

## CLINICAL STUDY

# The pharmacokinetics of acyl, des-acyl, and total ghrelin in healthy human subjects

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## Abstract

**Background:** Ghrelin stimulates GH secretion and regulates energy and glucose metabolism. The two circulating isoforms, acyl (AG) and des-acyl (DAG) ghrelin, have distinct metabolic effects and are under active investigation for their therapeutic potentials. However, there is only limited data on the pharmacokinetics of AG and DAG.

**Objectives:** To evaluate key pharmacokinetic parameters of AG, DAG, and total ghrelin in healthy men and women.

**Methods:** In study 1, AG (1, 3, and 5 µg/kg per h) was infused over 65 min in 12 healthy (8 F/4 M) subjects in randomized order. In study 2, AG (1 µg/kg per h), DAG (4 µg/kg per h), or both were infused over 210 min in ten healthy individuals (5 F/5 M). Plasma AG and DAG were measured using specific two-site ELISAs (study 1 and 2), and total ghrelin with a commercial RIA (study 1). Pharmacokinetic parameters were estimated by non-compartmental analysis.

**Results:** After the 1, 3, and 5 µg/kg per h doses of AG, there was a dose-dependent increase in the maximum concentration ( $C_{max}$ ) and area under the curve ( $AUC_{(0-last)}$ ) of AG and total ghrelin. Among the different AG doses, there was no difference in the elimination half-life, systemic clearance (CL), and volume of distribution. DAG had decreased CL relative to AG. The plasma DAG:AG ratio was ~2:1 during steady-state infusion of AG. Infusion of AG caused an increase in DAG, but DAG administration did not change plasma AG. Ghrelin administration did not affect plasma acylase activity.

**Conclusions:** The pharmacokinetics of AG and total ghrelin appears to be linear and proportional in the dose range tested. AG and DAG have very distinct metabolic fates in the circulation. There is deacylation of AG in the plasma but no evidence of acylation.

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## Introduction

Ghrelin is a 28-amino acid peptide secreted mainly from the neuroendocrine X/A-like cells in the gastric mucosa with smaller amounts derived from the enteroendocrine cells in the proximal small intestine (1, 2) and from pancreatic islets (3, 4). During synthesis, a significant proportion of the peptide undergoes posttranslational modification in which the serine<sup>3</sup> residue is covalently linked to a medium-chain fatty acid. This acylation process is required for the peptide to bind to its cognate receptor, the GH secretagogue receptor (GHSR) 1a (1), and most biological actions ascribed to ghrelin require the activation of GHSR-1a (5). The ghrelin isoform that has not been acylated or has had the acyl group enzymatically removed, des-acyl ghrelin (DAG), does not bind to the classical ghrelin receptor (1) but a

variety of GHSR-1a-independent effects on insulin secretion (6), osteoblast growth (7), and lipid metabolism in adipocytes (8) have been attributed to it. Given the 'antidiabetic' properties of DAG as opposed to the 'prodiabetic' effects of acyl ghrelin (AG) observed in preclinical and clinical studies, DAG analogs have been developed recently and their potential as therapeutics for type 2 diabetes is being investigated (9). Acylation of ghrelin is a specific process primarily mediated by the recently discovered enzyme ghrelin O-acyl transferase (GOAT) (10, 11). It is unclear what percentage of ghrelin is acylated intracellularly, but both AG and DAG are detectable in the circulation where they exist in reported ratios of 1:4 to 1:9 depending on the sample preservation methods, assay, species, or nutritional state, with AG being the less common species (1, 12).

Ghrelin is the only known circulating factor that promotes food intake. In healthy subjects, plasma ghrelin levels rise progressively before meals and fall to a nadir within 2 h after eating, with changes in plasma levels during meals that vary two- to fourfold (12, 13). Interestingly, total ghrelin levels do not increase significantly after long-term food deprivation, but the two isoforms follow distinct patterns: DAG remains at peak pre-fasting levels while AG concentrations settle near what is the usual nadir following a typical meal (12). Consistent with these changes in plasma ghrelin isoforms, expression of *Goat* (*Mboat4*) mRNA decreases in the stomach of fasting mice (14). These observations suggest that ghrelin secretion and acylation are processes that may be separately regulated. Detailed information about ghrelin elimination in healthy individuals is limited. Substantial hepatic extraction of AG has been reported (15), but it is not clear whether deacylation takes place primarily in the liver. In contrast, there is some evidence that renal clearance (CL) may be a major pathway for the CL of DAG (16, 17) typical for the CL of small peptides. In addition to hepatic and renal elimination, AG is deacylated to DAG by serum and tissue esterases such as butyrylcholinesterase (BuChE) (18). It is unclear whether exogenous ghrelin administration alters the deacylation process in humans.

Given many of the observed opposing metabolic effects of AG and DAG and suggestion of different routes of elimination, it is important to understand the metabolism of these two isoforms and their pharmacokinetic properties when designing physiology or pharmacology studies for clinical research. The objective of this study was to determine basic pharmacokinetic parameters of AG and DAG in healthy individuals with normal liver and kidney function. We focused on the relation of ghrelin dose to change plasma levels and systemic exposure as measured by area under the plasma concentration curve (AUC) and peak plasma levels ( $C_{max}$ ). We also examined the dynamic changes in AG and DAG concentration during continuous AG, DAG, or combined AG and DAG infusions in a separate study of ten healthy subjects. Finally, we assessed the effects of AG infusion on the serum activity of the ghrelin deacylation enzyme BuChE.

