Identification of activated protein C as a ghrelin endopeptidase in bovine plasma

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Abstract

Ghrelin is a natural GH secretagogue first identified in the stomach. The ghrelin peptide is 28 amino acids long with an octanoic acid attached to Ser^3 near the N-terminus. This lipid modification is essential for the interaction between ghrelin and the ghrelin-specific receptor GH secretagogue receptor type 1a (GHSR1a), whereas the five or more residues of the N-terminus seem to be sufficient to activate GHSR1a to the same level as those of full-length ghrelin. In this study, we found that ghrelin was converted into smaller fragments during incubation with animal plasma *in vitro* and in a mouse model. Mass spectrometric analysis revealed that both acyl and desacyl ghrelin were hydrolyzed at the peptide bond between Arg^{15} and Lys^{16}, generating an N-terminal peptide consisting of the first 15 residues. Next, we partially purified a ghrelin endopeptidase from bovine plasma and identified the enzyme as an anticoagulant serine protease-activated protein C. Octanoyl-truncated ghrelin(1–15) activated GHSR1a-dependent signaling similar to the full-length peptide, as assayed using the cell-based early-growth factor 1 reporter system. Moreover, administration of the protein C-activating agent, ProTac, to mice enhanced the production of octanoyl ghrelin(1–15) in circulation. These results indicate that ghrelin is processed into shorter peptides in circulation under thrombotic and inflammatory conditions, although high doses of the short-form or full-length ghrelin did not have any obvious effects on thromboplastin time or platelet aggregation in human plasma. Truncation of ghrelin might be responsible for altering structural characteristics such as stability, hydrophobicity, and affinity with circulating macromolecules.

Key Words
- ghrelin
- protein C
- plasma
- protein purification

Journal of Endocrinology (2015) **224**, 61–73

Introduction

Ghrelin was first identified by Kojima *et al.* (1999) as an endogenous ligand for the growth hormone secretagogue receptor type 1a (GHSR1a, Howard *et al.* 1996) present in the rat stomach. This hormone is a 28-amino acid peptide, containing a unique structure with an *n*-octanoyl ester at the third serine near the N-terminus. The peptide consisting of the first five residues of ghrelin, Gly-Ser-Ser(octanoyl)-Phe-Leu, is known as the 'active core'

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DOI: 10.1530/JOE-14-0529

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because of its ability to complete reactions (Bednarek *et al.* 2000, Moazed *et al.* 2009), and the lipid modification is required for binding to GHSR1a. Ghrelin exhibits orexigenic and endocrine actions owing to both central and peripheral mechanisms. In healthy human subjects, blood ghrelin concentrations change in response to changes in nutrient status, and even in response to changes in lifestyle (Adams *et al.* 2010). Changes in circulating ghrelin levels also correlate with metabolic abnormalities such as diabetes (Katsuki *et al.* 2004), obesity (Tschöp *et al.* 2001), and cachexia (Garcia *et al.* 2005).

Circulating ghrelin mainly comes from the stomach (Ariyasu *et al.* 2001, Dezaki *et al.* 2006), where ghrelin-secreting X/A-like cells have been identified (Date *et al.* 2000). Both acyl and desacyl ghrelins might be secreted into the circulation (Mizutani *et al.* 2009), as both forms are observed in gastric mucosal cells. Indeed, the ratio of circulating acyl:desacyl ghrelin can be changed by altering the activity of ghrelin *O*-acyltransferase in the stomach (Kirchner *et al.* 2009, Barnett *et al.* 2010). Rapid clearance of endogenous or i.v. administered acyl ghrelin from the circulation has been observed (Gauna *et al.* 2004, Mayorov *et al.* 2008). We previously reported that acyl protein thoesterase 1 (APT1) purified from bovine serum is a ghrelin deacylation enzyme that hydrolyzes the lipid moiety from ghrelin (Satou *et al.* 2010). Sera derived from septic rats exhibit higher ghrelin deacylation activity as compared with those of naive animals (Satou *et al.* 2010). These results may explain why the concentrations of desacyl ghrelin in circulation are higher than those of acylated ghrelin (Gauna *et al.* 2004, Marzullo *et al.* 2004). The evidence reported by several groups indicates that the concentrations of acyl and desacyl ghrelin in circulation are strictly regulated.

Although the acyl moiety at the third residue of ghrelin is unstable, the peptide bonds in the main chain of ghrelin remain stable after incubation with sera derived from cattle (Satou *et al.* 2010) or rats (De Vriese *et al.* 2004). As mentioned previously, the C-terminus of the ghrelin peptide is dispensable for the activation of GHSR1a *in vitro* (Bednarek *et al.* 2000, Van Craenenbroeck *et al.* 2004). However, it has been reported that the short form of ghrelin lacking its C-terminus was not sufficient to elicit GH release in neonatal rats (Torsello *et al.* 2002). Torsello and colleagues also suggested that the shorter fragments did not compete for binding of full-length ghrelin in the human hypothalamus or pituitary. In this study, we show that synthetic human ghrelin can be truncated into shorter fragments, i.e. a fragment consisting of the 15 N-terminal amino acids, in the presence of bovine plasma, and identify an anticoagulant protein, activated protein C (APC), as the ghrelin endopeptidase responsible for this degradation. Moreover, we show that protein C activation by snake venom led to the production of the shorter form of ghrelin from circulating ghrelin in mice. The resultant peptide preserves its ability to activate GHSR1a-dependent intracellular signaling pathways. These results provide, to our knowledge, the first direct evidence that circulating ghrelin might be processed into small fragments with distinct biological roles.

Materials and methods

Animals

All experimental procedures were approved by the Research Ethics Committee of Dokkyo Medical University (registration no. 05-486). Male C57BL/6J mice (12 weeks of age) were housed under controlled temperature (21–23 °C) and light conditions (12 h light:12 h darkness) with *ad libitum* access to chow and water.

Reagents

Human synthetic full-length ghrelin(1–28) (either the octanoylated form, o-ghrelin(28) or the des-octanoylated form, d-ghrelin(28)) was purchased from Peptide Institute, Inc. (Osaka, Japan), and truncated human o-ghrelin(5) (octanoylated ghrelin(1–5)) was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). The other truncated forms of human ghrelin, including o-ghrelin(15) (octanoylated ghrelin(1–15)), were custom synthesized by Scrum, Inc. (Osaka, Japan). Mouse o-ghrelin(28) and decanoylated ghrelin(28) were obtained from KNC Laboratories (Kobe, Japan). Human APC, as well as bovine protein C and APC, were purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Anact C was donated by Teijin Pharma (Tokyo, Japan) and Kaketsuken (Kumamoto, Japan). ProTac (0.6 unit/ml) was obtained from American Diagnostica (Greenwich, CT, USA). A Bradford protein assay kit was purchased from Bio-Rad. Chromatographic reagents were obtained from GE Healthcare Biosciences (Buckinghamshire, UK) unless otherwise indicated. Cell culture media and mass spectrometry grade trypsin were purchased from Wako (Tokyo, Japan). A rabbit polyclonal antibody against protein C was obtained from AssayPro (Saint Charles, MO, USA), and anti-rabbit IgG conjugated with peroxidase was purchased from Santa Cruz Biotechnology. All other chemicals used in this study were purchased from Wako or Sigma unless otherwise indicated.

