordance with the principles and guidelines for animal use issued by the National Institute of Livestock and Grassland Science Animal Care Committee, and which were formulated to comply with Japanese regulations.

Animals

Six lactating Holstein cows (calving number: 3.3 ± 0.8; days in milk: 57.2 ± 2.1; daily milk yield: 32.5 ± 0.4 kg; body weight: 687 ± 18 kg) were fed a diet (Table 1) according to the Japanese Feeding Standard for Dairy Cattle (Agriculture, Forestry, and Fisheries Research Council Secretariat 1999), twice daily (09.00 and 18.00 hours). The cows were allowed free access to the diet and water. Refusals were weighed daily before the morning feeding. The crude protein (CP) and total digestible nutrient (TDN) contents in the diet and the level of TDN offered were 13.0% and 69.8% on a dry matter (DM) basis, respectively. Cows were milked twice daily before each feeding. The daily DM intake and TDN sufficiency through the experiment were 23.1 ± 1.0 kg and 105.7 ± 3.5%, respectively. The cows were inserted with chronic catheters (Argyle 14 G CV catheter kit; Nippon Sherwood Medical Industries Ltd, Tokyo, Japan) into both sides of the jugular vein at least 1 day before the first treatment. These catheters were used for both reagent infusion and blood sampling.

Treatments and blood sampling

Cows were randomly given two infusion treatments with mixture solution of amino acids (AMI: Aminic (Ajinomoto Pharmaceuticals Co., Ltd, Tokyo, Japan) plus methionine dissolved into saline; total amino acids: 91.0 mg/mL; infusion rate: 4.0 mg/kg of metabolic body weight ($BW^{0.75}$)/min) or saline (CON) in a cross-over design. Each treatment was carried out with at least a 1 week interval to avoid measurement values being affected by the previous treatment. The amino acid composition of AMI is presented in Table 2. The infusion amount of AMI was equal to approximately 6% of the requirement of digestible CP. At 4 h after morning feeding (13.00 hour), a mixture solution of amino acids or saline was continuously infused into the jugular vein catheter on the left side. The solutions were infused with a peristaltic pump (Model 312/MP-4; Gilson, Inc., Middleton, France) for 4 h. At 2 h after the initiation of AMI and CON infusion, synthetic bovine ghrelin (1 μg/kg BW; KNC Laboratories Co., Ltd, Kobe, Japan) was single-dosed into the right side jugular vein through the catheter. This injection dose of ghrelin was chosen based on Itoh et al. (2005). After ghrelin injection, 10 mL of saline was injected for flushing the inside of the catheter. Blood samples were taken at −130, −120, −10, 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150 and 180 min relative to the ghrelin injection from the catheter of the right side jugular vein and collected into heparinized tubes with aprotinine (500 KIU/mL of blood; Trasyrol, Bayer, Leverkusen, Germany). The obtained blood samples were centrifuged at 1500 × g for 20 min at 4°C. Harvested plasma was stored at −80°C prior to assay.

Table 1 Composition of total mixed ration used in this study

Item	DM (%)
Corn silage	28.0
Italian ryegrass silage	17.0
Alfalfa hay cubes	10.0
Sudan grass hay	5.0
Mixed concentration	40.0

Chemical analyses

Plasma ghrelin, GH and insulin levels were measured with time-resolved fluoro-immunoassay. Assay for bioactive ghrelin and GH was conducted as described previously (Sugino et al. 2004). The ghrelin concentration was measured by competitive solid-phase immunoassay using europium (Eu)-labeled synthetic bovine ghrelin and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and inter-assay coefficients of variation were 1.3 and 1.5%, respectively. Least detectable dose in this assay system was 0.025 ng/mL. The GH concentration was measured by competitive solid-phase immunoassay using Eu-labeled synthetic bovine GH and polystyrene microtiter strips coated with anti-rabbit γ-globulin. Intra- and inter-assay coefficients of variation were 2.6 and 3.6%, respectively. Least detectable dose in this assay system was 0.158 ng/mL. Insulin assay was conducted as described previously (Takahashi et al. 2006). The insulin concentration

