Characterisation of proghrelin peptides in mammalian tissue and plasma

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Abstract

Ghrelin is a 28 amino acid stomach peptide, derived from proghrelin(1–94), that stimulates GH release, appetite and adipose deposition. Recently, a peptide derived from proghrelin(53–75) – also known as obestatin – has been reported to be a physiological antagonist of ghrelin in the rat. Using four specific RIAs, we provide the first characterisation of proghrelin(1–94) peptides in human plasma, their modulation by metabolic manipulation and their distribution in mammalian tissues. ghrelin(1–28) immunoreactivity (IR) in human plasma and rat plasma/stomach consisted of major des-octanoyl and minor octanoylated forms, as determined by HPLC/RIA. Human plasma ghrelin(1–28) IR was significantly suppressed by food intake, oral glucose and 1 mg s.c. glucagon administration. ghrelin(1–28) IR and proghrelin(29–94) IR peptide distributions in the rat indicated that the stomach and gastrointestinal tract contain the highest amounts of the peptides. Human and rat plasma and rat stomach extracts contained a major IR peak of proghrelin(29–94)-like peptide as determined by HPLC/RIA, whereas no obestatin IR was observed. Human plasma proghrelin(29–94)-like IR positively correlated with ghrelin(1–28) IR, was significantly suppressed by food intake and oral glucose and shared with ghrelin(1–28) IR a negative correlation with body mass index. We found no evidence for the existence of obestatin as a unique, endogenous peptide. Rather, our data suggest that circulating and stored peptides derived from the carboxyl terminal of proghrelin (C-ghrelin) are consistent in length with proghrelin(29–94) and respond to metabolic manipulation, at least in man, in similar fashion to ghrelin(1–28).

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Introduction

The discovery of ghrelin as an endogenous, stomach-derived ligand that can activate the growth hormone secretagogue receptor-1a (GHS-R1a) at physiological concentrations has provided novel insights into regulation of growth hormone (GH) secretion, appetite and metabolism (Kojima *et al.* 1999, Korbonits *et al.* 2004, Sun *et al.* 2004, van der Lely *et al.* 2004). ghrelin is formed by enzymatic cleavage between amino acids Arg^{28}/Ala^{29} of proghrelin(1–94) and the novel post-translational addition of an *n-octanoic* moiety to Ser^{3} (Kojima *et al.* 1999). This addition is thought to account for approximately 50% of stored and 10% of circulating ghrelin in humans and rodents (Hosoda *et al.* 2000, Shiiya *et al.* 2002), suggesting that the *n-octanoic* acid moiety is rapidly degraded upon secretion from stomach cells. In the rat (Hosoda *et al.* 2000), immunoreactive (IR) ghrelin(1–28) peptides are most abundant in the stomach, followed by the duodenum, ileum and jejunum. Deposits of ghrelin IR are also found in the submaxillary gland, kidney, thyroid, hypothalamus and pituitary.

Early reports suggested that Ser^{3}-octanoylation was obligatory for ghrelin induced increases in GH release and adipose deposition (Kojima *et al.* 1999, Tschop *et al.* 2000, Wren *et al.* 2000), but des-octanoyl ghrelin, which does not stimulate GH-release, may also influence feeding and metabolism *in vivo* (Broglio *et al.* 2004, Thompson *et al.* 2004, Toshinai *et al.* 2006). The total plasma concentrations of ghrelin in humans are negatively correlated with body mass index (BMI; Tschop *et al.* 2001, Shiiya *et al.* 2002) and are suppressed by nutritional intake (Cummings *et al.* 2001) or glucose administration alone (Shiiya *et al.* 2002). However, the *in vivo* physiological contributions of putative ghrelin secretagogues, including insulin, glucagon and leptin remain unclear or controversial (Korbonits *et al.* 2004, Soule *et al.* 2005).

We have previously described immunoreactive peptide(s) derived from the carboxyl terminus (C) of proghrelin(1–94) (C-ghrelin) in the human circulation (Pemberton *et al.* 2003). More recently, a putative stomach peptide derived from the carboxyl terminus of rat C-ghrelin, named obestatin, was reported (Zhang *et al.* 2005). The structure of obestatin was deduced by amino acid sequencing of a purified 20 residue peptide sequence, combined with mass spectrometry data, to generate a 23 amino acid sequence (proghrelin(53–75)). Amidation of obestatin was assumed, but not verified, on the basis of a C-terminal Gly–Lys motif (Zhang *et al.* 2005).

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Subsequent *in vitro* and *in vivo* analysis suggested that amidated obestatin could suppress food intake, inhibit jejunal contraction and decrease body weight gain in rats via activation of the G-protein-coupled receptor GPR39. Surprisingly, plasma concentrations of obestatin were not modified by fasting–feeding manipulations in rats (Zhang *et al.* 2005) and GPR39 receptor transcripts show variable expression in hypothalamus tissue across species (Zhang *et al.* 2005, Jackson *et al.* 2006, Nogueiras *et al.* 2006).

The existence of obestatin in the human circulation has not been reported and whether it responds to metabolic manipulations in similar fashion to ghrelin is also unknown. Furthermore, the distribution and molecular forms of obestatin and other putative carboxyl terminal proghrelin-derived peptides in mammalian tissues and plasma has not been reported. Accordingly, we provide here: (i) documentation of the distribution and molecular forms of IR peptides derived from proghrelin(1–94) in rat tissues and plasma, (ii) the first description of plasma levels and molecular forms of proghrelin(1–94) peptides in the human circulation and (iii) the first human studies documenting the response of circulating carboxyl terminal proghrelin/obestatin-like peptides to fasting/feeding and metabolic manipulations.

Materials and methods

All human protocols were approved by the Upper South Regional Ethics Committee of the Ministry of Health, New Zealand functioning in accordance with the Guidelines on the Practice of Ethical Committees in Medical Research, 3rd ed. and all subjects gave full, informed consent. All animal investigations conformed to the UFAW Handbook on the Care and Management of Laboratory Animals.

Chemicals

Synthetic human and rat proghrelin(63–94) and proghrelin(53–75) (obestatin) peptides were obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Peptides derived from human proghrelin(29–40) were obtained from Auspep (Parkville, Australia). All buffer reagents were purchased from BDH and/or Sigma.

Human endocrine studies

Four human studies were performed: (i) IR proghrelin reference range study, (ii) overnight fasting/feeding replacement study, (iii) oral glucose loading and (iv) s.c. glucagon stimulation. For all studies, healthy human volunteers presented to the endocrine clinic at 0800 h after an overnight 12-h fast. Exclusion criteria were previous gastric surgery, cardiovascular, endocrine or psychiatric illness, use of prescribed medications, including H_2-receptor antagonists, proton pump inhibitors and diabetes mellitus.