## Materials and methods

### Study design

**Study 1** This was a randomized, single-blinded study designed to examine the effect of ghrelin on insulin secretion and glucose tolerance in healthy subjects (19). Briefly, healthy volunteers between the ages of 18 and 55 years with a BMI between 18 and 29 kg/m<sup>2</sup> were recruited from the greater Cincinnati area. Subjects with a history or clinical evidence of impaired fasting

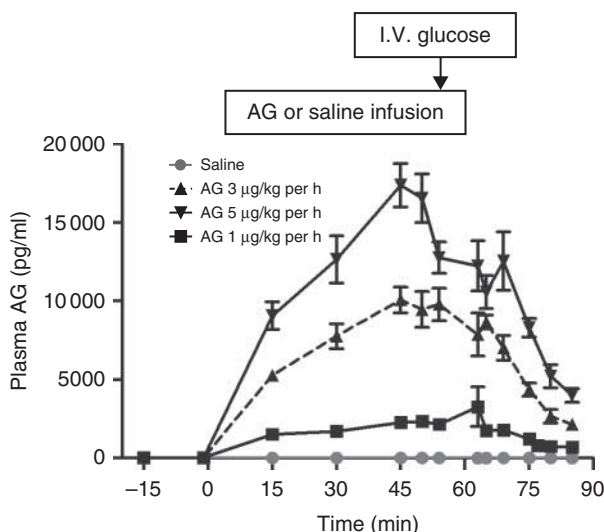
glucose or diabetes mellitus, recent myocardial infarction, congestive heart failure, active liver or kidney disease, GH deficiency or excess, neuroendocrine tumor, anemia, or those who were on medications known to alter insulin sensitivity were excluded. Study procedures were performed at the Cincinnati Children's Medical Center Clinical and Translational Research Center (CTRC). All study participants signed informed consent documents that had been approved by the University of Cincinnati Institutional Review Board.

After a 10- to 12-h fast, either synthetic human AG (Bachem AG, Rubendorf, Switzerland) at doses of 1, 3, and 5 µg/kg per h or 0.9% saline was infused for a total of 65 min on four separate days. An i.v. bolus of glucose (11.4 g/m<sup>2</sup> body surface area) was given at 55 min as part of the intravenous glucose tolerance test (IVGTT) to assess acute insulin response to i.v. glucose. After ghrelin infusion was stopped at 65 min, seven additional blood samples were taken at 2- to 5-min intervals for 20 min to measure ghrelin CL rate. Blood samples were stored on ice and plasma separated by centrifugation within an hour. The plasma samples were stored at -80 °C until further analysis.

**Study 2 Subjects.** Healthy volunteers, similar to those in study 1, between the ages of 18 and 50 years with a BMI between 18 and 29 kg/m<sup>2</sup> were recruited from the greater Cincinnati area. All study participants signed informed consent documents that had been approved by the University of Cincinnati Institutional Review Board.

Subjects received: i) synthetic human AG (Bachem Americas, Torrance, CA, USA; 0.28 µg/kg bolus followed by 1 µg/kg per h rate of infusion); ii) human DAG (C S Bio Co., Menlo Park, CA, USA; 1.1 µg/kg bolus followed by 4 µg/kg per h continuous infusion); iii) a combined infusion of AG and DAG (same rate as single infusions); or iv) saline for a total of 210 min on four separate study days in randomized order. A frequently sampled IVGTT (FSIVGTT) designed to quantify insulin secretion and insulin sensitivity began 30 min after the infusions were started and continued for 180 min. Ghrelin measurements were obtained at 0, 5, 15, 25, 30, 60, 90, 150, and 210 min during the infusion period.

**Studies 1 and 2 Sample analyses.** Blood samples were collected into 3 ml EDTA-plasma tubes containing 0.06 ml AEBSE, a protease and esterase inhibitor (4 mM final concentration). Following centrifugation, 200 µl 1 N (0.2 mM) HCl was added to every milliliter of plasma. Plasma total immunoreactive ghrelin was measured by RIA using a commercially available kit (Millipore, Billerica, MA, USA). Lower and upper limits of detection were 40 and 2560 pg/ml with intra- and interassay CV of 4 and 14.7% respectively. AG and DAG levels were measured using separate sensitive and specific two-site sandwich ELISAs. The sensitivity of the AG assay was 6.7 pg/ml with an intra- and interassay CV of ~14 and 18% (12). The sensitivity of



**Figure 1** Plasma acyl ghrelin (AG) levels after 1, 3, and 5 µg/kg per h doses of AG or saline continuous i.v. infusion (0–65 min) in healthy men and women in study 1. Data are presented as mean  $\pm$  S.E.M.

the DAG assay was 4.6 pg/ml with an intra- and interassay CV of  $\sim 13$  and 20% (12). BuChE activity was determined by colorimetric assay following established methods (20). Assay results are reported as enzyme units relative to the activity of a purified bovine BuChE standard (Sigma–Aldrich #C1057) run on each plate. The assay showed an intra-assay CV of 3.6% and an interassay CV of 7.2%. All samples were run in duplicates, and all specimens from a given participant were run in the same assay.

**Pharmacokinetic analysis.** The pharmacokinetic data were analyzed with the non-compartmental model using WinNonlin 6.2 (Pharsight, Inc., Cary, NC, USA). Peak plasma levels ( $C_{max}$ ), time required to achieve  $C_{max}$  ( $T_{max}$ ), mean residence time (MRT), and volume of distribution ( $V_d$ ) were estimated (21). The elimination half-life ( $t_{1/2}$ ) of ghrelin was calculated as the ratio of  $\ln 2/\lambda_z$  ( $\lambda_z$ , slope of the terminal portion of the concentration vs time curve after the termination of ghrelin infusion).  $AUC_{0-last}$  was computed using the linear trapezoidal rule. Average of the two plasma ghrelin levels before ghrelin or saline administration served as baseline. Systemic CL was estimated by dose/ $AUC_{0-last}$ .

