Ghrelin-induced GH secretion in domestic fowl *in vivo* and *in vitro*

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Abstract

Although avian and mammalian species differ significantly in their regulation of GH secretion, preliminary studies have demonstrated *in vivo* GH responses to ghrelin in chickens, as in mammals. However, the relative potency of ghrelin as a GH-releasing hormone (GHRH) in birds is uncertain, as is its site of action.

The intravenous administration of human ghrelin to immature chickens promptly increased the circulating GH concentration (within 10 min), although this was transitory and was only maintained for 20 min. This GH response was dose-related with an EC_{50} of approximately $3.0 \mu\text{g/kg}$, comparable with the reported potency of human GHRH in chickens. When incubated with dispersed pituitary cells, human ghrelin induced dose-dependent GH release over a range of 10^{-6} to 10^{-9} M, with an EC_{50} of 7.0×10^{-8} M, comparable with that induced by human GHRH (EC_{50} 6.0×10^{-8} M),

although it was less effective at doses of 10^{-6} to 10^{-8} M. This was due to direct effects on pituitary somatotrophs, since human ghrelin increased GH release (determined by the reverse hemolytic plaque assay) from individual pituitary cells. The incubation of these cells with human ghrelin induced a dose-dependent increase in the numbers of somatotrophs secreting GH and in the amount of GH released by each cell.

In summary, these results demonstrated that ghrelin is a dose-related GH-releasing factor in chickens with a potency comparable with that induced by human GHRH. The GH-releasing action of ghrelin is due, at least in part, to stimulatory actions on the numbers of somatotrophs induced to release GH and upon the amount of GH released from individual somatotrophs.

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Introduction

The discovery that ghrelin is the endogenous ligand of the growth hormone (GH) secretagogue receptor (Kojima *et al.* 1999) led to a re-evaluation of the neuroendocrine regulation of GH secretion (Kojima *et al.* 2001). Indeed, ghrelin was shown to strongly stimulate GH release in humans (Arvat *et al.* 2000, Peino *et al.* 2000, Takaya *et al.* 2000), in whom it was found to be more potent than GH-releasing hormone (GHRH) *in vivo* (Arvat *et al.* 2000, 2001). It is also more effective than GHRH in stimulating *in vivo* GH release in rats (Seoane *et al.* 2000, Tolle *et al.* 2001), although its GH-releasing activity *in vitro* is only comparable with (Kojima *et al.* 1999) or less than (Yamazaki *et al.* 2002, Pinilla *et al.* 2003) that induced by GHRH.

The hypothalamic regulation of GH secretion in birds differs significantly from that in mammals (Harvey 1993a), but ghrelin has recently been shown to increase plasma GH concentrations in immature chickens, although not (as yet) in a dose-related way (Ahmed & Harvey 2002, Kaiya *et al.* 2002). The *in vivo* potency of ghrelin in birds is thus unknown, as is its possible site of action. Although ghrelin

directly stimulates GH release from mammalian pituitary cells (Kojima *et al.* 1999, Yamazaki *et al.* 2002), it also has hypothalamic sites of action (Date *et al.* 2000, Tannenbaum & Bowers 2001, Tolle *et al.* 2001, Tamura *et al.* 2002, Wren *et al.* 2002). Moreover, while pituitary sites of ghrelin action have been demonstrated, it is still uncertain if this is due to the direct stimulation of somatotrophs or the stimulation of other cell types that might indirectly regulate somatotroph function. Indeed, other secretagogues are thought to affect pituitary function by inducing paracrine cascades between heterologous cell types (e.g. Robberecht *et al.* 1992, Tilemans *et al.* 1992, Schwartz 2000, 2001). The GH-releasing activity of ghrelin has, therefore, been further examined *in vivo* and *in vitro* in domestic fowl and direct effects on isolated chicken somatotrophs have been determined.