For the healthy volunteer reference range study, blood samples were obtained from 56 healthy volunteers (35 women) with an average age of 47 ± 2 years (range 19–73 years) and BMI of $25{\cdot}7 \pm 0{\cdot}7$ kg/m^2. For the fasting/feeding replacement study, eight healthy volunteers (four women) with an average age of 47 ± 8 years and BMI of $22{\cdot}8 \pm 2{\cdot}6$ kg/m^2 received at 0830 h a test meal of 450 calories containing 50% carbohydrate, 30% fat and 20% protein. Blood samples were drawn at -15 and 0 min pre-meal, then $+30, +60, +90, +120$ and $+150$ min post-meal for the measurement of proghrelin peptides, insulin and glucose. All samples were kept on ice until centrifugation at 4 °C with plasma then stored at -80 °C until RIA. For oral glucose tolerance testing (OGTT), 11 volunteers (5 women) with a mean age of 47 ± 3 years and BMI of $23{\cdot}5 \pm 0{\cdot}8$ kg/m^2 drank a 200 ml solution containing 75 g glucose or 200 ml water on separate days, between 0800 and 0830 h. Venous blood was drawn at $t = -10$, 0, 15, 30, 45, 60, 90 and 120 min, centrifuged at 4 °C and plasma stored at -80 °C until specific assay for proghrelin peptides. Glucagon stimulation testing was carried out as previously described (Soule *et al.* 2005). Briefly, nine volunteers (three women) of mean age 47 ± 4 years and BMI $24{\cdot}1 \pm 0{\cdot}9$ kg/m^2 presented at 0800 h and received, in a non-randomised unblinded fashion, 1 mg glucagon in 1 ml saline or 1 ml saline s.c. Venous blood samples were taken at $t = 0$, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min post-administration. Plasma samples for RIA were immediately processed and stored at -80 °C.

Rat tissue and plasma collection

Six adult male Sprague–Dawley rats (250–325 g) were housed under controlled conditions and fasted for 12-h overnight. Animals were anaesthetised with 50 mg/kg sodium pentobarbital i.p., decapitated and trunk blood was collected into chilled Na^3-EDTA tubes. Plasma was then prepared by centrifugation and stored at -80 °C prior to RIA. Thyroid, submaxillary gland, atrium, left ventricular free wall, stomach, duodenum, pituitary, colon, kidney, thymus and adrenal tissue samples were rinsed in the ice-cold saline, weighed and quickly frozen at -80 °C prior to extraction and RIA.

Tissue and plasma extraction

Rat tissue extracts were prepared as previously described (Pemberton *et al.* 2004). Briefly, rat tissue samples were thawed on ice, diced and boiled gently in ten volumes of distilled water for 4–5 min to inactivate intrinsic proteases. After cooling on ice, samples were adjusted to 1 M acetic acid/20 mM HCl and homogenised for 1 min at high speed. Supernatants obtained from centrifugation at 4000 r.p.m./4 °C for 20 min were lyophilised and frozen at -80 °C prior to RIA and/or HPLC. Recovery of synthetic ghrelin(1–28), obestatin and proghrelin(63–94) added to tissue samples prior to boiling/homogenisation was 72 ± 3, 81 ± 4 and 68 ± 8% respectively ($n = 4$ for each peptide). All plasma samples

(rat and human) were extracted on SepPak cartridges as previously described (Pemberton *et al.* 2004), dried and stored at −20 °C prior to RIA and HPLC. The recovery of synthetic human and rat obestatin, ghrelin(1–28) and proghrelin(63–94) added to plasma and extracted using our procedures was 83±10, 73±5 and 66±4% respectively (n=5 for each peptide).

Hormone concentration analysis

Plasma insulin was determined by the Roche Elecsys two-site system (Roche Diagnostics). The detection limit for this assay was 2·5 pmol/l and the inter-assay coefficient of variation (CV) <5% between 64 and 860 pmol/l. Plasma glucose was determined on an Abbott Aeroset Analyser (Abbott Systems). GH was determined by IRMA (Bioclone, Marrickville, NSW, Australia) with a detection limit of 0·19 μg/l and inter-assay CV <10% between 1·8 and 5·9 μg/l.

In order to determine the concentrations of multiple potential peptides derived from proghrelin(1–94), we employed four specific RIAs that utilised antisera directed against four regions of the proghrelin(1–94) sequence (Fig. 1A). Cross-reactivity data for each of these assays are given in Table 2.

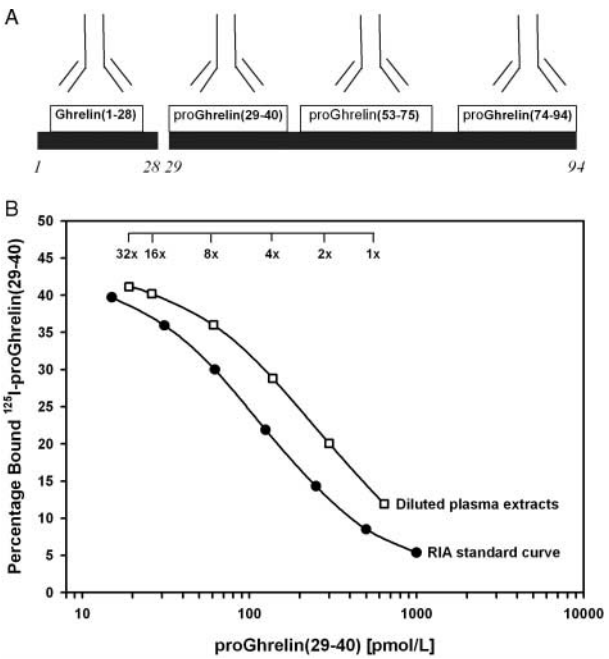

Figure 1 (A) Four antibody/RIA approach to the study of putative proghrelin(1–94) peptides. Antibodies generated against the regions shown were used to establish specific RIAs for the detection of putative proghrelin(1–94) peptides in tissues and plasma. (B) Representative dilution curve of a concentrated plasma extract drawn from a healthy volunteer (open squares, numbers above square indicate dilution factor) measured against the proghrelin (29–40) RIA standard curve (filled circles). Both curves are drawn on $\log_{10}$ scale.