Table 2 Composition of amino acid mixture used in this study

Item	w/v (%)
L-Val	1.167
L-Leu	1.075
L-Lys	0.833
L-Ile	0.758
L-Arg	0.750
L-Thr	0.625
L-Ala	0.592
Gly	0.583
L-Phe	0.583
L-Pro	0.417
L-His	0.417
L-Met	0.867
L-Ser	0.142
L-Trp	0.108
L-Asn	0.083
L-Gln	0.042
L-Tyr	0.033
L-Cys	0.029
Total	9.104
Total N (mg/mL)	12.667

Val, valine; Leu, leucine; Lys, lysine; Ile, isoleucine; Arg, arginine; Thr, threonine; Ala, alanine; Gly, glycine; Phe, phenylalanine; Pro, proline, His, histidine; Met, methionine; Ser, serine; Cys, cysteine; Gln, glutamine; Tyr, tyrosine.

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was measured by competitive solid-phase immunoassay using Eu-labeled synthetic bovine insulin and polystyrene microtiter strips coated with anti-guinea pig γ-globulin. Intra- and inter-assay coefficients of variation were 2.2 and 1.8%, respectively. Least detectable dose in this assay system was 0.016 ng/mL.

Plasma total glucagon levels were measured using commercially available radioimmunoassay kit (glucagon assay kit; Daiichi Radioisotope Laboratories Ltd, Tokyo, Japan). Glucagon concentrations were measured in the same assay, and the intra-assay coefficient of variation was 3.4%. Least detectable dose was 0.015 pg/mL.

Plasma glucose concentrations were determined using a glucose analyzer (GA-1151; Arkray, Inc., Kyoto, Japan). Plasma AAN levels were analyzed following the procedures of Goodwin (1968). Plasma NEFA concentrations were determined with the commercially available kit (NEFA C-test Wako; Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Calculations and statistics

The values of plasma hormones and metabolites were expressed as means of six cows with standard error (SE). The area under the curve (AUC) was calculated for hormones and metabolites after ghrelin injection over 0–60 min, 60–120 min and 120–180 min terms. The values were expressed as means of six cows with SE. Data were analyzed as a cross-over design with a mixed linear model using restricted maximum likelihood by the JMP program package (version 5.01 for Windows; SAS Institute, Inc., Cary, NC, USA). Dependent variables were summarized as least squares means and least squares SE. The model included the treatment, time and the interaction between treatment and time as the fixed effects, and cow and period as the random effects. The temporal changes of the concentrations for each infusion treatment were evaluated by multiple comparisons with an alpha level of 0.05 using the LSMEANS statement specifying TUKEY (JMP program). The differences in plasma concentrations at each time and AUC at each term between CON and AMI were evaluated by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

In rats, ghrelin expression and secretion were enhanced by feeding of a high protein diet (Vallejo-Cremades *et al.* 2004, 2005). Knerr *et al.* (2003) reported that oral low-dose essential amino acid mixture bolus elicited hyper-aminoacidemia and an increased ghrelin secretion in fasting humans. In sheep, plasma ghrelin concentrations were increased by abomasal infusion of casein plus starch (Sugino *et al.* 2010). Thus, the absorption of amino acids may be one of the stimulating factors of ghrelin secretion in both ruminant and non-ruminant animals. In addition, our recent study demonstrated that the