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Quantification of ghrelin by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed essentially as described in our previous work (Satou *et al*. 2010). Briefly, 2 μl of enzyme and 1 μl (68 pmol) of diluted ghrelin were dissolved in 7 μl of 20 mM Tris–HCl, pH 7.5, containing 0.02% (w/v) Triton X-100. The reaction mixtures were incubated 37 °C for the indicated times. The samples were loaded onto a MALDI-TOF MS instrument, Voyager DE Biospectrometry (Life Technologies). The assays were performed in the linear positive mode, with a 20 kV accelerating voltage, 93.5% grid voltage, 0.03% guide wire, and a delay time of 200 ns, and all spectra were obtained by averaging 100 laser shots. The relative intensities of o-ghrelin(28) ($m/z=3370$), d-ghrelin(28) ($m/z=3245$), and o-ghrelin(15) ($m/z=1880$) were calculated, and ghrelin degradation activity was determined from the percentage of decomposed fragment intensity relative to total ghrelin intensity (acylated ghrelin+decomposed fragments) from each spectrum data set.

Separation of ghrelin by reverse-phase HPLC

For analysis of ghrelin degradation *in vitro*, 5 μl of enzyme were incubated with 1 nmol synthetic o-ghrelin(28) or d-ghrelin(28) in a final volume of 100 μl (0.02% Triton X-100, 20 mM Tris–HCl, pH 8.0) at 37 °C for 30 min. Enzyme reactions were terminated by the addition of acetic acid to a final concentration of 0.1 M. Samples obtained from the enzyme reaction were analyzed by reverse-phase HPLC (RP-HPLC) as described below.

For the detection of degraded and truncated forms of ghrelin circulating *in vivo*, animals received injections of 0.03 unit/100 μl of ProTac, a protein C activator, or saline into the tail vein. Fifteen minutes later, blood samples were collected through cardiac puncture (approximately 1 ml/animal). Then, plasma was prepared as previously described (Nishi *et al*. 2005). Mouse plasma obtained from ProTac-treated mice ($n=23$) or saline-treated mice ($n=20$) was combined and transferred to polypropylene tubes containing aprotinin followed by centrifugation at 4 °C. The separated plasma was acidified by adding one tenth volume of 1 M HCl and stored at −80 °C until use. Each plasma mixture (14 ml) was semi-purified and condensed using Sep-Pak C18 cartridges (Sep-Pak Plus Cartridge, Waters Corp., Milford, MA, USA), followed by vacuum freeze-drying, and then reconstituted with 0.5 ml of 1 M acetic acid solution (AcOH). Each sample (from ProTac- or saline-treated mice) prepared as described above was subsequently analyzed by RP-HPLC. RP-HPLC (Gilson, Villiers le Bel, France) equipped with a C18-cartridge (3.9×150 mm; Symmetry 300, Waters Corp.) (C18-RP-HPLC) was performed using a linear gradient from 10 to 60% CH_3CN–0.1% trifluoro-acetic acid at a flow rate of 1 ml/min for 40 min, and each 500 μl fraction (fractions 1–80) was collected and lyophilized. The lyophilized sample from each fraction was dissolved in 1 ml of RIA buffer to perform o-ghrelin RIA as described below. The concentration of immunoreactivity for o-ghrelin (including both full-form and the N-terminal peptide fragment of o-ghrelin) in each fractionated sample was measured after serial dilution with RIA buffer to achieve optimal concentration (within 30–70%-maximal binding of the radio-labeled tracer to antiserum for o-ghrelin) for the measurement. The elution point of full-length or truncated forms of o-ghrelin that were naturally produced, secreted, and circulating *in vivo* was determined based on the retention times (or eluted fractions) of synthetic o-ghrelin(28) (retention time, 20.5 min and eluted into fractions 41–42) or N-terminal fragments of o-ghrelin including o-ghrelin(15) (retention time, 23.5 min and eluted into fractions 47–48). The recovery rates of respective synthetic ghrelin-peptides, including o-ghrelin(28), o-ghrelin(5), o-ghrelin(15), d-ghrelin(28), or d-ghrelin(15), were all over 90% when pre-treated with the Sep-Pak Plus cartridge.

RIA

The specific ghrelin RIA for the N-terminus (N-RIA) or C-terminus (C-RIA) was performed as described elsewhere (Hosoda *et al*. 2002, Nishi *et al*. 2005). All assays were performed in duplicate. The measurement of intact o-ghrelin(28) was performed by the 'sandwich-type' ELISA (Active Ghrelin ELISA) described below.

ELISA

An ELISA for full-form ghrelin, which detects only o-ghrelin(28) with intact N- and C-terminal sequences (Active ghrelin ELISA Kit, Mitsubishi Kagaku Iatron, Tokyo, Japan), was performed according to the manufacturer's instructions. Human plasma (1 ml) was treated with 0–100 μl of ProTac diluted in saline and added to reach a final volume of 1.1 ml, then incubated at 37 °C for 10 min. Enzyme reactions were terminated by the addition of acetic acid to a final concentration at 0.1 M,

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and 50 µl of each sample were subsequently treated with the Active Ghrelin ELISA Kit.

Amidolytic assay

Protein C was incubated with or without ProTac at 1 unit/6 mg protein before its use in an assay. A 5 µg sample of the enzyme was then incubated with 0.2 µg of a synthetic substrate, Boc-Leu-Ser-Thr-Arg-pNA (Peptide Institute, Inc.), in a final volume of 100 µl (20 mM Tris–HCl, pH 8.0 containing 0.02% Triton X-100) for 12 min at room temperature. The change in absorbance was recorded at 405 nm.

Purification of ghrelin endopeptidase

All procedures were carried out at 4 °C, and Triton X-100 was added to buffers at a final concentration of 0.02%. A total of 20 ml bovine plasma was dissolved in an equal volume of a buffer containing 20 mM Tris–HCl, pH 8.0, and filtered through a 0.45 µm PVDF disc filter (Merck Millipore, Darmstadt, Germany) following brief centrifugation. The sample was sequentially passed through Cellulofine-sulfate (10 ml, Seikagaku Corp., Tokyo, Japan) and Blue sepharose (20 ml). The unadsorbed fractions were applied to an ANX sepharose FF column (20 ml, 1.6×10 cm) equilibrated with 20 mM Tris–HCl, pH 8.0. The column was washed with ten bed volumes of the same buffer containing 250 mM NaCl and then eluted with a linear gradient to 500 mM NaCl. The active fractions were combined and loaded on a Sephacryl S-300 column (0.48×15 cm) equilibrated with 20 mM Tris–HCl, pH 7.2. The active fraction was passed through a mannose-conjugated sepharose 4B column (0.5 ml), and the unadsorbed fraction was separated using a UnoQ column (0.7×3.5 cm) with a linear gradient from 0 to 500 mM NaCl, followed by passage through a Blue sepharose column (0.5 ml).

Peptide mass fingerprinting analysis

Partially purified fractions were separated by SDS–PAGE and stained with EZ Stain Silver (ATTO, Tokyo, Japan). The major protein bands were excised for in-gel digestion with mass spectrometry-grade trypsin (Wako). The mass spectra of extracted peptides were obtained by MALDI-TOF MS as described previously (Satou *et al*. 2010). The spectral data were analyzed using the MS-Fit program (http://prospector.ucsf.edu/) for protein identification.