Ghrelin(1–28) and proghrelin(74–94) RIAs Plasma ghrelin concentrations in human plasma were determined as previously reported (Pemberton *et al.* 2003, Soule *et al.* 2005), using an antibody that was 100% cross-reactive with octanoyl and des-octanoyl human ghrelin(1–28). Inter- and intra-coefficient of variation between 50 and 220 pmol/l for this assay were 11·4 and 9·2% respectively. Rat ghrelin(1–28) concentrations were determined in the same RIA format as for human samples, utilising a commercial anti-rat ghrelin(1–28) antibody and standards (Bachem, Lausanne, Switzerland) that had 1% cross-reactivity with human ghrelin(1–28). Human proghrelin(74–94) IR was determined as previously described (Pemberton *et al.* 2003). For rat samples, proghrelin(74–94) levels were determined against rat proghrelin(74–94) standards (0–28 000 pmol/l) using our previously described antiserum A14 (Pemberton *et al.* 2003) at a final dilution in the ratio of 1:3000. For this assay, inter- and intra-coefficient of variations between 100 and 3000 pmol/l were 8·2 and <5% respectively.

Proghrelin(29–40) RIA For the measurement of putative proghrelin(29–40) IR peptides, we generated a novel and specific RIA directed against amino acids 29–40 of the human proghrelin(1–94) sequence.

Proghrelin(29–40)-*Cys*[41] was coupled to malemide treated/N-e-maleimid D caproyloxy sulfosuccinimide ester (EMCS) derivatised BSA in PBS (pH 7·0) by gentle mixing at room temperature. Coupled peptide was emulsified with Freund's adjuvant and injected subcutaneously in two New Zealand white rabbits over four to five sites at monthly intervals. Rabbits were bled 12 days after injection to assess antibody (Ab) titres until adequate levels were achieved. For RIA, proghrelin(29–40) IR was determined using antiserum I32 at a final dilution in the ratio of 1:15 000.

Proghrelin(29–40)-*Tyr*[41] was iodinated via the Chloramine T method and purified on reverse phase HPLC as previously described (Pemberton *et al.* 2004). All samples, standards, radioactive traces and antiserum solutions were diluted in sodium-based assay buffer (Pemberton *et al.* 2003). The assay incubate consisted of 100 μl sample or standard (0–1000 pmol human proghrelin(29–40)) combined with 100 μl antiserum which was vortexed and incubated at 4 °C for 24 h. Then, 100 μl traces (4000–5000 c.p.m.) were added and further incubated for 24 h at 4 °C. Free and bound immunoreactivities were finally separated by the solid-phase second antibody method (donkey anti-rabbit Sac-Cel) and counted in a Gammamaster counter (LKB, Uppsala, Sweden).

Obestatin/proghrelin(53–75) RIA measurements in human and rat samples Obestatin (proghrelin(53–75)) immunoreactivities in (1) human plasma and (2) rat plasma and tissue samples were determined using commercially available species appropriate RIAs, according to the manufacturer's instructions (Phoenix Pharmaceuticals, Belmont, CA, USA). All standards, radioactive tracer and

antiserum solutions were diluted in the RIA buffer provided by the manufacturer.

HPLC

Stomach tissue and plasma extracts were subjected to size-exclusion HPLC (SE-HPLC) at room temperature on a TSK-Gel G2000SW peptide column (Toyosoda, Tokyo, Japan) using isocratic conditions of 60% acetonitrile/0·1% trifluoro-acetic acid (TFA) at a flow rate of 0·25/ml per min. Fractions were collected at 1-min intervals and subjected to proghrelin RIA. The SE-HPLC column was calibrated using dextran blue (Vo), cytochrome C ($M_r \sim 12\,400$), aprotinin ($M_r \sim 6500$), obestatin(53–75; $M_r \sim 2500$), urotensin II ($M_r \sim 1600$) and glycine (Vt). proghrelin-derived peptides identified by SE-HPLC/RIA were then further characterised on a Brownlee C_{18} reverse-phase HPLC (RP-HPLC) column (Applied Biosystems, Foster City, CA, USA) with a linear eluting gradient from 20 to 60% acetonitrile/0·1% TFA over 40 min, at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, dried under an air stream and subjected to specific RIA as for SE-HPLC. RP-HPLC was calibrated using synthetic amidated obestatin(53–75), octa-noyl ghrelin(1–28) and des-octanoyl ghrelin(1–28).

Statistical analysis

All results are presented as means ± S.D. Comparison of tissue concentrations of proghrelin peptide means was carried out using paired, two-tailed Student's *t*-test. Time–course data from human endocrine studies were analysed using two-way ANOVA for repeated measurements followed by least significant difference *post hoc* testing. Correlation analysis of plasma hormone concentrations was carried out using a general linear regression model. In all analyses, a P value $< 0{\cdot}05$ was considered significant.

Results

Characteristics of proghrelin peptide RIAs

The newly developed RIA for human C-ghrelin (progh-relin(29–40)) had a zero binding of 51·6 ± 0·3%, detection limit of 9·8 ± 2·2 pmol/l, effective displacement $(ED)_{50}$ of 122·2 ± 4·6 pmol/l and a non-specific binding of 3·9 ± 0·1% over 12 assays. Serial doubling dilution of human plasma extracts was in parallel with the assay standard curve (Fig. 1B). Inter- and intra-assay coefficient of variations between 50 and 250 pmol/l for this assay were < 9 and 5% respectively. The RIA for human obestatin had a zero binding of 24·5 ± 0·2%, detection limit of 30·4 ± 2·0 pmol/l, ED_{50} of 241·8 ± 10·2 pmol/l and a non-specific binding of 1·3 ± 0·1% over three assays. The RIA for rat obestatin had a zero binding of 39·2 ± 1·4%, detection limit of 19·4 ± 1·4 pmol/l, ED_{50} of 336·3 ± 9·8 pmol/l and a non-specific binding of 1·2 ± 0·1% over four assays.

Molecular forms of proghrelin peptides in human plasma

Proghrelin(74–94) and proghrelin(29–40) SE-HPLC/RIA analysis of human plasma extracts detected a single, major IR peak with a molecular mass ~7000, consistent in size with full length human proghrelin(29–94) (Fig. 2A). The C-ghrelin(74–94) RIA also detected a minor IR peak at $M_r \sim 10\,000$. No IR was detected consistent in size with putative proghrelin(29–52) or proghrelin(76–94) peptides in SE-HPLC profiles. The obestatin(53–75) RIA detected a single IR peak at $M_r \sim 7000$ in SE-HPLC fractions but did not detect any IR peaks in the molecular mass range ~1000–3000 (Fig. 2B). Ghrelin(1–28) RIA analysis of plasma extracts detected two peaks: a minor component at $M_r \sim 10\,000$ and a major peak eluting consistent with synthetic ghrelin(1–28) at $M_r \sim 3000$ (Fig. 2B).