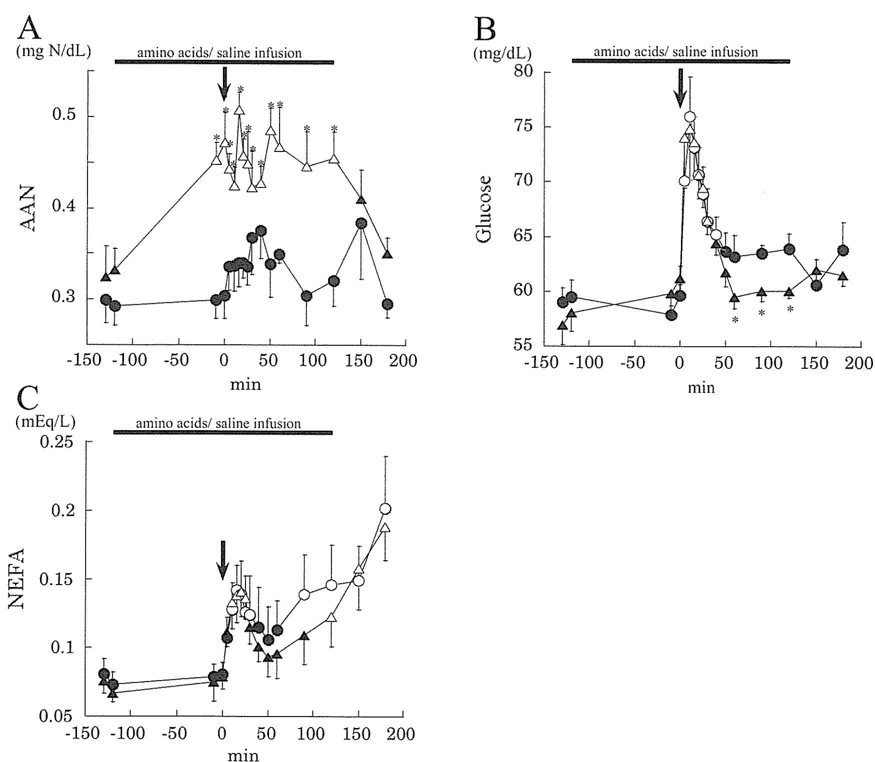

Figure 1 Plasma α-amino nitrogen (ANN) (A), glucose (B) and non-esterified fatty acids (NEFA) (C) concentrations by ghrelin injection in amino acids (AMI) and saline (CON) treatments. Values are expressed as mean ± SE (vertical bar, $n = 6$). Circles and triangles show the CON and AMI treatments, respectively. $*P < 0.05$ compared between CON and AMI at each time. Open plot: $P < 0.05$, difference between pre-amino acid or saline infusion (mean values from −130 and −120 min). The horizonal bar and arrow show the period of the amino acid or saline infusion and ghrelin injection time, respectively.

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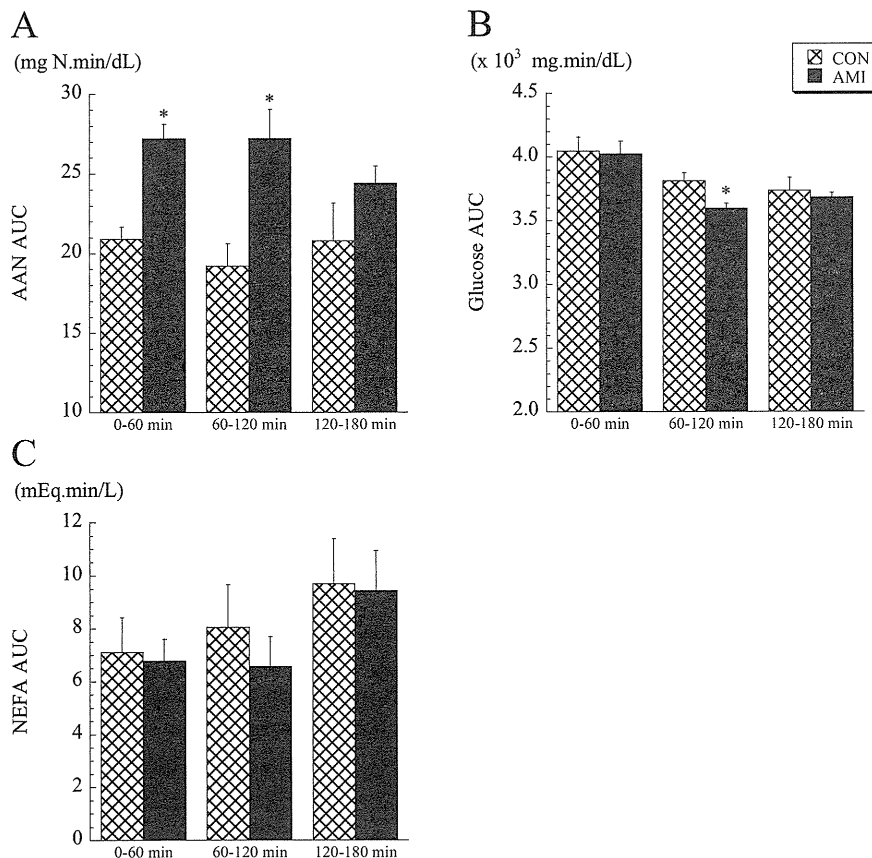