### Statistical analysis

All pharmacokinetic estimates are expressed as mean  $\pm$  S.D. Dose proportionality in  $C_{max}$  and AUC were assessed using both linear regression and power law. Pharmacokinetic parameters between low and high ghrelin doses were compared using a paired Student's

*t*-test. Repeated measures ANOVA was used to compare parameters across the three ghrelin doses. Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

## Results

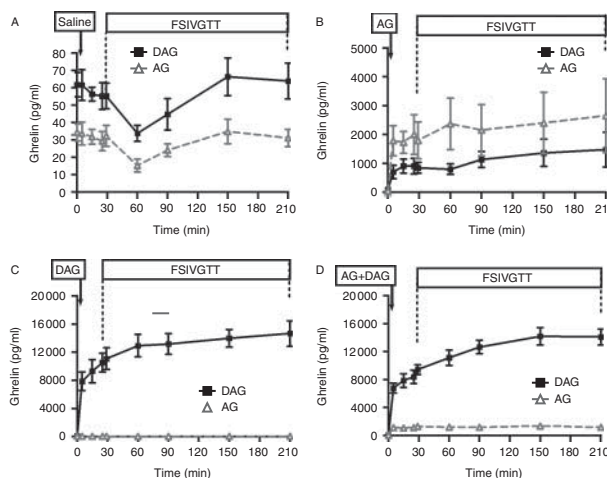
### Subject characteristics

Subject characteristics for study 1 were described in detail elsewhere (19). In brief, 12 healthy men and women (eight male/four female) with an average age of  $26.0 \pm 11.4$  years (mean  $\pm$  S.D.) and BMI of  $24.1 \pm 4.2$  kg/m<sup>2</sup> completed the study. In study 2, 17 subjects (nine male/eight female) completed the study. AG and DAG measures were obtained in 10 of the 17 subjects (five female/five male age  $25.9 \pm 6.1$  years, BMI  $24.5 \pm 3.4$  kg/m<sup>2</sup>).

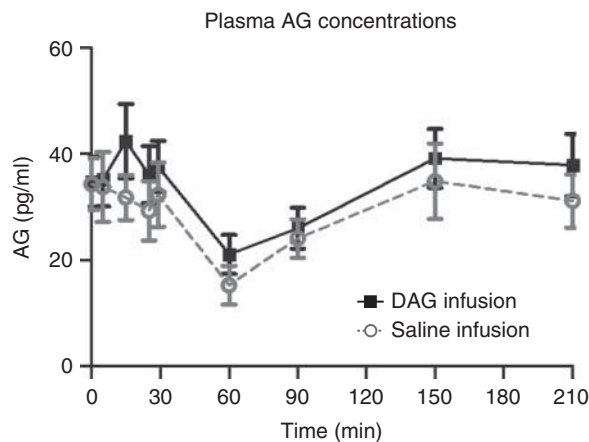
### Plasma concentrations of ghrelin during infusions

In study 1, infusions of AG at 1, 3, and 5 µg/kg per h raised plasma concentrations of AG to peak concentrations of  $3.9 \pm 3.5$ ,  $11.7 \pm 2.4$ , and  $19.6 \pm 2.3$  ng/ml, corresponding to 118-, 355-, and 594-fold increases from the mean baseline AG concentrations of  $0.033 \pm 0.024$  ng/ml respectively. A plasma steady state was reached at 45 min after the start of ghrelin infusion. Overall, the mean plasma concentration of AG demonstrated a dose–response increase during the continuous i.v. infusion (Fig. 1).

In study 2 (Fig. 2 A, B, C, and D), the 1 µg/kg per h AG infusion raised plasma AG concentrations from  $0.043 \pm 0.038$  to  $1.93 \pm 1.30$  ng/ml and DAG concentrations from  $0.078 \pm 0.045$  to  $1.29 \pm 1.12$  ng/ml



**Figure 2** (A, B, C, and D) Plasma AG and DAG concentrations during i.v. AG and DAG infusions in healthy men and women ( $n=10$ ) in study 2. Data are presented as mean  $\pm$  S.E.M.



**Figure 3** Plasma AG concentration during DAG and saline infusions in 10 healthy men and women (Study 2). Data are presented as mean  $\pm$  SEM.

respectively, corresponding to a 44- and 17-fold increase from baseline (Fig. 2B). Conversely, the 4  $\mu\text{g/kg per h}$  DAG infusion exclusively increased plasma DAG levels (from  $0.068 \pm 0.044$  to  $15.9 \pm 4.91$  ng/ml) corresponding to a 233-fold rise. AG levels did not change with administration of DAG (baseline  $0.036 \pm 0.014$  to  $0.050 \pm 0.021$  ng/ml) (Fig. 2C) and, in fact, were similar to those during the saline infusion (Fig. 3). The combined AG and DAG infusions raised plasma AG 54-fold, and DAG concentrations 272-fold, changes of similar magnitude to the individual infusions (Fig. 2D).

The DAG:AG ratio was  $1.85 \pm 0.07$  at baseline and did not change during the saline infusion (DAG  $\text{AUC}_{0-\text{last}}$ :AG  $\text{AUC}_{0-\text{last}} = 1.90 \pm 0.50$ ), remaining constant during the entire FSIVGTT (Table 1 and Fig. 2A). The 1  $\mu\text{g/kg per h}$  AG infusion reversed the DAG:AG ratio to 0.4:1 (DAG  $\text{AUC}_{0-\text{last}}$ :AG  $\text{AUC}_{0-\text{last}} = 0.6 \pm 0.3$ ; Table 1 and Fig. 2B), and although DAG increased significantly with the AG infusion, levels remained

lower than AG during the entire 210 min. DAG was the predominant plasma isoform with the combined AG and DAG infusion (Fig. 2D).