Materials and Methods

In vivo experiments

Immature, 4- to 5-week-old White Leghorn cockerels (approximately 400–500 g) were injected intravenously

(into a wing vein) with human ghrelin (031-30; Phoenix Peptides International, Belmont, CA, USA) at doses of 0.03–100.0 µg/kg in 0.9% (w/v) NaCl. Controls were similarly injected with the saline vehicle (1.0 ml/kg). Heparinized blood samples (0.5–1.0 ml) were then collected from the contralateral vein after 10 min (the time of the maximal GH response to other GH secretagogues; Harvey & Scanes 1984), or at additional intervals (20, 30 and 60 min) following injection, in accordance with previous *in vivo* studies (Harvey *et al.* 1978, Harvey & Scanes 1984, Scanes & Harvey 1984) and in compliance with the University of Alberta's Policy on Animal Welfare. Following centrifugation and separation, the plasma was stored at -20°C prior to GH radioimmunoassay, using NIADD reagents (Bethesda, MD, USA, kindly provided by Dr A F Parlow), as previously described (Ahmed & Harvey 2002). Because the blood volume of a chick is approximately 10% of its body weight and as the birds were killed (by cervical dislocation) at the end of the experiment, plasma was not replaced following blood collection.

In vitro experiments

Dispersed pituitary cells The GH-releasing activity of human ghrelin was assessed using freshly dispersed pituitary cells, as outlined by Harvey *et al.* (1978) for the GH-releasing activity of thyrotrophin-releasing hormone (TRH). Briefly, anterior pituitary glands from the heads of 6-week-old broiler fowl (obtained from Lillydale, Edmonton, Alberta, Canada) were collected into ice-cold Medium 199 (Gibco, Burlington, Ontario, Canada) and pituitary cells were obtained after a 2-h incubation at 39°C in 0.02% (w/v) collagenase (type III, fraction A; Sigma Chemical Company, St Louis, MO, USA). The cells were collected by centrifugation (100 *g* for 10 min) and twice resuspended in fresh media, after filtration through a nylon gauze. The cells were dispersed at a final concentration of 1 pituitary equivalent/0.5 ml, using a magnetic stirrer, and 0.5 ml aliquots were dispersed into 2 ml polystyrene tubes, together with the test solutions, dissolved in an equal volume of Medium 199. The resulting cells had a viability of >95%, as assessed by trypan blue exclusion. The tubes ($n=6$ per group) were then oxygenated with 95% (v/v) O_2 :5% (v/v) CO_2 , sealed with parafilm and incubated in a shaking water bath at 39°C for 2 h. The tubes were then centrifuged (at 1000 *g* for 10 min at 4°C) and the supernatants collected and stored at -20°C until GH radioimmunoassay (Ahmed & Harvey 2002). The sensitivity of the assay was <1.0 ng GH/ml and hence the samples were diluted in radioimmunoassay buffer prior to assay. The GH-releasing activity of human ghrelin (Phoenix Peptides International) was determined at concentrations of 10^{-10} to 10^{-6} M, in comparison with similar concentrations of human GHRH₁₋₄₄ (Bachem, Torrance, CA, USA), an estab-

lished *in vitro* GH-releasing factor in birds (Harvey 1999). All the samples from these experiments were determined in a single assay to facilitate comparisons of GH-releasing activity.