Figure 2 The area under the curves (AUCs) of plasma α-amino nitrogen (ANN) (A), glucose (B) and non-esterified fatty acids (NEFA) (C) concentrations over 0–60 min, 60–120 min and 120–180 min following ghrelin injection in amino acids (AMI) and saline (CON) treatments. Values are expressed as mean ± SE (vertical bar, $n = 6$). $*P < 0.05$ compared between CON and AMI at each period.

intravenous infusion of amino acids increased plasma ghrelin concentrations in sheep (Sugino *et al.* 2010). These previous reports suggest that the increased circulating levels of amino acids may play a key role in enhanced ghrelin secretion. In this study, amino acid infusion increased the concentrations of plasma AAN (pre-infusion: 0.328 mg N/dL; post-infusion: 0.462 mg N/dL; $P < 0.05$, Fig. 1a) and AAN AUCs were greater in AMI compared with CON (0–60 min; CON: 20.9 mg N.min/dL; AMI: 27.2 mg N.min/dL and 60–120 min; CON: 19.2 mg N.min/dL; AMI: 27.2 mg N.min/dL; $P < 0.05$, Fig. 2a), but did not affect the plasma ghrelin levels (Figs 3a and 4a). Sugino *et al.* (2010) demonstrated that more than 66% rise of plasma AAN stimulated ghrelin secretion, but not in 27% rise of plasma AAN. In the present study, 41% rise of plasma AAN levels by amino acid infusion did not change plasma ghrelin levels in lactating cows. Kuhara *et al.* (1991), who infused intravenously 17 kinds of amino acids each at the dose of 3.0 mmol/kgBW over 30 min, observed that acidic amino acids stimulated GH secretion, neutral amino acids stimulated insulin secretion and neutral straight-chain amino acids stimulated glucagon secretion in sheep. We infused the mixture of amino acids with nearly equal levels (2.68 mmol/kgBW over 120 min) in the total amount, but the infusion level of each amino acid was lower than that by Kuhara *et al.* (1991), which might be the reason for no response of GH, insulin and glucagon to AMI observed in the present experiment. There may be a threshold level of plasma AAN and the kind of amino acid stimulating ghrelin and metabolic hormone secretion.

Plasma levels of ghrelin increased after ghrelin injection ($P < 0.05$, Fig. 3a) with no difference between CON and AMI (Figs 3a and 4a). Plasma GH concentrations were increased by ghrelin injection in both treatments ($P < 0.05$, Fig. 3b). Plasma GH concentrations peaked at 5 min after ghrelin injection, and then decreased to the basal level within 50 min in both treatments. GH secretory responses to ghrelin were not different between both treatments (Figs 3b and 4b). Takahashi *et al.* (2009) reported that ghrelin stimulated GH secretion more in the satiety state than in the fasting state in sheep. Thus, GH secretory responses to ghrelin might be affected by feeding states