Cell culture, transfection, and reporter gene assay

HEK293 cells were cultured in DMEM (high glucose, Wako) with 10% FBS (Life Technologies) and maintained at 37 °C in a 5% CO_2-humidified culture incubator. In the case of PC12 cells, DMEM supplemented with 5% FBS with 5% horse serum was used. Ghrelin-evoked cellular responses were determined using the methods described by Naito *et al*. (2000) with some modifications (Murakami *et al*. 2004). Two days after dispersion, HEK293 cells were transfected with *zif268* (early growth response gene 1 (*EGR1*))–firefly luciferase-pGL4.24, Renilla luciferase-pRL (Promega) and human *GHSR1a* (NM_198407.2)-pcDNA3.1 using Lipofectamine 2000 (Life Technologies). As a control, cells were transfected with empty pcDNA3.1 vector (pcDNA). Two days after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) following incubation of cells with various forms of ghrelins for 4 h. Ghrelin used in these experiments was dissolved in DMEM.

SDS–PAGE and immunoblot analysis

SDS–PAGE was carried out using the Laemmli method with 10% (w/v) acrylamide gels. Separated proteins were transferred onto a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad). The membranes were incubated with 150 mM NaCl and 50 mM Tris–HCl, pH 7.5, containing 5% skim milk overnight, and then incubated with the primary anti-protein C antibody for 1 h at room temperature. Subsequently, the membranes were washed and incubated with anti-IgG-conjugated with peroxidase for 1 h at room temperature, then washed with 0.05% Tween 20, 150 mM NaCl, and 50 mM Tris–HCl, pH 7.5. The immunoreacted proteins were detected by addition of the West Pico blotting reagent and X-ray film exposure (Thermo Fisher Scientific, Waltham, MA, USA).

RT-PCR analysis

PC12h cells were cultured as described previously. Plasmid transfection was conducted using the method mentioned previously. After stimulation for 1 h, total RNA was isolated from the cells with RNeasy mini reagents (Qiagen) according to the manufacturer's instructions. A 1 µg sample of the total RNA was reverse-transcribed using Transcriptor RT (Roche Diagnostics) in a total reaction volume of 20 µl. PCR amplifications were performed with GoTaq (Promega) by using GeneAmp 9700 PCR System (Applied Biosystems) for 25 cycles; denaturation 95 °C for 30 s, annealing 55 °C for 30 s, and extension at 72 °C for

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1 min. In the case of quantitative PCR analysis, transcripts were amplified by FastStart Universal SYBR Green Master (Roche Diagnostics) using 9700 Real-Time PCR System. PCR was performed with the following primers: 5′-ACCAGAACCACAAGCAAACC-3′ and 5′-TGATGGCAGCACTGAGGTAG-3′ for human *GHSR1a* (yielding a product of 349 bp); 5′-CTACCGGTCTTCTGCCTCAC-3′ and 5′-GATGAAAGCAAACACCACCA-3′ for rat *Ghsr1a* (yielding a product of 216 bp); 5′-TTGAGCCCAGAGCACCAGAAA-3′ and 5′-AGTTGCAGAGGAGGCAGAAGCT-3′ for rat ghrelin prepropeptide (yielding a product of 348 bp); 5′-AACAACCCTACGAGCACCTG-3′ and 5′-AGGCCACTGACTAGGCTGAA-3′ for rat *Egr1* (yielding a product of 202 bp); and 5′-TTGTAACCAACTGGGACGATATGG-3′ and 5′-GATCTTGATCTTCATGGTGCTAGG-3′ for rat *Actb* (yielding a product of 846 bp).

Determination of thromboplastin time and platelet aggregation

A 5 μl sample of human plasma was incubated with 190 μl of H-Kit solution including thromboplastin and calcium lactate (Sysmex, Hyogo, Japan) in the presence of 3.4 μM of various forms of ghrelins to a final volume of 200 μl. The activated partial thromboplastin time was measured with gentle agitation at room temperature.

Platelet aggregation induced by ADP was quantitatively measured in terms of the maximum total light scattering intensity by a laser light scattering system (PA-200c; Kowa, Tokyo Japan). Platelet-rich plasma was prepared from the citrated human blood by sequential centrifugation, and was preincubated with 5 μM of various forms of ghrelins followed by 2 μM ADP to the final volume of 200 μl.

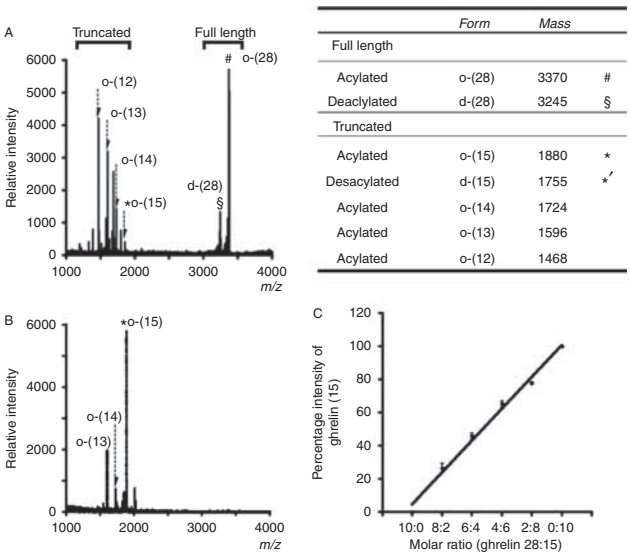
Statistical analyses

All values are expressed as the mean±S.E.M. ($n=3$ or more). Group means were compared using the Student's *t*-test or the Wilcoxon–Mann–Whitney *U* test to determine the significance of the differences between the individual means. Statistical significance was assumed at $P<0.05$. Each experiment was repeated at least three times with similar results.

Results

Degradation of synthetic human ghrelin in the presence of bovine plasma

A 68 pmol sample of o-ghrelin(28) was incubated with 2 μl of bovine or human plasma for 30 or 60 min, and subjected to MALDI-TOF MS. The input synthetic o-ghrelin(28) seemed to be rapidly processed into smaller peptide fragments, as well as being present in the deacylated form (Fig. 1A, Supplementary Figs 1 and 2, see section on supplementary data given at the end of this article; Satou *et al.* 2011). Each observed peak was compared with the theoretical molecular mass calculated from the peptide sequence as shown in the table (right upper panel). It can be speculated that o-ghrelin(28) was truncated in a stepwise manner after incubation with bovine plasma: o-ghrelin(28) ($m/z=3370$) was hydrolyzed first between the Arg^{15} and Lys^{16} residues, then the smaller product o-ghrelin(15) ($m/z=1880$) could be hydrolyzed from the C-terminus in a step-by-step manner. To confirm this hypothesis, synthetic human o-ghrelin(15) was incubated with bovine plasma. As shown in Fig. 1B, degraded peaks corresponding to o-ghrelin(14) ($m/z=1724$) and o-ghrelin(13) ($m/z=1596$) were observed from authentic o-ghrelin(15). The truncated forms of



	Form	Mass	
Full length			
Acylated	o-(28)	3370	#
Deaclylated	d-(28)	3245	§
Truncated			
Acylated	o-(15)	1880	*
Desacylated	d-(15)	1755	*′
Acylated	o-(14)	1724	
Acylated	o-(13)	1596	
Acylated	o-(12)	1468	

Figure 1
Degradation of ghrelin by bovine plasma as analyzed by MALDI-TOF MS. (A) Degradation of synthetic human octanoylated ghrelin, o-ghrelin(28), by bovine plasma. Synthetic human o-ghrelin(28) was incubated with bovine plasma for 30 min at 37 °C, and submitted to MALDI-TOF MS analysis. Acyl ghrelin, o-(28) (#) was degraded into its deacylated form, d-(28) (§) or into truncated short forms that include the first 15 residues of the N-terminus, denoted by o-(15) (*) (*′, see Fig. 4B). Right table, MALDI-TOF MS analysis. Measured values for the expected peptide derivatives of acylated or deacylated ghrelin are shown and the theoretical masses are indicated. Octanoylation and des-octanoylation are abbreviated as 'o-' and 'd-' respectively. (B) Degradation of synthetic human o-ghrelin(15) by bovine plasma. Synthetic human o-ghrelin(15) was incubated with bovine plasma. Human o-ghrelin(15) itself is sufficient to generate shorter forms of ghrelin, o-(14) and o-(13), in the presence of bovine plasma. (C) The percentage intensities of o-(15)/o-(15)+o-(28) values obtained by MALDI-TOF MA —.