### Pharmacokinetics of AG and DAG

The pharmacokinetic estimates of AG and total ghrelin for study 1 as determined by the non-compartmental analysis are summarized in Table 2. The mean  $t_{1/2}$  of AG was in the range of 9–11 min. The  $C_{\text{max}}$  achieved with the 3 and 5  $\mu\text{g/kg per h}$  dose AG infusions was approximately three and five times that with the 1  $\mu\text{g/kg per h}$  dose respectively.  $\text{AUC}_{0-\text{last}}$  also increased linearly with dose, and like  $C_{\text{max}}$ , it demonstrated a dose-proportional change. The observed differences in  $C_{\text{max}}$  and  $\text{AUC}_{0-\text{last}}$  were abolished when the measures were normalized to dose for AG. The MRT, CL, and the steady-state  $V_d$  were similar between the three doses (Table 2).

Plasma total ghrelin concentrations during AG infusion were reported previously (19). A summary of the pharmacokinetic properties of total ghrelin is shown in Table 2. In comparison with AG, total ghrelin had a longer  $t_{1/2}$  of  $\sim 35$  min. Consistent with the findings for AG,  $C_{\text{max}}$  for total ghrelin increased proportionally with higher doses of AG infusion ( $5.49 \pm 0.61$ ,  $17.13 \pm 4.50$ , and  $28.73 \pm 11.59$  ng/ml, from low to high respectively). Similarly, the  $\text{AUC}_{0-\text{last}}$  for total ghrelin during AG infusions was  $531.9 \pm 155.0$ ,  $1583.1 \pm 574.8$ , and  $2326.1 \pm 587.9$  min  $\times$  ng/ml from low to high doses respectively. There was no significant difference in MRT, CL, or  $V_d$  between the three doses (Table 2).

The relationship between dose and plasma total ghrelin and AG concentrations were examined using linear regression analysis. Both plasma AG and total ghrelin  $C_{\text{max}}$  and AUC increased linearly with increased doses of AG administration ( $R^2 = 0.86$  and  $0.94$  for AG, Fig. 4A and B;  $R^2 = 0.66$  and  $0.72$  for total ghrelin, Fig. 4C and D respectively) whereas the dose-adjusted  $C_{\text{max}}$  ( $C_{\text{max}}/D$ ) and  $\text{AUC}_{0-\text{last}}$  ( $\text{AUC}_{0-\text{last}}/D$ ) remained constant (Table 2).

**Table 1** Pharmacokinetic parameter estimates of plasma AG and DAG after administration of varying doses of AG or DAG or the combination of AG and DAG by continuous i.v. infusion in healthy men and women obtained by non-compartmental analysis. Results are presented as mean  $\pm$  s.d.

|                                                     | Saline infusion  | AG infusion (1 $\mu\text{g/kg per h}$ ) | DAG infusion (4 $\mu\text{g/kg per h}$ ) | AG + DAG infusion (1 + 4 $\mu\text{g/kg per h}$ ) |
|-----------------------------------------------------|------------------|-----------------------------------------|------------------------------------------|---------------------------------------------------|
| Pharmacokinetic parameters of AG                    |                  |                                         |                                          |                                                   |
| $T_{\text{max}}$ (min)                              |                  | $148 \pm 89$                            | $65 \pm 66$                              | $101 \pm 63$                                      |
| $C_{\text{max}}$ (ng/ml)                            | $0.045 \pm 0.02$ | $1.93 \pm 1.30$                         | $0.05 \pm 0.021$                         | $1.65 \pm 0.63$                                   |
| $\text{AUC}_{(0-\text{last})}$ (min $\times$ ng/ml) | $5.67 \pm 3.10$  | $267.86 \pm 180.83$                     | $7.7 \pm 1.88$                           | $253.57 \pm 90.86$                                |
| MRT (min)                                           | $107 \pm 11$     | $108 \pm 10$                            | $106 \pm 8$                              | $107 \pm 7$                                       |
| CL (ml/min per kg)                                  |                  | $5.4 \pm 2.8$                           |                                          | $4.5 \pm 2.0$                                     |
| Pharmacokinetic parameters of DAG                   |                  |                                         |                                          |                                                   |
| $T_{\text{max}}$ (min)                              |                  | $109 \pm 74$                            | $170 \pm 49$                             | $175 \pm 32$                                      |
| $C_{\text{max}}$ (ng/ml)                            | $0.078 \pm 0.03$ | $1.29 \pm 1.12$                         | $15.94 \pm 4.91$                         | $15.48 \pm 3.6$                                   |
| $\text{AUC}_{(0-\text{last})}$ (min $\times$ ng/ml) | $11.04 \pm 5.05$ | $172.35 \pm 132.2$                      | $2696.53 \pm 953.3$                      | $2556.56 \pm 714.6$                               |
| MRT (min)                                           | $107 \pm 10$     | $107 \pm 11$                            | $106 \pm 8$                              | $110 \pm 9$                                       |
| CL (ml/min per kg)                                  |                  |                                         | $1.7 \pm 0.9$                            | $1.7 \pm 0.6$                                     |

**Table 2** Pharmacokinetic parameter estimates of plasma acyl and total ghrelin after administration of 1, 3, and 5  $\mu\text{g/kg}$  per h dose AG by continuous i.v. infusion (study 1) in healthy men and women obtained by non-compartmental analysis. Results are presented as mean  $\pm$  s.d.