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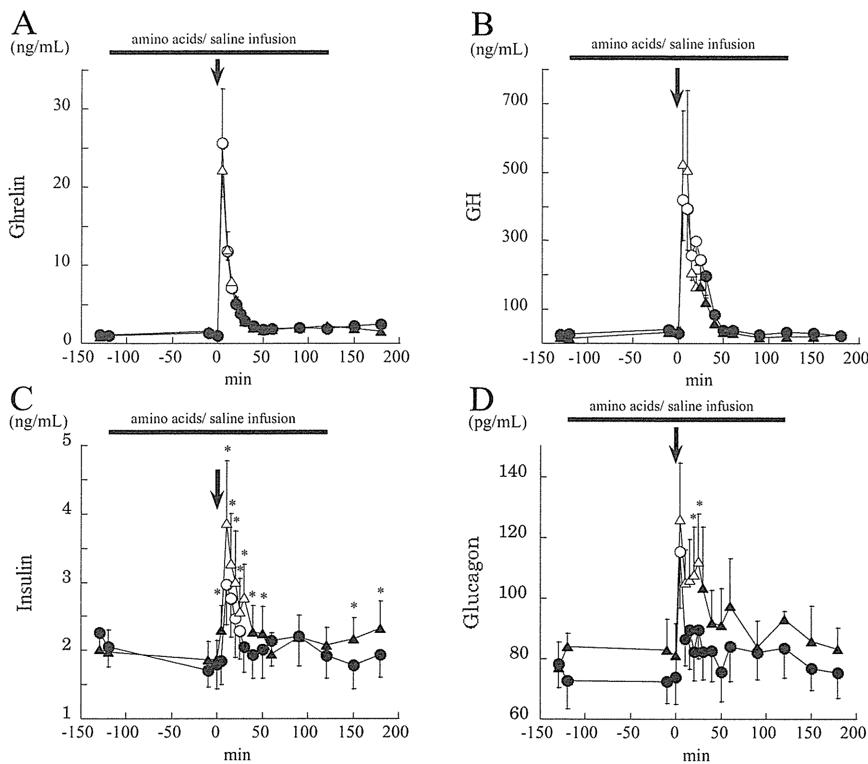

Figure 3 Plasma ghrelin (A), growth hormone (GH) (B), insulin (C) and glucagon (D) concentrations by ghrelin injection in amino acids (AMI) and saline (CON) treatments. Values are expressed as mean ± SE (vertical bar, $n = 6$). Circles and triangles show the CON and AMI treatments, respectively. *$P < 0.05$ compared between CON and AMI at each time. Open plot: $P < 0.05$, difference between pre amino acids or saline infusion (mean values from −130 and −120 min). The horizonal bar and arrow show the period of the amino acids or saline infusion period and ghrelin injection time, respectively.

or energy balance. We investigated GH secretory responses to ghrelin under the same feeding state in this study. Thus, we speculated that GH secretion by ghrelin could not be affected by the difference in the level of the circulating amino acids.

Plasma glucagon concentrations increased after ghrelin injection in both treatments ($P < 0.05$, Fig. 3d). Other scientists have reported that ghrelin enhanced glucagon secretion only in lactating cows (Itoh *et al.* 2005) and that ghrelin induced pancreatic glucagon secretion in diabetic rats, but not in normal rats (Adeghate & Parvez 2002). This ghrelin action in stimulating glucagon secretion could be expressed when insulin resistance is enhanced under the conditions such as lactation or diabetes. A significant increase in glucagon was observed at 5 min after ghrelin injection in both treatments, and thereafter, plasma glucagon concentrations rapidly declined in CON, but not in AMI (25 min; CON: 89.6 pg/mL; AMI: 112.1 pg/mL; $P < 0.05$, Fig. 3d). Consequently, glucagon AUC from 0 to 60 min in AMI was greater compared with CON (0–60 min; CON: 5099.8 pg.min/mL; AMI: 6132.7 pg.min/mL, $P < 0.05$, Fig. 4d). In the current study, we found no effect of amino acid infusion on plasma glucagon levels before ghrelin injection. However, glucagon secretion was more enhanced by ghrelin injection in AMI than in CON. Thus, it may be possible that the infused amino acids enhance glucagon secretion through a ghrelin-dependent process. However, in this study such a mechanism was unclear and further investigations are suggested for more clarification.