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ghrelin degraded from synthetic o-ghrelin(28) could not be detected in the presence of bovine serum (data not shown), in agreement with previous reports (De Vriese *et al*. 2004, Satou *et al*. 2010). In the absence of bovine plasma, synthetic ghrelin is stable for hours at 37 °C (data not shown).

To certify the accuracy of detection of truncated ghrelin by MALDI-TOF MS, several different molar ratios of o-ghrelin(28) and o-ghrelin(15) were simply mixed and analyzed. The percentage intensities of the o-ghrelin(15) peaks, namely (o-ghrelin(15) peak)×100/(o-ghrelin(28) peak+o-ghrelin(15) peak), as determined by MALDI-TOF MS, were consistent with what was observed for controlled mixtures of o-ghrelin(28) and o-ghrelin(15) (Fig. 1C). On the basis of this result, we calculated the endopeptidase activity, i.e. we were able to determine a rate of production of o-ghrelin(15) from o-ghrelin(28).

Trypsin, which is one of the major proteinases and is abundant in plasma, seemed to be a good candidate for an enzyme responsible for truncation of ghrelin. In Fig. 2A, the human ghrelin peptide sequence is represented, with two potential tryptic cleavage sites indicated by open and filled arrowheads. As shown in Fig. 2B, upper left panel, o-ghrelin(28) was degraded into two derivatives, corresponding to o-ghrelin(11) and o-ghrelin(15), after incubation with trypsin. This indicates that the 11th residue of o-ghrelin(28) is more preferable for trypsin recognition. However, after incubation with bovine plasma, o-ghrelin(28) was degraded into o-ghrelin(15), and o-ghrelin(11) was not detected (Fig. 2B, upper right panel). This result was inconsistent with the activity of trypsin. Moreover, the production of o-ghrelin(15) from o-ghrelin(28) by bovine plasma was abolished by incubation with benzamidine, a serine protease inhibitor, or with EDTA (Fig. 2B, lower panels). Therefore, we concluded that a serine protease other than trypsin, and present in plasma, may be responsible for the ghrelin endopeptidase activity that produces o-ghrelin(15), and that the presence of divalent ions is important.

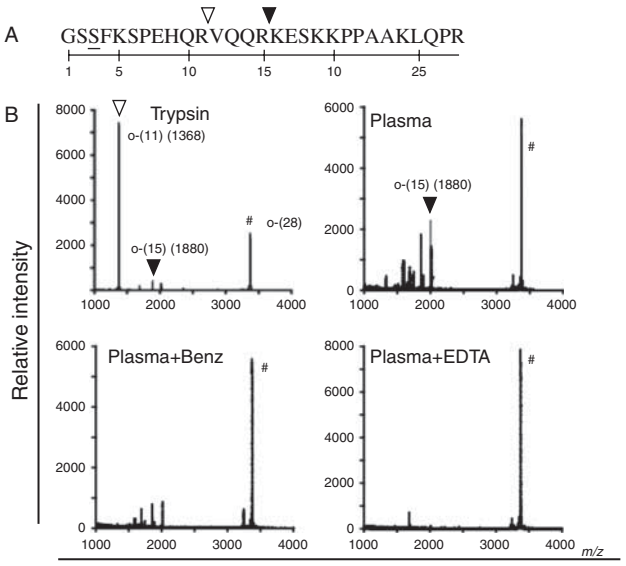

Figure 2
Presence of a ghrelin endopeptidase in bovine plasma. (A) The amino acid sequence of human ghrelin. Open and filled arrowheads indicate potential trypsin cleavage sites. The octanoylated serine is represented by an underlined 'S'. (B) Degradation of o-ghrelin(28) (#) by trypsin and bovine plasma. Authentic trypsin mixed with full-length human o-ghrelin(28) preferentially generated more of the shorter fragment o-(11) (open arrowhead) as compared with the o-(15) form (filled arrowhead) (upper left panel). Under the same conditions, o-ghrelin(15) was clearly detected in the reaction mixture of o-ghrelin(28) and bovine plasma although o-(11) was obscure (upper right panel). The enzymatic properties of plasma against o-ghrelin(28) disappeared in the presence of benzamidine (Benz) (lower left panel) or EDTA (lower right panel).

Identification of a ghrelin endopeptidase from bovine plasma

Fractions corresponding to the highest peaks of ghrelin endopeptidase activity against human octanoylated ghrelin were sequentially purified by column chromatography from 20 ml bovine plasma as described in the 'Materials and methods' section. After three rounds of column chromatography, the fraction corresponding to peak enzyme activity was applied to a Sephacryl S-300 column for further separation (Fig. 3A). As shown in the left panel of Fig. 3B, the flow-through fractions from the mannose-conjugated sepharose 4B column were further separated using a UnoQ column with a linear gradient of NaCl. The right panel shows a silver-stained gel separation of corresponding fractions obtained from the UnoQ column. The filled triangles indicate the fraction with peak enzyme activity. Finally, the active fractions were passed through Blue sepharose, followed by SDS–PAGE separation (Fig. 3C). Two major bands were identified by peptide mass fingerprinting (PMF) analysis, protein C heavy chain (upper band), and adiponectin (lower band). Condensed bands in eluted fractions from a Sephacryl S-300 column were also confirmed to be protein C by western blotting analysis. The peak of enzyme activity coincided well with the density of the bands (Fig. 3A, inset). From these results, we concluded that APC is an important candidate for the ghrelin endopeptidase. Protein C is a vitamin-K-dependent plasma protease that regulates blood coagulation through inactivation of factors Va and VIII (Griffin *et al*. 2007). Protein C is a zymogen in circulation, and is converted to APC via partial proteolysis by thrombin after blood coagulation. These results indicate

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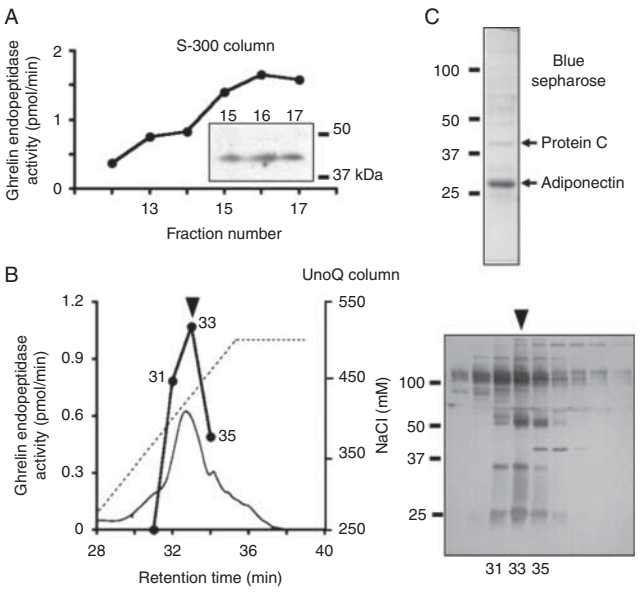