RP-HPLC analysis of IR C-ghrelin observed on SE-HPLC also detected a single peak that was immunoreactive with both proghrelin(29–40) and proghrelin(74–94) RIAs (Fig. 2C). The small peak on SE-HPLC at $M_r \sim 10\,000$ that was immuno-reactive with proghrelin(74–94) and ghrelin(1–28) RIAs was not analysed further and assumed to be proghrelin(1–94)-like material. obestatin immunoreactivity identified on SE-HPLC eluted as two peaks on RP-HPLC, the first (and major) of which eluted consistent with IR C-ghrelin identified in Fig. 2C, with the second peak eluting three fractions later (Fig. 2D). No peaks eluting consistent with synthetic human obestatin (progh-relin(53–75)) were observed. ghrelin(1–28) IR of $M_r \sim 3000$ SE-HPLC almost totally comprised des-octanoylated ghre-lin(1–28) on RP-HPLC, with a small component eluting consistent with the octanoyl form (Fig. 2D).

Plasma concentrations of proghrelin peptides in fasted, healthy human volunteers

Since the proghrelin(29–40) RIA detected a single molecular species of C-ghrelin in human plasma and did not cross-react with the proghrelin(1–94)-like peptide observed by the proghrelin(74–94) RIA, all C-ghrelin measurements in human studies were performed with our 'in house' proghrelin(29–40) assay.

The mean plasma concentration of ghrelin(1–28) IR in 56 healthy, fasted volunteers (293 ± 171 pmol/l) was significantly higher than that of proghrelin(29–40) IR (114 ± 71 pmol/l, $P < 0{\cdot}01$ versus ghrelin(1–28)). However, IR ghrelin(1–28) and IR C-ghrelin concentrations both held significant negative correlations with BMI ($P < 0{\cdot}05$, Fig. 3A and B), but not with age or gender. Furthermore, plasma C-ghrelin and ghrelin(1–28) IR had a strong positive correlation ($P < 0{\cdot}001$, Fig. 3C).

Concentrations of proghrelin peptides in human plasma: response to endocrine manipulation

Plasma glucose and insulin concentrations were significantly increased within 30 min of feeding and returned to near

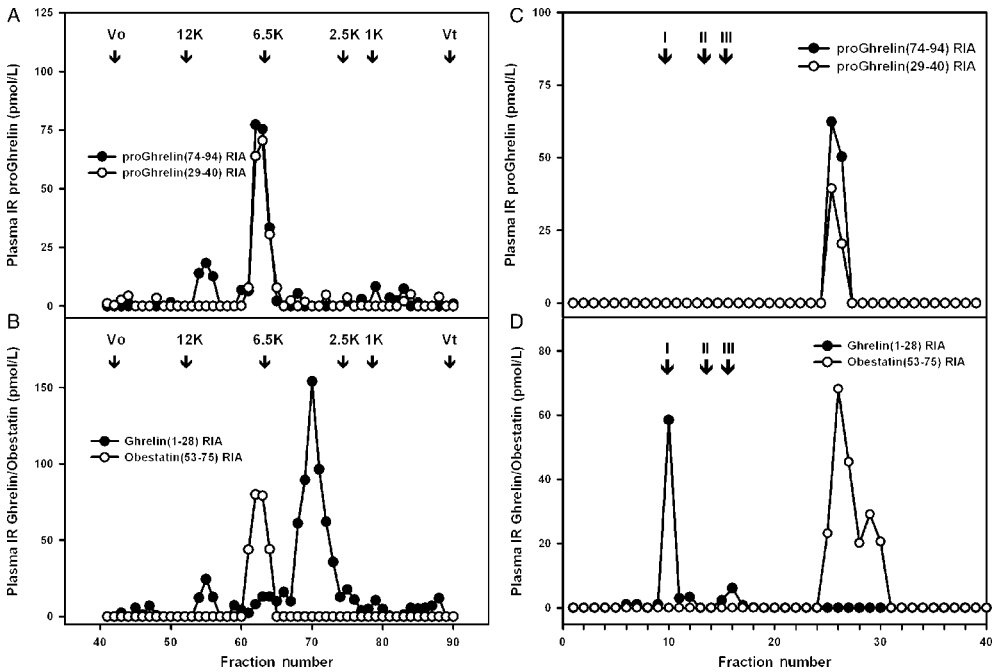

Figure 2 SE-HPLC/RIA and RP-HPLC/RIA profiles of IR proghrelin peptides in human plasma. The proghrelin(29–40) and proghrelin(74–94) RIAs both detected a major IR peak at $M_r \sim 7000$ on SE-HPLC (A) which eluted as a single peak on subsequent RP-HPLC (C). The obestatin(53–75) RIA detected a single IR peak at $M_r \sim 7000$ on SE-HPLC (B) which eluted as two peaks on RP-HPLC (D), at similar times to those observed in (C). No obestatin(53–75) IR was observed on SE-HPLC or RP-HPLC profiles. IR ghrelin(1–28) eluted on SE-HPLC as a major peak at $M_r \sim 3000$ and a minor peak at $M_r \sim 10\,000$ (B). The major $M_r \sim 3000$ ghrelin(1–28) peak eluted on RP-HPLC as a major peak at the position of des-octanoyl ghrelin with a minor component at the octanoyl form position (D). The minor IR $M_r \sim 10\,000$ ghrelin(1–28) peak on SE-HPLC eluted consistent with that observed by the proghrelin(74–94) RIA (A and B) and was concluded to be proghrelin(1–94)-like material. In C and D I, II and III are the elution positions of synthetic human des-octanoyl ghrelin, obestatin and octanoyl ghrelin peptides respectively.

fasting levels by 150 min in healthy human volunteers (Fig. 4A). Mean fasting plasma concentrations of ghrelin (364±212 pmol/l) and C-ghrelin IR (151±108 pmol/l) decreased significantly within 60 min of feeding and remained suppressed for the entire 150 min of the study (maximum 35±12% reduction in plasma ghrelin at $t=$ 90 min; maximum 38±14% reduction in C-ghrelin at $t=$ 120 min, both $P<0{\cdot}01$, Fig. 4B). Consistent with this observation, oral glucose administration also significantly suppressed fasting plasma ghrelin(1–28) (Fig. 5A) and C-ghrelin IR (Fig. 5B) over 120 min compared with control water administration ($P<0{\cdot}05$). As we have previously described (Soule *et al.* 2005), 1 mg i.m. glucagon significantly suppressed plasma ghrelin(1–28) concentrations compared with control saline administration ($P<0{\cdot}05$, Fig. 5C). In contrast, plasma C-ghrelin IR was not significantly suppressed by glucagon (Fig. 5D) although there was a trend for proghrelin(29–94)-like IR in the glucagon group to be lower than controls ($P=0{\cdot}10$). Plasma GH concentrations peaked at 150-min post-glucagon administration, an effect that was absent in saline controls (Fig. 5C and D).