Plasma glucose and NEFA concentrations increased after ghrelin injection in both treatments (Fig. 1b,c). These responses were preceded by increased GH and glucagon levels by ghrelin injection. It is well known that GH and glucagon enhance glucose and NEFA release into the blood circulation through their gluconeogenic, glycogenolytic and lipolytic actions. Because ghrelin inhibits insulin signaling in skeletal muscle, liver and fat tissue (Murata *et al.* 2002; Barazzoni *et al.* 2008), ghrelin-induced hyperglycemia may also be related to the limited peripheral glucose uptake and/or to the enhanced gluconeogenesis. Although there has been no information about an acute response to ghrelin on plasma NEFA levels, long-term infusion of ghrelin in lactating cows increased plasma NEFA levels with no changes in plasma GH level (Roche *et al.* 2008). In the present study therefore, ghrelin injection possibly increased plasma glucose and NEFA concentrations via GH and glucagon.

Plasma insulin concentrations began to increase at 5 min after ghrelin injection with an observed higher peak at 10 min in AMI compared with CON (CON: 2.967 ng/mL; AMI: 3.875 ng/mL; $P < 0.05$, Fig. 3c), although insulin AUC was not significantly differed between the two infusion treatments (Fig. 4c). Although the increased plasma glucose and NEFA

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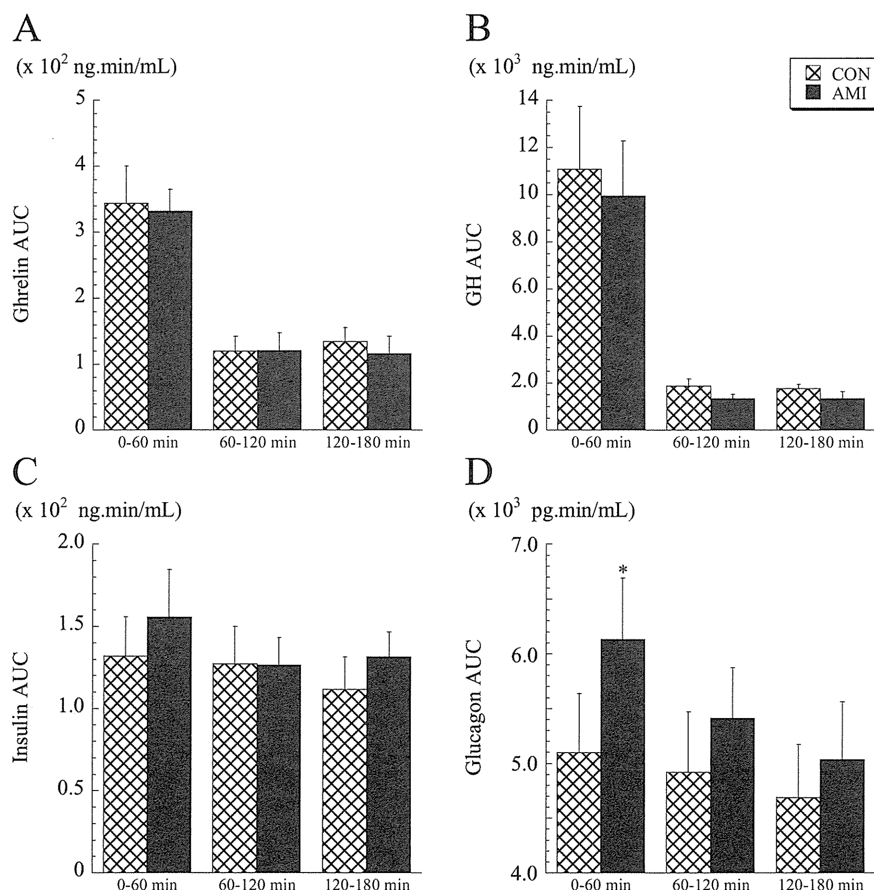

Figure 4 The area under the curves (AUCs) of plasma ghrelin (A), growth hormone (GH) (B), insulin (C) and glucagon (D) concentrations over 0–60 min, 60–120 min and 120–180 min following ghrelin injection in amino acids (AMI) and saline (CON) treatments. Values are expressed as mean ± SE (vertical bar, $n = 6$). *$P < 0.05$ compared between CON and AMI at each period.