Figure 3
Purification and identification of protein C as a ghrelin endopeptidase from bovine plasma. (A) Sephacryl S-300 chromatography. The results for the fourth column of seven sequential rounds of chromatographic purification of a ghrelin endopeptidase are shown. Protein C was detected by western blotting in the fractions corresponding to the highest peaks of enzyme activity (inset). (B) Typical chromatographic pattern of the sixth column following UnoQ separation (left). Enzyme activity is represented by a thick continuous line with filled circles. The thin and dotted lines indicate protein and NaCl concentrations respectively. Corresponding fractions were subsequently separated by SDS–PAGE followed by silver staining (right). The positions of molecular size markers (kDa) are indicated. The arrowheads indicate the corresponding highest peak of enzyme activity. (C) Identification of partially purified proteins after purification. Partially purified proteins from UnoQ column separation were applied onto a Blue sepharose column, and unadsorbed proteins were separated by SDS–PAGE followed by silver staining. The two distinctive major protein bands were subjected to PMF analysis and identified as protein C and adiponectin.

that protein C in bovine plasma may be activated by contact with devices during purification procedures and even during the incubation period of the enzyme activity assay. To confirm that APC is the ghrelin endopeptidase in circulation, acyl and desacyl forms of ghrelin(28) were incubated with commercially available APC and protein C purified from human and bovine plasma (Figs 4 and 5 respectively).

Characterization of APC as a ghrelin endopeptidase

Commercially obtained human APC incubated with human o-ghrelin(28) gave results in agreement with results observed for partially purified bovine APC in the same type of assay. As shown in Fig. 4A, human APC clearly hydrolyzed human o-ghrelin(28) into o-ghrelin(15), as detected by RP-HPLC (upper panel) and MALDI-TOF MS (lower panel). Moreover, the desacyl form of ghrelin(28), d-(28), was also clearly cleaved by the protease, as detected by RP-HPLC (upper panel) and MALDI-TOF MS (lower panel) (Fig. 4B). The endopeptidase activity of human APC against the desacyl form of ghrelin was about half of that against acylated ghrelin (Fig. 4C). These results are consistent with the MS spectra showing that o-ghrelin(28) is more susceptible to hydrolysis of its peptide bonds than the deacylated form (Supplementary Fig. 2). The acyl moiety of ghrelin can contribute to substrate specificity, and APC preferentially hydrolyzed acylated ghrelin (Supplementary Figs 3 and 4). A diagnostic APC, Anact C, which was obtained from Teijin Pharma and Kaketsuken, also clearly hydrolyzed acylated ghrelin (Fig. 4D). These results clearly demonstrate that APC has ghrelin endopeptidase activity. As shown in Supplementary Fig. 1A, fresh human plasma generated no obvious truncated forms of ghrelin except for the deacylated form. Protein C in healthy human plasma could not be clearly activated like that in bovine and rodent plasma. However, human plasma was found to degrade ghrelin into shorter fragments after stimulation with biochemical processes, such as dialysis or salt-precipitation (Supplementary Fig. 1B). Further studies are needed to elucidate these points.

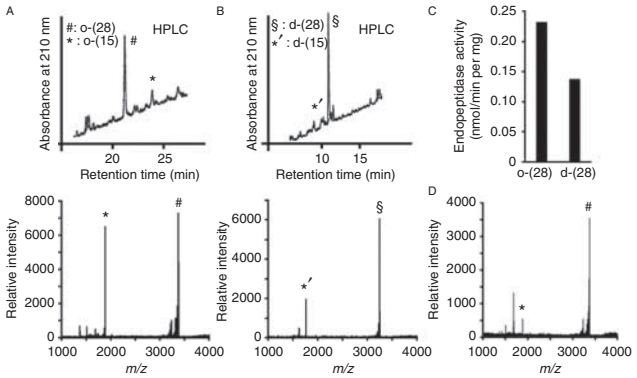

Figure 4
Activated protein C (APC) has ghrelin endopeptidase activity. (A) APC has endopeptidase activity against o-ghrelin(28). Purified human activated protein C obtained from a commercial vendor was incubated with synthetic human o-ghrelin(28) (#) for 30 min, and then subjected to RP-HPLC followed by RIA (upper panel) or MALDI-TOF MS (lower panel) analysis. Digestion of the input peptide, o-(28) (#), resulted in a form containing the 15 residues of the N-terminus (*), denoted o-(15) as in Fig. 1. (B) APC has endopeptidase activity against des-acylated ghrelin(28), d-(28). The desacyl form of ghrelin (§) was incubated with APC as in (A). The digestion resulted in a form containing the 15 residues of N-terminus, d-(15) (*′). (C) The calculated endopeptidase activities of human APC against acyl and desacyl ghrelin(28) are 0.231 and 0.137 nmol/min per mg protein, respectively, based on RP-HPLC data from (A) and (B). (D) One unit of Anact C, a diagnostic APC derived from human plasma, was incubated with synthetic human o-ghrelin(28) (136 pmol) for 30 min. Anact C hydrolyzed mature o-ghrelin(28) into o-(15) (*), similar to the results observed for APC in (A).

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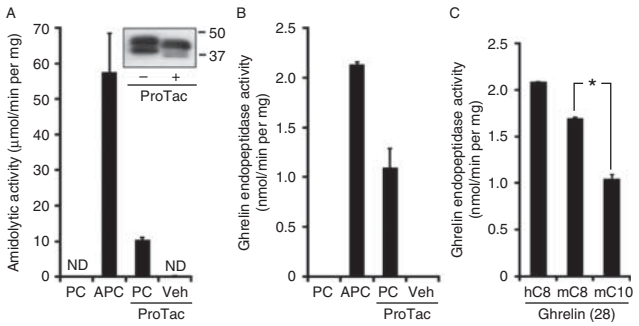

Figure 5
Characterization of APC and ProTac-treated protein C (PC) as a ghrelin endopeptidase. (A) APC and ProTac-treated PC have amidolytic activity. Commercially available purified bovine protein C, the zymogen of APC, was confirmed to have no amidolytic activity against a synthetic APC substrate, Boc-Leu-Ser-Thr-Arg-pNA, whereas thrombin-treated bovine PC, APC, sufficiently hydrolyzed the substrate (57.3 μmol/min per mg protein). Treatment with ProTac activated PC and led to release of the active peptide from PC, as revealed by western blotting (inset). ProTac-treated PC acquired amidolysis activity. Vehicle (Veh) contains only distilled water. (B) APC and ProTac-treated PC have ghrelin endopeptidase activity. The same experiment was conducted using human o-ghrelin(28) as a substrate. APC and ProTac-treated PC also had ghrelin endopeptidase activity, as revealed by MALDI-TOF MS analysis, but the specific activity was quite low (2.13 and 1.09 nmol/min per mg protein respectively) compared with amidolytic activity. (C) Bovine APC was incubated with human o-ghrelin(28) (hC8), mouse o-ghrelin(28) (mC8), or mouse decanoyl ghrelin(28) (mC10). The endopeptidase activity observed for the substrate mC10 (1.04 nmol/min per mg protein) was almost half that observed for mC8. $*P<0.05$.