| Parameter                                  | AG infusion dose         |                          |                          |
|--------------------------------------------|--------------------------|--------------------------|--------------------------|
|                                            | 1 $\mu\text{g/kg}$ per h | 3 $\mu\text{g/kg}$ per h | 5 $\mu\text{g/kg}$ per h |
| Acyl ghrelin                               |                          |                          |                          |
| Half-life (min)                            | 11 $\pm$ 4               | 10 $\pm$ 3               | 9 $\pm$ 2                |
| $C_{\text{max}}$ (ng/ml)                   | 3.87 $\pm$ 3.46          | 11.72 $\pm$ 2.39         | 19.64 $\pm$ 2.34         |
| $C_{\text{max}}/D$                         | 3.87 $\pm$ 3.46          | 3.91 $\pm$ 0.79          | 3.92 $\pm$ 0.47          |
| $\text{AUC}_{(0-90)}$ (min $\times$ ng/ml) | 142 $\pm$ 47             | 549 $\pm$ 100            | 896 $\pm$ 174            |
| $\text{AUC}_{(0-90)}/D$                    | 142 $\pm$ 47             | 183 $\pm$ 33             | 179 $\pm$ 35             |
| MRT (min)                                  | 45 $\pm$ 3               | 45 $\pm$ 2               | 45 $\pm$ 2               |
| CL (ml/min per kg)                         | 7.66 $\pm$ 2.36          | 5.73 $\pm$ 0.91          | 5.47 $\pm$ 0.69          |
| $V_d$ (ml/kg)                              | 126 $\pm$ 58             | 79 $\pm$ 24              | 78 $\pm$ 22              |
| Total ghrelin                              |                          |                          |                          |
| Half-life (min)                            | 39 $\pm$ 17              | 32 $\pm$ 10              | 34 $\pm$ 13              |
| $C_{\text{max}}$ (ng/ml)                   | 5.49 $\pm$ 0.61          | 17.13 $\pm$ 4.5          | 28.73 $\pm$ 11.59        |
| $\text{AUC}_{(0-90)}$ (min $\times$ ng/ml) | 323 $\pm$ 61             | 955 $\pm$ 202            | 1550 $\pm$ 492           |
| $\text{AUC}_{(0-90)}/D$                    | 323 $\pm$ 61             | 318 $\pm$ 67             | 310 $\pm$ 98             |
| MRT (min)                                  | 85 $\pm$ 19              | 91 $\pm$ 49              | 47 $\pm$ 14              |
| CL (ml/min per kg)                         | 2.27 $\pm$ 1.003         | 2.26 $\pm$ 0.77          | 2.48 $\pm$ 0.71          |
| $V_d$ (ml/kg)                              | 114 $\pm$ 21             | 127 $\pm$ 56             | 119 $\pm$ 65             |

BuChE is the enzyme that accounts for the majority of deacylation of AG in the circulation of humans (18). Plasma BuChE activity was measured during saline and ghrelin infusion in six subjects at 0, 44, and 65 min into the AG infusion. Neither infusion time nor treatment assignment affected plasma BuChE levels ( $P=0.95$ , two-way repeated measures ANOVA), suggesting that neither the exogenous AG infusion nor the dose of AG altered the ghrelin des-acylation process.

## Study 2

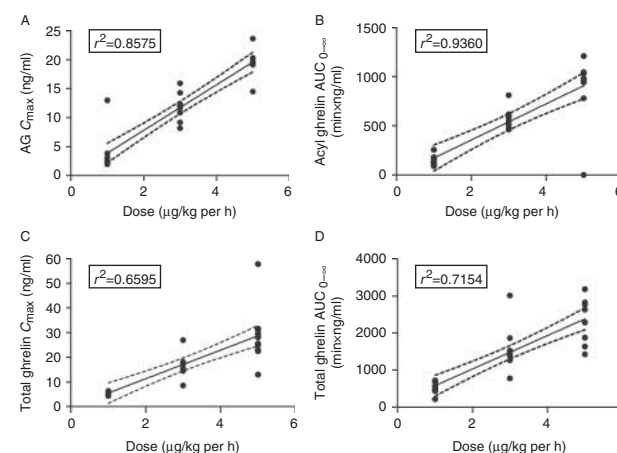
The pharmacokinetic estimates of AG and DAG obtained by non-compartmental analysis are summarized in Table 1. Both  $C_{\text{max}}$  and  $\text{AUC}_{0-\text{last}}$  for AG and DAG in the plasma increased during the 1  $\mu\text{g/kg}$  per h AG infusion, while the DAG infusion only increased  $C_{\text{max}}$  and  $\text{AUC}_{0-\text{last}}$  for DAG. MRTs were similar across all infusions. The systemic CL was similar between single-peptide and combined-peptide (AG+DAG) infusions but the CL of DAG was approximately three times smaller than AG when they were infused alone (Table 1). Unlike study 1, a direct measure of AG or DAG  $t_{1/2}$  could not be achieved because ghrelin was infused at a constant rate till the end of the study.

## Discussion

Recent experimental findings indicate that ghrelin plays an important role in the regulation of energy balance and glucose metabolism (22). The available information suggests that the two ghrelin isoforms, AG and DAG, have distinct metabolic effects (23), and analogs of both compounds are being developed for potential therapeutic

application (9, 24). Despite the accumulation of evidence supporting physiological roles for ghrelin, little is known about its metabolism and CL. In this study, we analyzed data from studies with AG and DAG infusions to determine pharmacokinetic parameters for the two isoforms in healthy, nonobese individuals with normal liver and kidney function. Our findings suggest that AG and DAG have different metabolic rates in the circulation with distinct rates of CL. Moreover, the results presented here indicate that AG is actively deacylated in the plasma.