Distribution of proghrelin peptides in rat tissue and plasma

Ghrelin(1–28), rat proghrelin(74–94) and obestatin(53–75) RIA IR concentrations in tissues and plasma from six fasted rats are summarised in Table 1. Both ghrelin(1–28) and proghrelin(74–94) IR had the same distribution profile, with highest concentrations observed in the stomach, followed by the duodenum and colon. proghrelin(74–94)-like IR was below assay detection limits in atrium, thyroid, ventricle and adrenal tissues. Surprisingly, rat obestatin(53–75) IR above detection limits was not observed in tissue extracts, including stomach (Table 1). Rat plasma ghrelin(1–28) IR was approximately one-third that of proghrelin(74–94) IR and one-half that of obestatin(53–75) IR (Table 1).

Molecular forms of proghrelin peptides in rat stomach and plasma

Ghrelin(1–28) IR in stomach extracts eluted as a single peak on SE-HPLC (Fig. 6A) consistent in size with previous reports (Kojima *et al.* 1999, Hosoda *et al.* 2000). In contrast, rat stomach proghrelin(74–94) IR consisted of two peaks; the first and major peak eluted at $M_r \sim 7000$, consistent in size with proghrelin(29–94), whereas the second and minor peak eluted

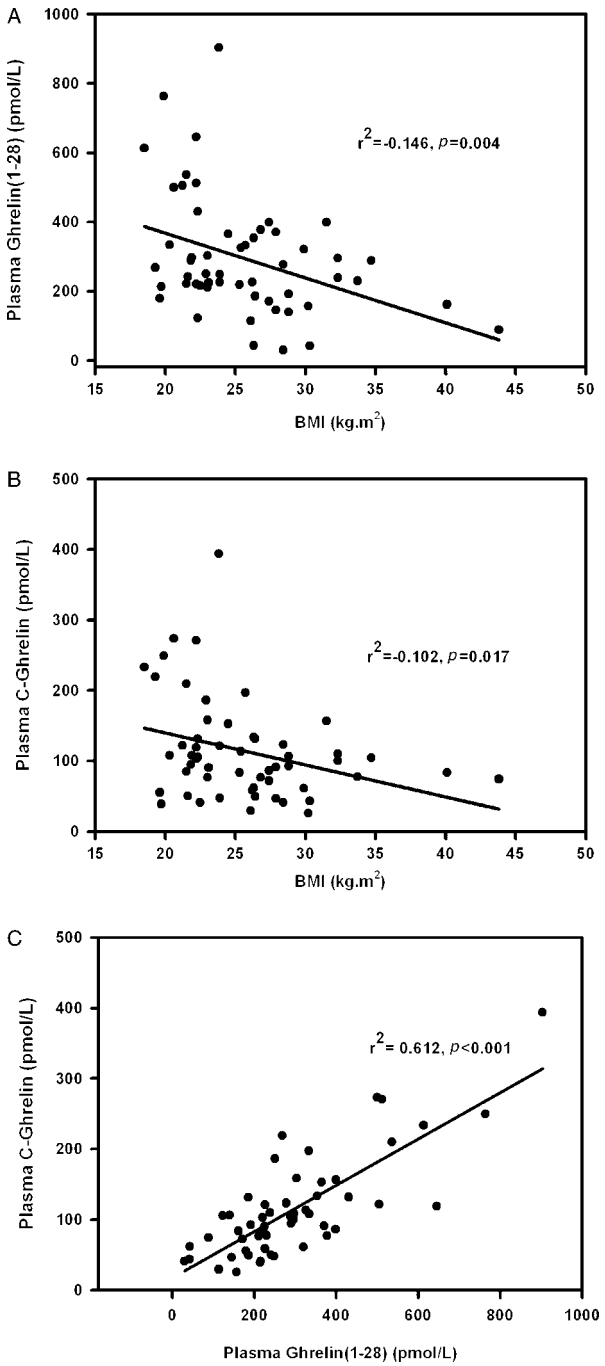

Figure 3 Plasma concentrations of ghrelin(1–28) IR (A) and C-ghrelin IR (B) in healthy humans both had a negative correlation with BMI ($P<0{\cdot}05$ for both). Accordingly, plasma ghrelin(1–28) IR and C-ghrelin IR had a strong positive correlation with each other ($P<0{\cdot}01$, C).

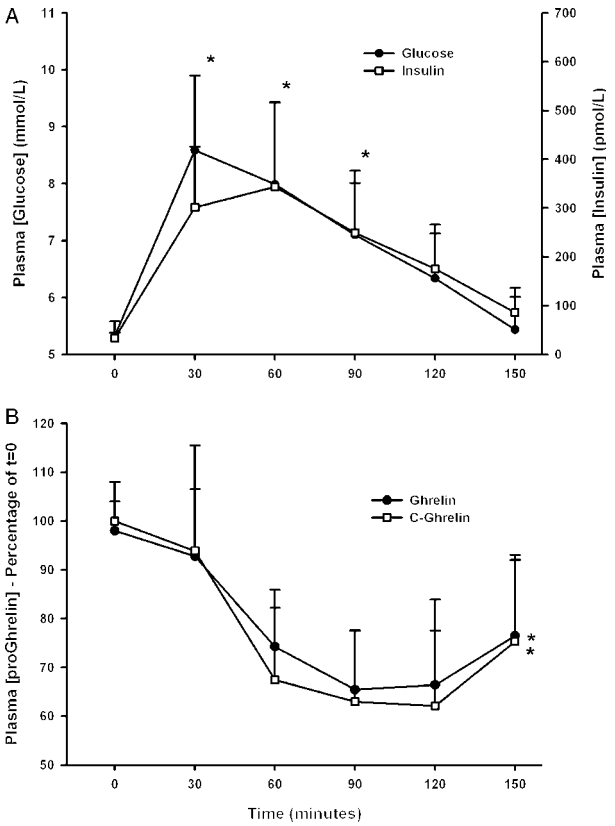

Figure 4 Response of plasma glucose and insulin (A) and ghrelin and C-ghrelin concentrations in humans ($n=8$) for 150 min after ingestion of a standardised meal given at time 0. Glucose and insulin concentrations significantly increased within 30 min (*both $P<0{\cdot}01$, A), whereas concomitant ghrelin and C-ghrelin concentrations significantly decreased (*both $P<0{\cdot}01$, B).

at $M_r \sim 1600$ (Fig. 6A). obestatin(53–75) IR in these same SE-HPLC profiles was not detected (Fig. 6A). Subsequent RP-HPLC analysis of ghrelin(1–28) IR in rat stomach extracts identified three IR peaks, the largest of which eluted consistent with octanoyl ghrelin(1–28), a minor peak of des-octanoyl ghrelin(1–28) and a third eluting three fractions after octanoyl ghrelin (Fig. 6C). The major IR proghrelin(74–94) peak on SE-HPLC ($M_r \sim 7000$) eluted on RP-HPLC as a single peak, later than all obestatin and ghrelin markers (Fig. 6C). The minor IR proghrelin(74–94) SE-HPLC peak ($M_r \sim 1600$) did not appear on RP-HPLC and was not investigated further.