Bovine APC, but not its zymogen protein C, showed amidolytic activity (57.3 μmol/min per mg), determined using a chromogenic substrate (Fig. 5A). Pretreatment with ProTac, a protein C activator isolated from snake venom, cleaved small fragments from protein C and released the activation peptide from protein C (the inset in Fig. 5A shows western blotting analysis with anti-protein C). ProTac-APC also exhibited amidolytic activity, whereas ProTac itself did not have any effect. Likewise, ghrelin endopeptidase activity against human acylated ghrelin was found in APC (2.13 nmol/min per mg) and ProTac-APC (1.09 nmol/min per mg) (Fig. 5B), although the specific activity was quite low as compared with its amidolytic activity. As mentioned previously, lipid modification of ghrelin seems to have an influence on endopeptidase activity. As shown in Fig. 5C, bovine APC preferentially hydrolyzed the major form of octanoylated ghrelin(28), i.e. about twice as much hydrolysis as was observed for a longer acyl chain of decanoylated ghrelin(28) (Fig. 5C).

Generation of truncated ghrelin *in vitro* and *in vivo*

To our knowledge, to date none of the short forms of ghrelin shown in this study have been observed in the plasma of several animals, including humans. We had the idea that o-ghrelin(15) could not be determined using a sandwich ELISA *in vitro* because the assay is dependent on the presence of intact N- and C-terminal ghrelin peptides (Fig. 6A). Increasing the dose of ProTac in incubation mixtures containing human plasma *in vitro* augmented APC activity (Fig. 6B). Under the same conditions, the amount of endogenous intact ghrelin estimated by the sandwich ELISA was reduced with increasing doses of ProTac (Fig. 6C, left panel). However, there were no significant changes in ghrelin immunoreactivities detected using N-RIA and C-RIA (Fig. 6C, middle and right panels respectively). These results indicate that the endogenous intact o-ghrelin(28) *in vitro* was processed into o-ghrelin(15) and its

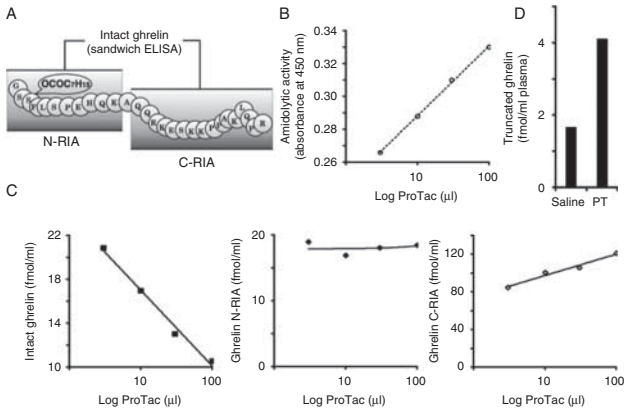

Figure 6
Endogenous ghrelin is degraded into shorter forms following activation of protein C *in vitro* and *in vivo*. (A) Schematic representation of ghrelin immunoassays. N-RIA recognizes the octanoylated N-terminus of ghrelin, and C-RIA recognizes the C-terminal portion of ghrelin (Date *et al*. 2000, Nishi *et al*. 2005). On the other hand, in the sandwich ELISA, the immobilized antibody corresponding to N-RIA captures octanoylated ghrelin and subsequently the C-terminus of ghrelin is recognized by the HRP-conjugated antibody. Thus, ghrelin processed by the endopeptidase, for example between Arg^{15} and Lys^{16}, could not be detected by the ELISA system. (B) Activation of endogenous protein C in human plasma by ProTac treatment. Amidolytic activity of human plasma was determined as shown in Fig. 5, and increased in proportion to the amount of added of ProTac. Conversion of plasma protein C to APC by ProTac was dose dependent. (C) Determination of endogenous ghrelin in human plasma after protein C activation *in vitro* by sandwich ELISA and RIA. ProTac-activated human plasma prepared in (B) was subjected to ghrelin immunoassays to determine the amount of endogenous ghrelin. In response to the increase in ProTac, a reduction in the levels of intact ghrelin was observed with sandwich ELISA (left panel) whereas the amount of each end of ghrelin, as determined by N-RIA (middle panel) or C-RIA (right panel), remained constant. (D) Determination of endogenous truncated forms of ghrelin in mouse plasma. Quantification of circulating truncated forms of ghrelin including o-ghrelin(15) of saline- or ProTac (PT)-treated mice by N-RIA following RP-HPLC. Truncated forms of ghrelin, including o-ghrelin(15), were identified in control mouse plasma and the amount of o-ghrelin(15) was increased within 15 min after i.v. administration of ProTac.

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counterpart in response to ProTac treatment. The sandwich ELISA is likely to be unsuitable for the detection of truncated ghrelin.

Next, we examined the conversion of circulating endogenous o-ghrelin(28) to o-ghrelin(15) in the presence of a protein C activator in a mouse model. As shown in Fig. 6D, the concentration of truncated forms of ghrelin including o-ghrelin(15) in control mouse plasma was estimated as 1.66 fmol/ml by combination analysis with N-RIA following HPLC. ProTac administration rapidly increased the level of truncated forms of ghrelin in plasma, with an increase of more than twofold (4.1 fmol/ml) as compared with the control group (Fig. 6D). The ratio of o-ghrelin(15) to intact ghrelin was also elevated in ProTac-treated animals (0.52) as compared with controls (0.28) (data not shown). Collectively, these results indicate that o-ghrelin(15) is present in mouse plasma, and furthermore, that endogenous intact o-ghrelin(28) in circulation *in vivo* can be processed into o-ghrelin(15) in response to ProTac treatment.

Functional significance of truncated ghrelin

To elucidate the biological activity of o-ghrelin(15), a reporter gene assay was carried out using HEK293 cells overexpressing the ghrelin receptor hGHSR1a. As previously shown by others (Holst *et al.* 2005), exogenous ghrelin receptor exhibits higher basal constitutive activity when assayed using the EGR1-driven reporter system (Fig. 7A). The addition of 10 nM of o-ghrelin(28), but not d-ghrelin(28), enhanced reporter activity (Fig. 7A, left panel). o-Ghrelin(28) and o-ghrelin(15) enhanced *EGR1* promoter-driven luciferase activity of HEK293 cells overexpressing GHSR1a, although neither form of acylated ghrelin had obvious effects in pcDNA-transfected cells (Fig. 7A, right panel). o-Ghrelin(5) did not significantly enhance *EGR1* promoter-driven luciferase activity of HEK293 cells overexpressing GHSR1a. As shown in Fig. 7B, both o-ghrelin(28) and o-ghrelin(15) enhanced *EGR1* promoter-driven luciferase activity of HEK293 cells overexpressing hGHSR1a in a dose-dependent manner (1–33 nM). These results indicated that o-ghrelin(15) could be a functional ghrelin receptor agonist.