In study 1, the wide range of ghrelin doses and frequent measures of AG and DAG allowed us to examine the dose proportionality of i.v. ghrelin administration. Relative to the 1  $\mu\text{g/kg}$  per h dose, the  $C_{\text{max}}$  for AG resulting from the 3 and 5  $\mu\text{g/kg}$  per h dose infusions increased by three- and fivefold while AUC



**Figure 4** Relationships of observed  $C_{\text{max}}$  and  $\text{AUC}_{(0-\infty)}$  values for acyl ghrelin (AG) and total ghrelin vs AG infusion dose with linear regression (bold line) and the 95% CI (dashed line)

increased by four- and sixfold respectively. Consistent with this, the dose-normalized  $C_{\max}$  and AUC values were not different between doses (Table 2). These observations demonstrate a clear and strong linear relationship between plasma AG concentration and treatment dose (Fig. 2A and B). In keeping with the dose proportionality of the pharmacokinetics, the elimination characteristics of AG, as reflected in the  $t_{1/2}$ , MRT, systemic CL, and  $V_d$ , were largely unchanged with different administration rates (Table 2). Thus, our findings demonstrate that, for AG, the increase in plasma levels can be reliably predicted based on the observed linear relationship for the dose range of 1–5  $\mu\text{g/kg}$  per h. A similar positive linear relationship was also observed between total ghrelin  $C_{\max}$ , AUC, and AG dose (Fig. 2C and D) (12). Of note, the  $C_{\max}$ , MRT, and CL estimates were quite different for comparable AG doses in study 1 and 2 (Tables 1 and 2). The ghrelin ELISA platform was changed between study 1 and 2 to improve assay sensitivity (these assays have very different specificities). This could partially explain the difference of pharmacokinetic parameters between the two studies. Differences in subject characteristics (i.e. body weight) between the two studies could also contribute to the pharmacokinetic variances. However, the fold increase in  $C_{\max}$  and AUC from saline to 1  $\mu\text{g/kg}$  per h dose ghrelin infusion was quite similar between studies.

As the majority of the studies using exogenous ghrelin administer AG, the pharmacokinetics reported here provides some guidance for the appropriate dose and route of administration for further research. In addition to the novel observation of a dose proportionality, our findings on other pharmacokinetic parameters for ghrelin are consistent with those reported previously. For example, the first-order elimination  $t_{1/2}$  we determined using non-compartmental analysis was 9–11 min for AG and 30–34 min for total ghrelin using doses ranging from 1 to 5  $\mu\text{g/kg}$  per h. This is comparable to the findings of Akamizu *et al.* (25) who reported a  $t_{1/2}$  of 9–13 min for AG and 27–31 min for total ghrelin following ghrelin bolus injections 1 and 5  $\mu\text{g/kg}$  using a one-compartment model analysis. Vestergaard *et al.* (26) employed a two-compartmental model to describe the pharmacokinetics of total ghrelin following administration of AG using a dose of 5 pmol/kg per min (equivalent to 1  $\mu\text{g/kg}$  per h) for 180 min. Their observed pharmacokinetic parameters for total ghrelin ( $C_{\max}$  of  $4.41 \pm 0.29$   $\mu\text{g/l}$ , initial  $t_{1/2}$  of  $24.2 \pm 2.5$  min, and MRT of  $92.7 \pm 16.3$  min) are in general agreement with the ones we report here. Likewise, the results of Paulo *et al.* (27) of a mean  $t_{1/2}$  of  $36 \pm 2.4$  min for total ghrelin estimated from the 1  $\mu\text{g/kg}$  AG injection is very similar to our estimates. However, their  $t_{1/2}$  for AG of  $21 \pm 3.0$  min is longer than our estimate of  $11 \pm 4$  min. In contrast to our observations, the metabolic CL and the  $t_{1/2}$  of AG and total ghrelin were reported to be increasing with higher doses of ghrelin in that study.

If these results are accurate, it would suggest that the CL/inactivation of AG is nonlinear and concentration-dependent. Differences in study design, study population, and assay methods are likely to explain the apparent discrepancy between our findings and those from Paulo *et al.*

DAG does not bind to the GHSR-1a and its biological role has been questioned since no cognate receptor has been identified (1). Some investigators have reported that DAG can exert beneficial effects on insulin secretion and glucose tolerance that, in general, tend to be opposite those of AG (28, 29, 30). DAG analogs are being developed as therapeutic agents for metabolic diseases such as type 2 diabetes (9). However, the pharmacokinetics of synthetic human DAG has not been well characterized. We found that when AG (1  $\mu\text{g/kg}$  per h) alone was infused, both plasma AG and DAG levels increased significantly showing a 47- and 16-fold increase in  $C_{\max}$  from baseline respectively. Conversely, the DAG (4  $\mu\text{g/kg}$  per h) alone infusion preferentially increased DAG concentration in the circulation without altering the levels of AG relative to saline infusion (Fig. 2A, B, and C). These findings are consistent with observations made by Vestergaard *et al.* (31) and suggest that AG is metabolized to DAG in peripheral circulation while little acylation of exogenous DAG is occurring. The combined AG and DAG infusion raised plasma levels of AG and DAG to the same extent as that observed with individual administration. The pharmacokinetic parameters such as CL and  $V_d$  of both AG and DAG were similar whether given as single agents or in combination.

It is not known what percentage of the ghrelin is acylated when secreted from ghrelin-producing cells. Both duration of fasting and dietary medium-chain fatty acid composition can impact ghrelin acylation (12, 14). Using a highly sensitive and specific two-site sandwich assay to measure AG and DAG, we found that DAG:AG ratio was  $1.8 \pm 0.7$  at baseline (after an overnight fast) and  $1.9 \pm 0.5$  during saline infusion (DAG  $\text{AUC}_{0-\text{last}}$ :AG  $\text{AUC}_{0-\text{last}}$ ). This ratio remained quite constant during the FSIVGTT (Fig. 2A) and is consistent with a previous report by Liu *et al.* (12) using the same ghrelin assay. Following AG infusion, plasma concentrations of both AG and DAG increased substantially but the ratio of DAG:AG in plasma remained constant during the infusion period (Fig. 2B and C). This would suggest that in addition to the production of 'new' DAG from AG breakdown, DAG elimination was also increased in proportion to load resulting in a steady state with a constant DAG:AG ratio. Data on the route of elimination of ghrelin is limited and seems to differ for the two isoforms as AG is extracted substantially by the liver (15, 32) while DAG appears to undergo significant renal CL (16, 17). Several enzymes have been identified as responsible for the removal of the octanoyl group from the AG peptide. BuChE is the main deacylating enzyme in humans (18). In our study, BuChE activity was not altered by AG infusion. We did not measure renal CL of ghrelin in this study.