Individual pituitary cells To ensure that the GH-releasing action of ghrelin *in vitro* was due to direct effects on pituitary somatotrophs, rather than mediated via actions on other pituitary cell types (Schwartz 2000, 2001), its GH-releasing activity was determined in individual somatotrophs, using the reverse hemolytic plaque assay (RHPA; Neill & Frawley 1983, Frawley & Neill 1984). In brief, dispersed pituitary cells (10^6 cells/ml) in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 0.1% (w/v) IgG-free bovine serum albumin (Fraction V; Sigma) and antibiotics (penicillin G and streptomycin sulfate (Invitrogen, Burlington, Ontario, Canada), both at 100 U/ml), were mixed with an equal volume of a 20% (v/v) suspension of ovine red blood cells (oRBCs) conjugated with *Staphylococcus aureus* protein-A (Sigma). This mix was then infused by capillary action into 30 µl Cunningham chambers, previously constructed on poly-L-lysine-coated microscopic slides (Sigma). The cells were allowed to attach as a monolayer (1 h at 39°C) in a 5% CO_2 incubator. The chambers were then washed with DMEM to remove all unattached cells. DMEM containing anti-chicken GH antiserum (1:50; kindly provided by Dr T Porter, University of Maryland, USA) was then infused into the chambers with or without secretagogues (human ghrelin (Phoenix Peptides International) or human GHRH₁₋₂₉ (Bachem) at a final concentration of 10^{-10} to 10^{-6} M) and the cells were incubated for 60 min, since preliminary studies established that plaque-forming cells are readily detectable at this time. After a wash with DMEM, guinea pig complement (Cedarlane, Ontario, Canada) was then infused into the chambers (at a dilution of 1:120 in DMEM). The cells were fixed, 30 min later, with 2% (w/v) glutaraldehyde (Sigma) and stained with toluidine blue to facilitate identification of the large pituitary cells dispersed amongst the smaller oRBCs. Approximately 1000 pituitary cells in each chamber were identified and the percentage of plaque-forming cells was determined using a light microscope. Plaque areas were determined using Image-Pro software (Carsson, Toronto, Ontario, Canada). The specificity of plaque formation was demonstrated by their absence when (i) the GH antiserum or (ii) the guinea pig complement were omitted, or when (iii) the GH antiserum was replaced by normal rabbit serum or (iv) by GH antiserum that had been preabsorbed with excess recombinant chicken GH (1 mg/ml for 2 h; Amgen, Thousand Oaks, CA, USA).

Statistical differences were determined by Student's paired *t*-test or analysis of variance, where appropriate, and data were correlated by linear regression and analyzed for EC_{50} for dose-response relationships.

Figure 1 GH concentration in the plasma of 4- to 5-week-old White Leghorn cockerels at intervals after an intravenous injection (indicated by the arrow) of human ghrelin (10 µg/kg). Values are means ± S.E.M. ($n=8$). * $P<0.05$ in comparison with pretreatment concentration (Student's paired t -test).

Results

In vivo experiments

As expected (Ahmed & Harvey 2002, Kaiya *et al.* 2002), the plasma GH concentration in immature chickens was increased ($P<0.01$) almost threefold 10 min after the intravenous administration of ghrelin, at a dose of 10 µg/kg (Fig. 1). The GH concentration remained elevated after 20 min ($P<0.01$, paired Student's t -test), but had returned to the pretreatment level by 30 min. In a second study, similar threefold increases in the GH concentration were observed 10 min after the administration of ghrelin at 10 or 100 µg/kg ($P<0.01$ in both cases, in comparison with the concentration (38.3 ± 4.3 µg/l) in the vehicle-injected controls; Fig. 2). Lower doses of ghrelin (1 µg/kg and 3 µg/kg) also increased the GH concentration ($P<0.05$), but to levels lower ($P<0.05$) than those induced by the higher doses (analysis of variance). At 0.1 µg/kg and 0.3 µg/kg, ghrelin had no effect on the plasma GH concentration. The GH concentration 10 min after ghrelin administration was, therefore, dose-related ($r=0.50$ ($n=72$), $P<0.001$), with an EC_{50} of approximately 3.0 µg/kg.

Figure 2 GH concentrations in the plasma of 4- to 5-week-old White Leghorn cockerels 10 min after an intravenous injection of human ghrelin at concentrations of 0.1–100 µg/kg. Controls were injected with the 0.9% (w/v) NaCl vehicle (1 ml/kg). Values are means ± S.E.M. ($n=12$). * $P<0.05$ (analysis of variance) in comparison with the controls (38.3 ± 4.3 µg/l). Groups with different letters are significantly different ($P<0.05$, analysis of variance).