SE-HPLC analysis of rat plasma extracts identified single IR peaks for both ghrelin(1–28) and proghrelin(74–94) IR, consistent with the major peaks observed in stomach extract SE-HPLC (Fig. 6B). The minor proghrelin(74–94) peak observed in rat stomach extracts ($M_r \sim 1600$) was not observed in plasma extracts. Plasma ghrelin(1–28) IR comprised a single peak of $M_r \sim 3000$ (Fig. 6B). obestatin(53–75) IR in rat plasma extracts comprised interfering material at $M_r \sim 300$, with no true obestatin IR peak occurring at $M_r \sim 2500$ (Fig. 6B). RP-HPLC analysis of IR ghrelin(1–28) and proghrelin(74–94) in rat plasma extracts provided data consistent with that observed in RP-HPLC analysis of rat stomach extracts (Fig. 6D).

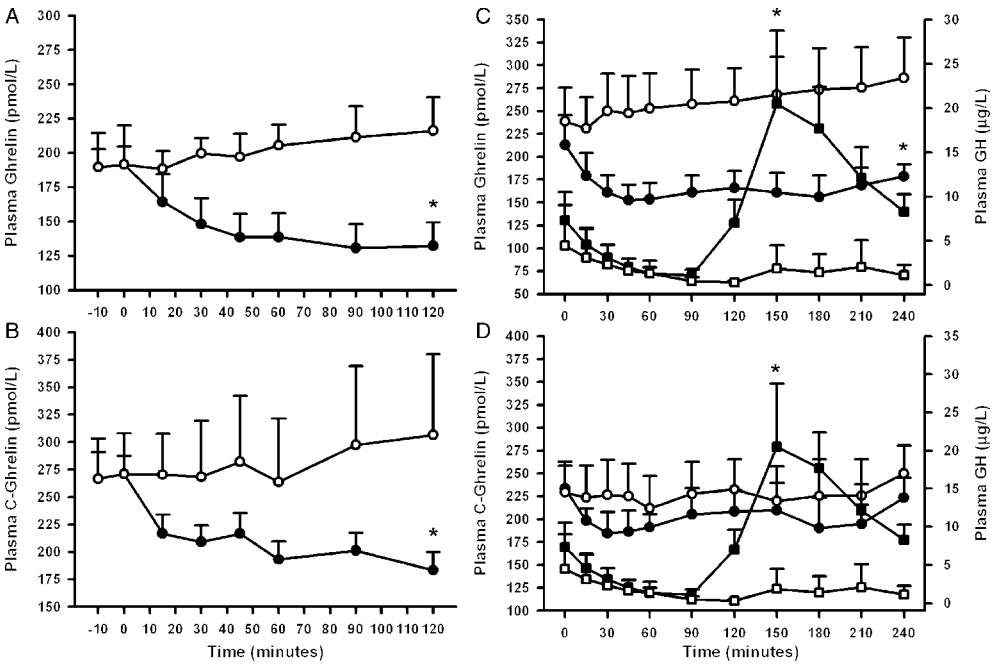

Figure 5 Plasma ghrelin (A and C) and C-ghrelin (B and D) concentrations in humans in response to oral glucose (A and B) and s.c. glucagon (C and D) stimulation. Both ghrelin (A) and C-ghrelin (B) concentrations were significantly suppressed by oral ingestion of 75 g glucose (filled circles, $*P<0{\cdot}05$) compared with control water ingestion (open circles). Plasma ghrelin concentrations (filled circles, C) were significantly suppressed within 60-min s.c. glucagon administration compared with control saline administration (open circles, C). In contrast, glucagon administration did not alter plasma C-ghrelin concentrations (filled circles) compared with control saline administration (open circles, D). GH concentrations peaked 150 min after glucagon administration ($*P<0{\cdot}05$, filled squares, C and D), whereas saline controls showed no significant change (open squares, C and D).

Discussion

The GH releasing and appetite-promoting hormone ghrelin comprises the first 28 amino acids of the proghrelin(1–94) peptide and has been intensively studied since its discovery in 1999 (Kojima *et al.* 1999, Korbonits *et al.* 2004, Kojima & Kangawa 2005). However, little is known about the circulating and tissue forms of putative peptides derived from the carboxyl terminus of proghrelin(1–94) (C-ghrelin).

Prior to performing endocrine manipulation studies, it was essential to define the circulating proghrelin species identified by each RIA. Therefore, consistent with previous reports

Table 1 Distribution and concentrations of proghrelin peptides in rat tissues and plasma using ghrelin(1–28), C-ghrelin(74–94) and obestatin(53–75) RIAs. Data are pmol/g per wet weight, mean±S.D.

	ghrelin(1–28) RIA	C-ghrelin(74–94) RIA	obestatin(53–75) RIA
Organ			
Stomach	454·90±107·50	325·44±241·30	ND
Duodenum	11·80±3·90	91·42±44·90	ND
Pituitary	4·1±1·3	2·4±1·1	ND
Colon	0·80±0·60	9·70±8·70	ND
Kidney	0·20±0·05	3·20±1·40	ND
Thymus	0·20±0·16	0·40±0·70	ND
Submaxillary gland	0·20±0·04	12·40±9·47	ND
Atrium	0·10±0·07	ND	ND
Thyroid	0·10±0·0·05	ND	ND
Left ventricle	0·10±0·03	ND	ND
Adrenal gland	0·10±0·03	ND	ND
Plasma (pmol/l)	57·70±7·70	177·70±20·90	ND

ND, not detected.