Even though the  $t_{1/2}$  could not be directly determined in study 2 due to the study design (bolus injection followed by continuous infusion), the observed difference between the CL of these compounds (DAG  $1.7 \pm 0.9$  vs AG  $5.4 \pm 2.8$  ml/min per kg, Table 1) suggests that the half-life of DAG is approximately threefold longer than that of AG, assuming that other parameters such as  $V_d$  between AG and DAG are similar. This may be the primary factor contributing to the observed longer  $t_{1/2}$  of total ghrelin. In all likelihood, the  $t_{1/2}$  of DAG reflects CL of the peptide from the circulation, while the much shorter  $t_{1/2}$  of AG is principally due to deacylation in the circulation and conversion to DAG. In fact, the conversion of AG to DAG is relatively slow compared with other regulatory peptides that are metabolized intravascularly such as glucose-dependent insulinotropic polypeptide (33) and glucagon-like peptide 1 (34). Moreover, the CL of DAG is also slower than peptides like insulin and glucagon (35). Thus, based on our analyses, the ghrelin isoforms are relatively long lived in the circulation, which may have implications for their biological effects.

In conclusion, this study is the first to examine the proportionality of pharmacokinetic parameters of AG and total ghrelin in healthy humans. The pharmacokinetic parameters of AG reported in this study provided useful information for investigators who conduct clinical research on ghrelin physiology or pharmacology. This understanding is important as both AG and DAG appear to have therapeutic properties and are currently being investigated in the clinical setting for pharmacological activity.

## 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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## References

- 1 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999 **402** 656–660. (doi:10.1016/j.lfs.2004.09.039)
- 2 Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000 **141** 4255–4261. (doi:10.1210/en.141.11.4255)
- 3 Wierup N, Svensson H, Mulder H & Sundler F. The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regulatory Peptides* 2002 **107** 63–69. (doi:10.1097/SMJ.0b013e3181b5771d)
- 4 Andralojc KM, Mercalli A, Nowak KW, Albarello L, Calcagno R, Luzi L, Bonifacio E, Doglioni C & Piemonti L. Ghrelin-producing epsilon cells in the developing and adult human pancreas. *Diabetologia* 2009 **52** 486–493. (doi:10.1016/0003-2697(76)90527-3)
- 5 van der Lely AJ, Tschöp M, Heiman ML & Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocrine Reviews* 2004 **25** 426–457. (doi:10.1016/j.metabol.2004.11.003)
- 6 Gauna C, Delhanty PJ, van Aken MO, Janssen JA, Themmen AP, Hofland LJ, Culler M, Broglio F, Ghigo E & van der Lely AJ. Unacylated ghrelin is active on the INS-1E rat insulinoma cell line independently of the growth hormone secretagogue receptor type 1a and the corticotropin releasing factor 2 receptor. *Molecular and Cellular Endocrinology* 2006 **251** 103–111. (doi:10.1161/01.STR.0000206458.58142.f3)
- 7 Delhanty PJ, van der Eerden BC, van der Velde M, Gauna C, Pols HA, Jahr H, Chiba H, van der Lely AJ & van Leeuwen JP. Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. *Journal of Endocrinology* 2006 **188** 37–47. (doi:10.1074/jbc.M413684200)
- 8 Muccioli G, Pons N, Ghè C, Catapano F, Granata R & Ghigo E. Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. *European Journal of Pharmacology* 2004 **498** 27–35. (doi:10.1095/biolreprod65.3.814)
- 9 Julien M, Kay RG, Delhanty PJ, Allas S, Granata R, Barton C, Constable S, Ghigo E, van der Lely AJ & Abribat T. *In vitro* and *in vivo* stability and pharmacokinetic profile of unacylated ghrelin (UAG) analogues. *European Journal of Pharmaceutical Sciences* 2012 **47** 625–635. (doi:10.1016/j.plipres.2008.11.001)
- 10 Yang J, Brown MS, Liang G, Grishin NV & Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 2008 **132** 387–396. (doi:10.1210/en.2012-1508)
- 11 Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE & Hale JE. Ghrelin octanoylation mediated by an orphan lipid transferase. *PNAS* 2008 **105** 6320–6325. (doi:10.1113/jphysiol.2008.167072)
- 12 Liu J, Prudom CE, Nass R, Pezzoli SS, Oliveri MC, Johnson ML, Veldhuis P, Gordon DA, Howard AD, Witcher DR *et al.* Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 1980–1987. (doi:10.1210/jc.2007-2235)
- 13 Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE & Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001 **50** 1714–1719. (doi:10.1042/BJ20051578)
- 14 Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schürmann A, Joost HG, Jandacek RJ, Hale JE *et al.* GOAT links dietary lipids with the endocrine control of energy balance. *Nature Medicine* 2009 **15** 741–745. (doi:10.1016/S0898-6568(01)00211-X)
- 15 Banks WA, Burney BO & Robinson SM. Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood-brain barrier. *Peptides* 2008 **29** 2061–2065. (doi:10.1210/jc.2006-2403)