concentrations after ghrelin injection were not different in the peak values between CON and AMI (Fig. 1b,c), the decline in plasma glucose concentrations from 60 to 120 min was greater in AMI compared with CON ($P < 0.05$, Figs 1b and 2b). The reason might be related to a greater insulin peak level in AMI. The insulin response followed the increases in glucagon and glucose. Glucagon stimulates insulin secretion directly by a paracrine manner in the pancreas or via hyperglycemia by enhancing glucose release from the liver (Johnson *et al.* 1972). Takahashi *et al.* (2006, 2007) demonstrated that intrajugular ghrelin administration stimulated glucose-induced insulin secretion in meal-fed sheep. In the present study, ghrelin might increase plasma glucagon and glucose, thereby enhancing insulin secretion. Sugino *et al.* (2010) demonstrated that the intravenous glucose infusion in hyper-aminoacidemia and hyper-ghrelinemia enhanced insulin secretion in sheep. Thus the high values of plasma insulin in AMI might be the resulted of high values of plasma glucose by ghrelin compared with CON.

In conclusion, the increase in plasma amino acids level may enhance ghrelin action to increase insulin and glucagon secretions into blood circulation during the lactating period in cows.

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Plasma ghrelin concentration is decreased by short chain fatty acids in wethers

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Abstract

To examine the effects of short chain fatty acids (SCFAs) on plasma ghrelin concentration, 4 wethers were injected intravenously with SCFA solutions [acetate (ACE), propionate (PRO), and butyrate (BUT) (0.8 mmol/kg BW)] and saline. The experiment was conducted after a 4 × 4 Latin square design. Each solution was injected into the jugular vein catheter with blood samples taken at −10, 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min relative to the injection time also from this catheter. Plasma ghrelin concentrations decreased after injection with ACE, PRO, and BUT. Although plasma glucose concentrations increased after injection with PRO and BUT ($P < 0.05$), the increment areas were greater with BUT than with PRO. Plasma insulin concentrations increased after injection with PRO and BUT ($P < 0.05$). The decrement areas in plasma ghrelin concentrations were equal in ACE, PRO, and BUT. These data suggest that SCFAs inhibit ghrelin secretion in wethers and not through increased circulating glucose and insulin as previously proposed.
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Keywords: Ghrelin; Short chain fatty acids; Wethers

1. Introduction

Ghrelin can stimulate GH secretion [1] and is a hormone mainly secreted by the abomasum in ruminants [2]. Plasma ghrelin concentration is related to the feeding and nutritional status of sheep [3]. The phenomenon showing increases in plasma ghrelin concentration before meals and decreases after meals [4] is well known. However, the mechanism of ghrelin secretion is not well known in ruminants. In some reports, a negative energy balance increases periprandial ghrelin concentration in dairy and beef cattle [5,6]. Sugino et al [7] demonstrated that cholinergic neurons are involved in the ghrelin secretory response to feeding in sheep. However, metabolites and other hormones can also affect plasma ghrelin concentrations in nonruminants. Nonruminants are observed to have postprandial increases of plasma glucose and insulin concentrations. Circulating insulin can suppress plasma ghrelin concentration in rats [8] and humans [9], and both oral and intravenous doses of glucose decreased plasma ghrelin concentration in humans [10]. However, ruminants absorb feed-derived carbohydrates as short chain fatty

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acids (SCFAs) from the rumen, and there are virtually no postprandial increases of plasma glucose concentration. Thus, it is probable that the inhibiting factor for ghrelin secretion may be different between nonruminants and ruminants. In ruminants, plasma SCFA concentrations increased immediately after feeding and have been theorized to be related to postprandial changes in plasma metabolic hormone (insulin and GH) concentrations [11]. Thus, SCFAs may suppress plasma ghrelin concentration. However, because some SCFAs in circulating plasma increase plasma glucose and insulin concentrations in ruminants, the effects of SCFAs on plasma ghrelin concentration may include the effects of glucose and insulin. The objective of this study was to investigate how some major SCFAs [acetate (ACE), propionate (PRO), and butyrate (BUT)] affect plasma ghrelin concentration and to consider the relationship between glucose, insulin, and ghrelin in wethers.