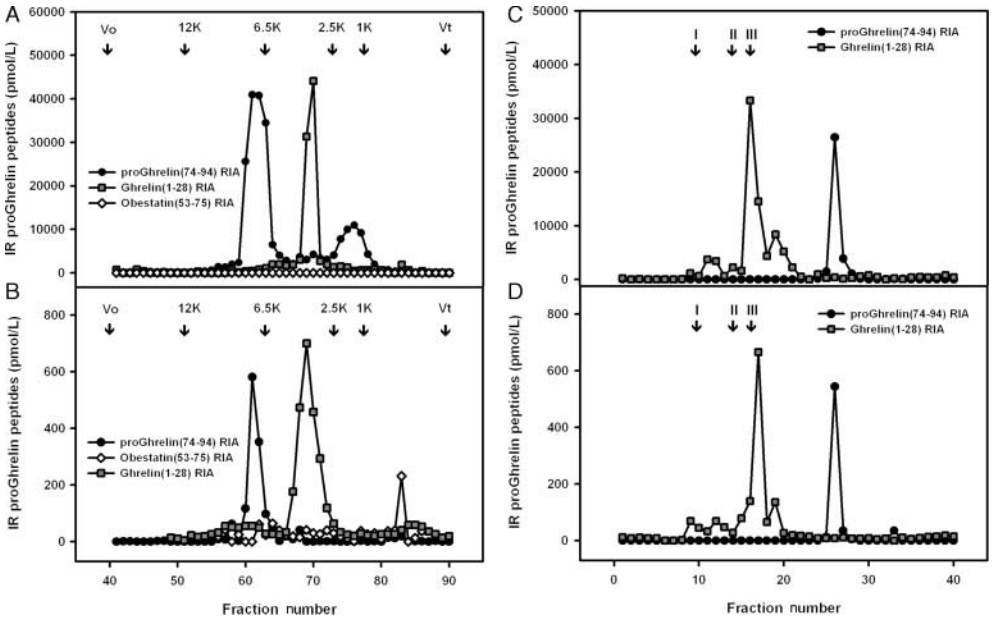

Figure 6 (A) In rat stomach, proghrelin(74–94) IR eluted as two peaks at $M_r \sim 7000$ and 1600 on SE-HPLC. IR ghrelin(1–28) eluted as a single peak at $M_r \sim 3000$. No IR obestatin(53–75) peaks were observed. (B) Rat plasma extracts contained a single peak of proghrelin(74–94) IR at $M_r \sim 7000$, ghrelin(1–28) IR at $M_r \sim 3000$ and obestatin(53–75) IR at $M_r \sim 300$ on SE-HPLC. Note that neither proghrelin(74–94) IR at $M_r \sim 1600$ nor proghrelin(1–94) IR at $M_r \sim 10\,000$ was observed. (C) RP-HPLC analysis of (A) IR ghrelin(1–28) identified des-octanoyl and octanoyl ghrelin eluting consistent with synthetic standards. IR proghrelin(74–94) from (A) eluted as a single peak, much later than column standards (I, des-octanoyl ghrelin; II, obestatin(53–75); III, octanoyl ghrelin). Note that proghrelin(74–94) IR at $M_r \sim 1600$ from (A) did not resolve on RP-HPLC. (D) Rat plasma ghrelin(1–28) IR from (B) eluted as des-octanoyl and octanoyl forms consistent with those observed in (C). Rat plasma proghrelin(74–94) IR from (B) also eluted a single peak consistent with that observed in (C). obestatin(53–75) IR from (B) did not bind to the RP-HPLC column.

(Kojima *et al.* 1999, Hosoda *et al.* 2000), the results from SE-HPLC/RP-HPLC coupled with specific RIA suggest that in human plasma IR ghrelin(1–28) at $M_r \sim 3000$ comprises two molecular species, with des-octanoyl ghrelin(1–28) being the major form. All three assays directed to the proghrelin(29–94) sequence, namely proghrelin(29–40), proghrelin(53–75) and proghrelin(74–94), detected the same single major peak on SE-HPLC at $M_r \sim 7000$, strongly suggesting that the entire C-ghrelin peptide (proghrelin(29–94)) circulates in humans. We found no evidence for the existence of smaller peptides, including obestatin. Indeed, this latter assay only detected the proghrelin(29–94) peak on SE-HPLC at $M_r \sim 7000$. Both the ghrelin(1–28) and the proghrelin(74–94) RIAs detected a high molecular weight peak on SE-HPLC at a position consistent with the molecular weight of proghrelin(1–94), $M_r \sim 10\,000$. The lack of cross-reactivity of our proghrelin(29–94) RIA with this peak is likely due to the antiserum requiring a free amino terminal group on residue 29, evidenced by the lack of cross-reactivity of this antiserum with the N-terminally extended peptide proghrelin(20–40) in cross-reactivity studies (Table 2). Taken together, our results show that ghrelin(1–28) and C-ghrelin(29–94) are the major peptides derived from the proghrelin precursor in the human circulation, with a minor circulating proghrelin component also present. Previously, we reported that circulating C-ghrelin in the human circulation ranged between $M_r \sim 3500$ and 7000 (Pemberton *et al.* 2003). However, these preliminary results were based on a single C-ghrelin RIA (proghrelin(74–94)) and utilised a lower concentration of acetonitrile on SE-HPLC. Combination of proghrelin(29–40), proghrelin(53–75) and proghrelin(74–94) RIAs together with increasing the SE-HPLC acetonitrile concentration (from 20 to 60%) resolved this discrepancy, suggesting that C-ghrelin can interact non-specifically with SE-HPLC matrices unless sufficient organic solvent is present.

Fasting plasma levels of ghrelin(1–28) and C-ghrelin(29–94) IR were positively correlated with one another and both were negatively correlated with BMI. We previously reported that plasma C-ghrelin IR did not appear to have any association with BMI (Pemberton *et al.* 2003), based upon measurements with our C-ghrelin(74–94) RIA which includes a small amount of proghrelin-like material in its measurement. In contrast, our present results – utilising our novel proghrelin(29–40) RIA, which detects only C-ghrelin(29–94)-like peptides – show that plasma C-ghrelin concentrations may have the same relationship with BMI as for ghrelin (Tschop *et al.* 2001, Shiiya *et al.* 2002). Consistent with previous reports (Ariyasu *et al.* 2001,