- 16 Yoshimoto A, Mori K, Sugawara A, Mukoyama M, Yahata K, Suganami T, Takaya K, Hosoda H, Kojima M, Kangawa K *et al.* Plasma ghrelin and desacyl ghrelin concentrations in renal failure. *Journal of the American Society of Nephrology* 2002 **13** 2748–2752. (doi:10.2337/diabetes.48.11.2204)
- 17 Jarkovská Z, Hodková M, Sazamová M, Rosická M, Dusilová-Sulková S, Marek J, Justová V, Lacinová Z, Haluzík M, Haas T *et al.* Plasma levels of active and total ghrelin in renal failure: a relationship with GH/IGF-I axis. *Growth Hormone & IGF Research* 2005 **15** 369–376. (doi:10.1371/journal.pone.0033370)
- 18 De Vriese C, Gregoire F, Lema-Kisoka R, Waelbroeck M, Robberecht P & Delporte C. Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. *Endocrinology* 2004 **145** 4997–5005. (doi:10.1159/000242635)
- 19 Tong J, Prigeon RL, Davis HW, Bidlingmaier M, Kahn SE, Cummings DE, Tschöp MH & D'Alessio D. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. *Diabetes* 2010 **59** 2145–2151. (doi:10.2337/157016109789043973)
- 20 Ellman GL, Courtney KD, Andres VJR & Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 1961 **7** 88–95.
- 21 Muller EE, Rigamonti AE, Colonna Vde G, Locatelli V, Berti F & Cella SG. GH-related and extra-endocrine actions of GH secretagogues in aging. *Neurobiology of Aging* 2002 **23** 907–919. (doi:10.1210/jc.2002-021332)
- 22 Castañeda TR, Tong J, Datta R, Culler M & Tschöp MH. Ghrelin in the regulation of body weight and metabolism. *Frontiers in Neuroendocrinology* 2010 **31** 44–60. (doi:10.1016/j.placenta.2006.01.017)
- 23 Heppner KM, Tong J, Kirchner H, Nass R & Tschöp MH. The ghrelin O-acyltransferase-ghrelin system: a novel regulator of glucose metabolism. *Current Opinion in Endocrinology, Diabetes, and Obesity* 2011 **18** 50–55. (doi:10.1016/j.placenta.2007.05.002)
- 24 Müller TD, Perez-Tilve D, Tong J, Pfluger PT & Tschöp MH. Ghrelin and its potential in the treatment of eating/wasting disorders and cachexia. *Journal of Cachexia, Sarcopenia and Muscle* 2010 **1** 159–167. (doi:10.1096/fj.08-116889)
- 25 Akamizu T, Takaya K, Irako T, Hosoda H, Teramukai S, Matsuyama A, Tada H, Miura K, Shimizu A, Fukushima M *et al.* Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. *European Journal of Endocrinology* 2004 **150** 447–455. (doi:10.2337/db09-0824)
- 26 Vestergaard ET, Hansen TK, Gormsen LC, Jakobsen P, Møller N, Christiansen JS & Jørgensen JO. Constant intravenous ghrelin infusion in healthy young men: clinical pharmacokinetics and metabolic effects. *American Journal of Physiology, Endocrinology and Metabolism* 2007 **292** E1829–E1836. (doi:10.2174/157340312801215773)
- 27 Paulo RC, Brundage R, Cosma M, Mielke KL, Bowers CY & Veldhuis JD. Estrogen elevates the peak overnight production rate of acylated ghrelin. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 4440–4447. (doi:10.2337/diabetes.47.5.847)
- 28 Broglio F, Gottero C, Prodham F, Gauna C, Muccioli G, Papotti M, Abribat T, Van Der Lely AJ & Ghigo E. Non-acylated ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin in humans. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 3062–3065. (doi:10.1194/jlr.M600236-JLR200)
- 29 Gauna C, Meyler FM, Janssen JA, Delhanty PJ, Abribat T, van Koetsveld P, Hofland LJ, Broglio F, Ghigo E & van der Lely AJ. Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 5035–5042. (doi:10.1161/CIRCULATIONAHA.104.520130)
- 30 Kiewiet RM, van Aken MO, van der Weerd K, Uitterlinden P, Themmen AP, Hofland LJ, de Rijke YB, Delhanty PJ, Ghigo E, Abribat T *et al.* Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes. *European Journal of Endocrinology* 2009 **161** 567–573. (doi:10.1900/RDS.2007.4.134)
- 31 Vestergaard ET, Buhl M, Gjedsted J, Madsen M, Jessen N, Nielsen S, Gaylinn BD, Liu J, Thorner MO, Møller N *et al.* Acute peripheral metabolic effects of intraarterial ghrelin infusion in healthy young men. *Journal of Clinical Endocrinology and Metabolism* 2011 **96** 468–477. (doi:10.1002/dmrr.1151)
- 32 Gauna C, Uitterlinden P, Kramer P, Kiewiet RM, Janssen JA, Delhanty PJ, van Aken MO, Ghigo E, Hofland LJ, Themmen AP *et al.* Intravenous glucose administration in fasting rats has differential effects on acylated and unacylated ghrelin in the portal and systemic circulation: a comparison between portal and peripheral concentrations in anesthetized rats. *Endocrinology* 2007 **148** 5278–5287. (doi:10.1016/j.placenta.2011.01.004)
- 33 Kieffer TJ, McIntosh CH & Pederson RA. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 *in vitro* and *in vivo* by dipeptidyl peptidase IV. *Endocrinology* 1995 **136** 3585–3596. (doi:10.1210/en.136.8.3585)
- 34 Drucker DJ. The biology of incretin hormones. *Cell Metabolism* 2006 **3** 153–165. (doi:10.1159/000014190)
- 35 Williams RF, Gleason RE & Soeldner JS. The half-life of endogenous serum immunoreactive insulin in man. *Metabolism* 1968 **17** 1025–1029. (doi:10.1038/oby.2011.43)

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