PC12h cells did not express endogenous rat ghrelin or GHSR1a. When *GHSR1a* was transfected into PC12h cells, its mRNA was clearly detected (Fig. 8A, upper left panel). As shown in Fig. 8A, upper right panel, o-ghrelin(28) could evoke endogenous *Egr1* gene expression in *GHSR1a*-transfected PC12h cells within 1 h. Consistent with the results obtained from HEK293

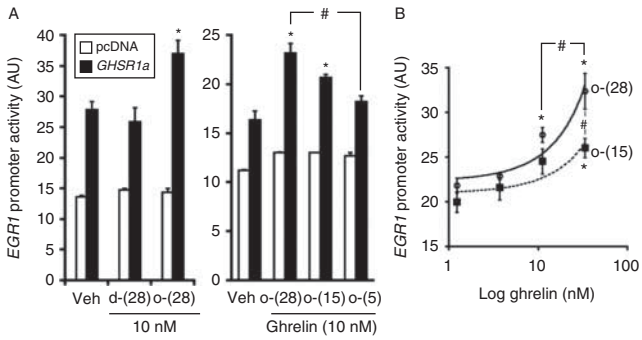

Figure 7
A short form of ghrelin, o-ghrelin(15), evokes cellular responses via GHSR1a in *GHSR1a*-transfected HEK293 cells. (A) o-Ghrelin(15), but not d-(28) or o-(5), enhanced luciferase reporter gene activity in a GHSR1a-dependent manner. A luciferase reporter gene assay was carried out in human *GHSR1a*-transfected HEK293 cells. A 10 nM dose of mature o-ghrelin(28) but not desacyl ghrelin(28), d-(28), enhanced expression of the *EGR1* promoter-driven reporter gene (left panel). Truncated forms of ghrelin, i.e. 15 residues of the N-terminus, o-(15), also could enhance luciferase activity driven by *EGR1* promoter, although less robustly as compared with the effects of o-ghrelin(28) (right panel). Note that higher constitutive activity was observed in *GHSR1a*-transfected cells as compared with pcDNA-transfected cells. Vehicle (Veh) contains only DMEM. $^{*}P<0.05$ versus vehicle control. $^{\#}P<0.05$ between the indicated results. (B) Dose–response experiments with o-ghrelin(28) (circles) and o-ghrelin(15) (squares) (1–33 nM) were assessed using *EGR1* promoter-driven reporter gene. $^{*}P<0.01$ versus 1 nM of o-ghrelin(28) or o-(15). $^{\#}P<0.05$.

cells, 50 nM o-ghrelin(15) significantly, evoked *Egr1* transcription, but to a lesser extent than o-ghrelin(28), in *GHSR1a*-transfected PC12h cells (Fig. 8A, bottom panels).

As APC is a major regulator of blood coagulation by inactivating factors Va and VIIIa, effects of o-ghrelin(15) on thrombotic events were evaluated. Neither the thrombin clotting time (Fig. 8B, upper panel) nor the maximum platelet aggregation (Fig. 8B, lower panel) was affected by o-ghrelin(28) or o-ghrelin(15) even at a concentration higher than physiological levels (approximately 100 – 150 nM in humans).

Discussion

Ghrelin can be deacylated (De Vriese *et al.* 2004, Satou *et al.* 2010) or degraded into smaller peptide fragments in blood and in various tissue homogenates (De Vriese *et al.* 2004). In this study, we used MALDI-TOF MS to confirm findings described in previous reports (Satou *et al.* 2010, 2011) that octanoyl ghrelin is rapidly degraded not only into desacyl forms but also into several smaller peptides when incubated with bovine plasma (Fig. 1 and Supplementary Fig. 2). These data are consistent with earlier observations (De Vriese *et al.* 2004, Gauna *et al.* 2004) that

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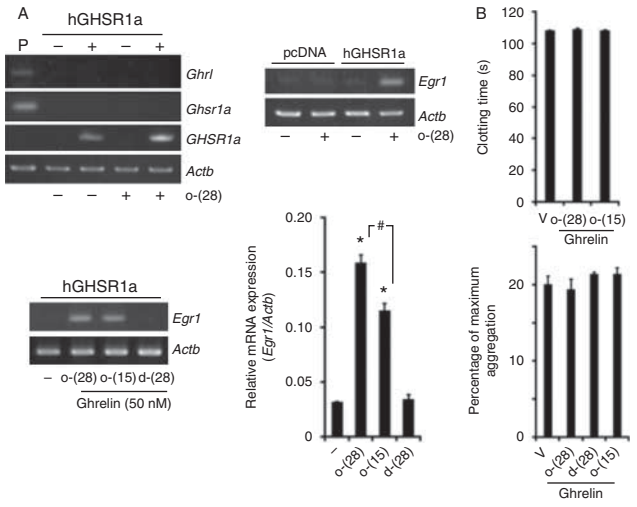

Figure 8

A short form of ghrelin, o-ghrelin(15), activates the expression of endogenous *Egr1* in hGHSR1a-transfected PC12 cells, but does not affect blood clotting. (A) o-Ghrelin(15) enhanced endogenous *Egr1* mRNA levels in a GHSR1a-dependent manner. Neither mRNA of rat prepro-ghrelin (*Ghrl*) nor GHSR1a (*Ghsr1a*) was endogenously expressed in PC12h cells, even in the presence of 100 nM o-ghrelin(28). 'P' indicates a rat pituitary sample, used as a positive control (upper left panel). The upper right panel shows that 50 nM of o-ghrelin(28) was sufficient to induce endogenous *Egr1* mRNA expression in hGHSR1a-transfected PC12h cells but not in pcDNA-transfected cells. Similar results were obtained with o-ghrelin(15)-exposed cells but not with desacyl ghrelin(28), d-(28)-exposed cells (bottom panels). Quantitative RT-PCR confirmed that o-ghrelin(15) significantly evoked transcription of *Egr1*, but to a lesser extent than o-ghrelin(28) (bottom right panel). $^{*}P<0.0002$ versus vehicle control and $^{\#}P<0.0005$ between the indicated results. (B) Coagulation was not affected by o-ghrelin(28) or o-(15). The thrombin clotting time of human plasma was determined in the presence of 3.4 μM of synthetic human o-ghrelin(28) or o-ghrelin(15) (upper panel). The lower panel shows the effects of 5 μM of synthetic o-ghrelin(28), o-ghrelin(15) or desacyl ghrelin(28), d-(28), on platelet aggregation induced by 2 μM ADP in human plasma. Neither coagulation step was affected by ghrelin or its derivatives, even at levels higher than physiological levels.

deacylated ghrelin in circulation is comparable with its acyl form. This has been attributed to the short lifetime of matured ghrelin because of deacylation by hydrolyzing enzymes in circulation such as APT1, butyrylcholinestease (De Vriese *et al.* 2007), and carboxypeptidase (De Vriese *et al.* 2004). As shown in Figs 1 and 2, ghrelin can also be hydrolyzed between its peptide bonds, with the largest digested form of ghrelin detected after incubation with plasma, o-ghrelin(15), resulting from cleavage between Arg^{15} and Lys^{16}.

The specific cleavage site resulting in production of o-ghrelin(15) from o-ghrelin(28) indicates that bovine plasma contains a previously unidentified ghrelin endopeptidase/protease. As shown in Fig. 2, the endopeptidase showed trypsin-like properties, as its catalytic activity was inhibited by benzamidine, a serine protease inhibitor, or EDTA (Fig. 2). However, the other potential tryptic cleavage site, Arg^{11}, was not cleaved during incubation with bovine plasma. These results indicated that trypsin itself can be excluded as a candidate ghrelin endopeptidase. In this study, we partially purified a ghrelin endopeptidase from bovine plasma using sequential column chromatography and identified the activity-containing protein as APC. We have confirmed that ghrelin could be digested between Arg^{15} and Lys^{16} in the presence of plasma, which is the supernatant of whole blood different from sera. Serum is prepared by removing coagulated elements from blood. These facts indicate that the enzymatic ghrelin processing requires activation of coagulation/anti-coagulation factors including APC. Tissue homogenates contain various cytosolic digestive enzymes. Therefore, distinctive degradation patterns observed by De Vriese *et al.* (2004) (N-terminal truncation) were probably due to these enzymes other than circulating peptidases. APC plays an important role in the anti-coagulation process by inactivating factors Va and VIII together with co-enzymes thrombomodulin and protein S (Griffin *et al.* 2007). We speculate that protein C in bovine plasma may be activated, at least in part, by contact with devices during purification and even during incubation of the enzyme activity assay.