2. Materials and methods

The procedures used in the present study were performed in accordance with the principles and guidelines for animal use set by Hiroshima University which were formulated to comply with regulations of the Japanese Ministry of Education, Culture, Sports and Technology. All experiments were approved by the Animal Care and Use Committee of Hiroshima University.

2.1. Animals

Four Suffolk wethers (1 year old; 58.2 ± 3.7 kg BW) had catheters (Argyle 14 G CV catheter kit; Nippon Sherwood Medical Industries Ltd, Tokyo, Japan) inserted into the jugular vein ≥1 d before the first treatment. The catheter was used for the injection of solution and for blood sampling. Wethers were fed a sufficient quantity of alfalfa hay cubes and barley (7:3) to meet dietary maintenance requirements on the basis of the Japanese Feeding Standards [12] and had free access to water and trace mineralized salt block. Diets were offered twice daily at 9:00 AM and 5:00 PM.

2.2. Treatments and blood sampling

The experimental design was a 4 × 4 Latin square that consisted of 4 intravenous injection treatments with saline (CON), ACE, PRO, or BUT. ACE, PRO, and BUT solutions were made by dissolving sodium acetate, sodium propionate, and sodium butyrate (Nacarai Tesque, Inc, Kyoto, Japan), respectively, with saline and adjusted to pH 7.6. Each SCFA solution was injected at the rate of 0.8 mmol/kg BW. The injection rate was determined by following Sano et al [13], and the injection amounts of ACE, PRO, and BUT were equal to approximately 0.31%, 0.47%, and 0.71% of daily ME intake, respectively. Each treatment was performed with ≥1 d apart to avoid measurement values being affected by the previous treatment. At 4 h after morning feeding (1:00 PM), each SCFA solution or saline was injected singly into the jugular vein catheter. After injection, 10 mL of saline was injected to flush the catheter. Blood samples (5 mL) were taken at −10, 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min relative to the injection time and collected into heparinized tubes with aprotinin (500 KIE/mL blood; Sigma-Aldrich, Inc, Tokyo, Japan). Catheters were flushed with heparinized saline (3 mL) to avoid blood clotting. The extracted blood samples were centrifuged at 2,330*g* for 15 min at 4°C. Harvested plasma was stored at −80°C before assay.

2.3. Chemical analyses

Plasma ghrelin and insulin concentrations were measured with time-resolved fluoro immunoassay. Assay for bioactive ghrelin was conducted as described previously by Sugino et al [3]. The ghrelin concentration was measured by competitive solid-phase immunoassay with the use of europium-labeled synthetic bovine ghrelin and polystyrene microtiter strips (Nalge Nunc Int, Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and inter-assay CVs were 6.9T and 5.5%, respectively. Least detectable dose and IC_{50} in this assay system were 0.025 and 0.831 ng/mL, respectively. Insulin assay was conducted as described previously by Takahashi et al [14]. The insulin concentration was measured by competitive solid-phase immunoassay with the use of europium-labeled synthetic bovine insulin and polystyrene microtiter strips coated with anti-guinea pig γ-globulin. Intra- and inter-assay CVs variation were 2.2% and 1.8%, respectively. Least detectable dose and IC_{50} in this assay system were 0.016 and 1.073 ng/mL, respectively.

Plasma glucose concentration was determined with a glucose analyzer (GA-1151; Arkray, Inc, Kyoto, Japan). Plasma acetate concentrations were determined with a commercially available kit (F kit acetate; R-Biopharm AG, Darmstadt, Germany). Plasma β-hydroxy butyrate (BHBA) concentration was determined with an automated biochemistry analyzer (Beckman Coulter K.K., Tokyo, Japan).