Table 2 Cross-reactivity data (in percent) for human and rat ghrelin(1–28), proghrelin(29–40), ghrelin(74–94) and obestatin(53–75) RIAs

	ghrelin(1–28) RIA	proghrelin(29–40) RIA	proghrelin(74–94) RIA	obestatin(53–75) RIA
Peptide				
ghrelin	100	<0·05	<0·01	<0·01
Des-octanoyl ghrelin	100	<0·01	<0·01	<0·01
proghrelin(29–40)	<0·05	100	<0·01	<0·01
proghrelin(20–40)	<0·10	<0·10	<0·02	<0·01
proghrelin(74–94)	<0·02	<0·01	100	<0·01
obestatin(53–75)	<0·01	<0·01	<0·01	100
Motilin	<0·01	<0·01	<0·01	<0·01
PYY(3–36)	<0·03	<0·01	<0·01	<0·01
Neuromedin U	<0·02	<0·01	<0·01	<0·01
Neuropeptide Y	<0·01	<0·01	<0·01	<0·01
Vasoactive intestinal polypeptide	<0·01	<0·01	<0·01	<0·01
Growth hormone-releasing hormone	<0·01	<0·01	<0·01	<0·01
Orexin	<0·01	<0·01	<0·01	<0·01
Glucagon-like peptide 1	<0·01	<0·01	<0·01	<0·01
Somatostatin	<0·01	<0·01	<0·01	<0·01

Cummings *et al.* 2001), our fasting–feeding study results show that human plasma concentrations of ghrelin are decreased after feeding. We extend this data to show that plasma C-ghrelin concentrations are also decreased in response to feeding and our subsequent OGTT study suggests that glucose loading may be a common mechanism underlying these observations. Glucose/caloric loading may inhibit the secretion of ghrelin from the X/A-like cells in stomach mucosa (Tschop *et al.* 2000, Ariyasu *et al.* 2001, Shiiya *et al.* 2002). However, the exact pathways mediating glucose-induced inhibition of ghrelin/C-ghrelin are presently unclear.

We have previously reported that increases in plasma ghrelin in humans are unlikely to be responsible for glucagon's induced increases in GH (Soule *et al.* 2005), although data from *in vitro* rat studies have demonstrated the ability of glucagon to stimulate ghrelin secretion from the stomach (Kamegai *et al.* 2004). In contrast with plasma ghrelin levels, C-ghrelin levels tended to be lower in the glucagons-treated group, but this did not reach significance ($P=0{\cdot}10$). This suggests that ghrelin secretion may be more sensitive to glucagon antagonism than C-ghrelin secretion or that it may have a shorter half-life in the circulation. Presumably, C-ghrelin is stored in the same stomach cell type (X/A) as ghrelin, but detailed secretion and clearance pattern studies as those done for ghrelin (Cummings *et al.* 2001) have not been reported. Our results do not preclude C-ghrelin from having a direct action upon GH-secretion; rather – like ghrelin – it is unlikely to be a direct stimulus for GH in the clinical setting of s.c. glucagon stimulation. Elucidation of any biological effects of C-ghrelin upon GH secretion and other hormonal/haemodynamic parameters will require yet more precise identification of the circulating peptide (including identification of putative post-translational modifications as known for ghrelin) followed by dose–response studies.

We found the distribution of IR C-ghrelin in rat tissues to be equivalent with that of ghrelin and that the stomach and gastrointestinal tract contain the highest concentrations on a pmol/g per wet weight basis. This is in agreement with previous reports describing the gene expression and immunoreactive peptide levels of ghrelin in rats (Kojima *et al.* 1999, Hosoda *et al.* 2000) and humans (Date *et al.* 2000, Ariyasu *et al.* 2001). Therefore, like ghrelin, it is probable that the primary source of circulating C-ghrelin is the stomach. We did not detect IR C-ghrelin in heart, thyroid or adrenal samples, but we cannot exclude the presence of the peptide in these tissues. To our surprise, we did not detect IR obestatin in rat stomach tissue extracts, instead observing only a proghrelin(29–94)-like peptide on SE-HPLC. The reason for this is unclear, as the rat obestatin RIA purchased from Phoenix Pharmaceuticals recognised synthetic amidated rat obestatin added to plasma samples and synthetic obestatin peptide was well recovered (>80%) through our extraction procedures. A recent report, utilising the same commercial obestatin RIA as we used, documented stomach concentrations of IR obestatin to be 0·2% those of IR ghrelin(1–28) in 21-day-old perinatal rats (Chanoine *et al.* 2006) although the molecular form was not described. These, and our, results contrast markedly with those of Zhang *et al.* (2005) who reported an approximate 2:1 ratio of IR ghrelin(1–28) to obestatin in rat stomach extracts subjected to G50 Sephadex chromatography.

Rat stomach and plasma ghrelin(1–28) IR eluted on HPLC/RIA consistent with the octanoyl form, with only a small amount of des-octanoyl form detected. This suggests our antibody was more specific to the octanoyl form as previous work has clearly shown that rat stomach extracts contain approximately a 1:1 ratio of octanoyl:des-octanoyl ghrelin(1–28), whereas rat plasma contains more than 90% of des-octanoyl form (Kojima *et al.* 1999, Hosoda *et al.* 2000). Therefore, it is possible that our rat tissue and plasma ghrelin concentration data underestimate true endogenous levels of the hormone.

Several recent studies have not been able to reproduce the originally reported anti-orexigenic effects of obestatin

(Gourcerol *et al.* 2006, Nogueiras *et al.* 2006, Samson *et al.* 2006), the ability of obestatin to activate GPR39 receptors has also not been reproduced (Holst *et al.* 2006) and GPR39 receptor transcripts have not been found in the hypothalamus (Jackson *et al.* 2006, Nogueiras *et al.* 2006), a logical target organ of obestatin. Therefore, in combination with our data which does not support the existence of obestatin peptide in humans or rats, the accumulating evidence does not support the concept of ghrelin and obestatin as physiological antagonists (Zhang *et al.* 2005).

In summary, utilising a four RIA approach, we have documented immunoreactive proghrelin(1–94)-derived peptides in mammalian tissues and plasma. We report that the stored and circulating form of C-ghrelin in rats and humans is C-ghrelin(29–94)-like material and that the tissue distribution of C-ghrelin matches that of ghrelin. Therefore, it is probable that the stomach is the main source of circulating C-ghrelin. We did not either find any evidence for obestatin peptides, circulating as distinct entities in the human and rat circulation, or as a secretable hormone in rat tissues. In agreement with our previous work (Soule *et al.* 2005), it appears unlikely that peptides derived from proghrelin(1–94) are responsible for exogenous glucagon stimulation-induced increases in plasma GH in humans. Finally, immunoreactive C-ghrelin in the human circulation is positively correlated with plasma levels of ghrelin, and fasting blood levels, also like ghrelin are negatively correlated with BMI. Both ghrelin and C-ghrelin in humans are reduced by nutritional intake, and glucose is one potential mechanism responsible for this.

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