Serum levels of protein C in human plasma (approximately 4 μg/ml) are much lower than the levels of fibrinogen (2–4 mg/ml), plasminogen (90–150 μg/ml), or other coagulant proteins such as prothrombin (approximately 100 μg/ml). The much lower concentration of protein C in circulation is suggestive of how protein C works, i.e. the enzyme should be concentrated in those restricted regions where clotting or inflammation occurs and prevent excess coagulation. Conversely, anticoagulation is associated with a risk of excess bleeding. In addition to the fraction of the protein in circulation, protein C can also be detected on the surface of human umbilical vein endothelial cells (HUVECs; Tanabe *et al.* 1991), in which the enzyme probably binds to its receptor (Fukudome & Esmon 1994) and forms anti-coagulation complexes (Griffin *et al.* 2007). It can be postulated that ghrelin processed by APC on HUVECs could exert endothelial functions on the cell surface (Riewald *et al.* 2002, Finigan *et al.* 2005). It is noteworthy that plasma proteases frequently hydrolyze diverse substrates, including non-physiological substrates (Drucker 2007). The data presented here (Figs 5 and 6) indicate that protein C might have an important regulatory role as a ghrelin endopeptidase.

Both o-ghrelin(28) and the truncated form of ghrelin, o-ghrelin(15), appear to activate ghrelin signaling in a GHSR1a-dependent manner as shown in Figs 7 and 8

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(Bednarek *et al*. 2000, Holst *et al*. 2005, Moazed *et al*. 2009), whereas these peptides did not show any obvious effects on blood coagulation or platelet activation (Fig. 8B). In this study, the *EGR1* promoter served as a ghrelin-responsive reporter in HEK293 cells. Ghrelin and its short forms evoked reporter activity driven by the *EGR1* promoter in these cells. Our results also indicate that endogenous *Egr1* expression can be modulated by heterologously expressed GHSR1a in response to the addition of ghrelin derivatives in PC12h cells (Fig. 8A) although the mechanisms underlying *Egr1* activation by ghrelin observed in this study are not fully understood. Consistent with this, truncated forms of peptide hormones, including ghrelin (Bednarek *et al*. 2000) and pituitary adenylate cyclase-activating polypeptide (Bourgault *et al*. 2008), have been shown to bind and activate their specific receptors, even if the shortened peptides generally have reduced potency. However, short forms of ghrelin reportedly do not bind to ghrelin receptors or augment GH release from the pituitaries of neonatal rats. We speculate that the more hydrophobic character of o-ghrelin(15) as compared with o-ghrelin(28) might enhance its association with plasma macromolecules such as lipoproteins. This hypothesis is consistent with the observation that plasma ghrelin has been found in association with lipoproteins (De Vriese *et al*. 2007, Holmes *et al*. 2009). Interestingly, the two amino acid difference between human and mouse ghrelin appears to result in differences in delivery of the peptide across the blood–brain barrier (Banks *et al*. 2002).

We found that no positive signal was seen in response to d-ghrelin(28) (Fig. 7A), consistent with previous reports that desacyl ghrelin does not activate the ghrelin receptor (Bednarek *et al*. 2000, Johansson *et al*. 2008). In contrast, desacyl ghrelin has been reported to have synergistic or antagonistic effects on ghrelin-dependent physiological events (Filigheddu *et al*. 2007, Bulgarelli *et al*. 2009). Desacyl ghrelin fragments protect pancreatic β-cells from oxidative stress to prevent diabetes in streptozotocin-treated rats (Granata *et al*. 2012). The cellular response evoked by these forms of ghrelin seems to be due to an undefined pathway, distinct from the canonical GHSR1a signaling.

In the case of bioactive peptides, such as those in the glucagon/vasoactive intestinal peptide family of peptides, mature hormones are rapidly digested into inactive fragments by their specific peptidases (Drucker 2006). Dipeptidyl peptidase-IV (DPP-IV) is known to hydrolyze a variety of hormones, cytokines and enzymes, including glucagon-like peptides (GLPs or incretins) (Mentlein *et al*. 1993, Pauly *et al*. 1996, Hartmann *et al*. 2000) and GH-releasing hormone (Frohman *et al*. 1989). Whereas the majority of DPP-IV substrates are assumed to be 'pharmacological' but not 'physiological' targets (Drucker 2007), selective DPP-IV inhibitors have been useful for the treatment of type 2 diabetes mellitus by preventing GLP1 degradation (Holst *et al*. 2009, Mudaliar & Henry 2012). In contrast, for other hormone systems it has been shown that digestion of peptides in circulation can increase their ability to act as bioactive hormones (Schmaier & McCrae 2007). The renin–angiotensin system is one of the most famous examples of peptide processing machinery in circulation; the propeptide angiotensinogen is digested into bioactive angiotensin derivatives by endopeptidases, including rennin and angiotensin converting enzymes, and these processed forms go on to exert distinct cardiovascular actions (Fyhrquist & Saijonmaa 2008).

In this study, we have shown that protein C, a key regulator for anticoagulation, acts as a ghrelin endopeptidase. We also clearly demonstrate that truncated forms of ghrelin, in particular o-ghrelin(15), are present in mouse plasma. Moreover, we show that i.v. administration of ProTac facilitated o-ghrelin(15) production in mice (Fig. 6D) and found that digested forms of ghrelin exert cellular functions in a ghrelin-receptor-dependent fashion. Importantly, these results indicate that ghrelin might have diverse health-related effects under the influence of thrombotic/thrombolytic system, including anti-inflammatory (Li *et al*. 2004, Chorny *et al*. 2008, Waseem *et al*. 2008, Bulgarelli *et al*. 2009) and cardiovascular effects (Baldanzi *et al*. 2002, Rossi *et al*. 2007), in addition to its previously reported roles in GH release and appetite control (Wren *et al*. 2001, Asakawa *et al*. 2005, Drucker 2007, Sun *et al*. 2008). Synthetic human o-ghrelin(15) can also be cleaved into smaller peptide fragments, such as o-ghrelin(14) or o-ghrelin(13) (Fig. 1B). We speculate that an as yet unidentified carboxy-exopeptidase(s) in blood is responsible for generating shorter fragments from circulating o-ghrelin(15). Further study is necessary to develop a quantitative and highly sensitive assay for measuring the short forms of ghrelin and to elucidate the functional significance of these ghrelin derivatives. At the same time, we should not exclude the possibility that other ghrelin endopeptidases and/or exopeptidases are present in blood.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0529.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Funding

This work was supported by Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number 21770149).

Acknowledgements

The authors thank the Medical Research Center at Dokkyo Medical University School of Medicine (DMUSM) for help with DNA sequencing, and the Research Support Center at DMUSM for allowing us to use their facilities. They also thank Drs Ken Yanagisawa and Satoshi Ohta at Department of Biochemistry, Jichi Medical University for help with MALDI-TOF MS analysis, and Teijin Pharma (Tokyo, Japan) and Kaketsuken (Kumamoto, Japan) for supplying Anact C.

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Received in final form 11 October 2014
Accepted 27 October 2014
Accepted Preprint published online 27 October 2014

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