In vitro experiments

In the absence of a GH secretagogue, very little GH (0.90 ± 0.04 µg GH/pituitary equivalent per ml; means ± S.E.M. ($n=6$)) was present in the media of dispersed pituitary cells (data not shown). In marked contrast, the media GH concentration was dramatically increased ($P<0.05$, analysis of variance) in the presence of ghrelin at concentrations of 10^{-9} to 10^{-6} M, although ghrelin was ineffective at a dose of 10^{-10} M (Fig. 3). The GH response was clearly dose-related ($r=0.94$ ($n=30$), $P<0.001$) with an EC_{50} of 7.04×10^{-8} M. At the same doses, GHRH similarly induced GH release ($r=0.93$ ($n=30$), $P<0.001$), with an EC_{50} of 6.0×10^{-8} M (Fig. 3). The GH responses to GHRH at 10^{-6} , 10^{-7} and 10^{-8} M were greater ($P<0.05$, analysis of variance) than those to the same doses of ghrelin (Fig. 3).

Individual GH-secreting cells were identified by the formation of plaques in the RHPA (Fig. 4A–C). Qualitative studies showed that the numbers and sizes of these plaques were increased in the presence of 10^{-6} M

Figure 3 GH released from incubated dispersed pituitary cells (a) in response to human ghrelin and (b) in comparison with human GHRH. Asterisks indicate values significantly different ($P < 0.05$, analysis of variance) from the controls (0.90 ± 0.04 µg pituitary equivalent/ml). Values are means $\pm$ S.E.M. ($n=6$). Groups with different letters are significantly different ($P < 0.05$, analysis of variance).

ghrelin (Fig. 4D–F), similar to those induced by 10^{-6} M GHRH (Fig. 4G–I) and 10^{-6} M TRH (Fig. 4J–L). The numbers and sizes of the plaques produced after a 60-min incubation in 10^{-10} M ghrelin (Fig. 5) were not, however, significantly different from those in controls (data not shown). The numbers of the plaques were, however, increased ($P < 0.05$, analysis of variance) in response to 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M ghrelin (Fig. 5). This response was dose-related ($r=0.55$ ($n=20$), $P < 0.05$), with an EC_{50} of approximately 9×10^{-9} M. The size of the plaques was also increased ($P < 0.05$, analysis of variance) with ghrelin at 10^{-7} and 10^{-6} M (Fig. 5). The response

was also dose-related ($r=0.71$ ($n=20$), $P < 0.05$), with an EC_{50} of approximately 4×10^{-8} M.

Discussion

These results demonstrate that ghrelin is a GH secretagogue in chickens with *in vivo* and *in vitro* GH-releasing activity.

Human and chicken ghrelin have previously been shown to increase circulating GH concentrations in 8-day-old broiler chicks, at doses of 2 and 10 µg (at least

Figure 4 Photomicrographs of plaques from GH-secreting pituitary cells, determined by the RHPA. The plaques are clear zones of lysed oRBCs surrounding large individual pituitary cells. The plaques are produced after GH antiserum (1:50) binds to GH released from the pituitary cells, and lysis of the protein A-conjugated oRBCs occurs in the presence of guinea pig complement (1:120). GH plaques in the absence of secretagogues are shown in (A), (B) and (C) (at magnifications of $\times 50$, $\times 100$ and $\times 400$ respectively). GH plaques in response to 10^{-6} M ghrelin are shown in (D), (E) and (F) at similar magnifications, whereas (G), (H) and (I) show GH plaques induced by 10^{-6} M human GHRH and (J), (K) and (L) show GH plaques produced by 10^{-6} M TRH, also at magnifications of $\times 50$, $\times 100$ and $\times 400$ respectively. Panels (C), (F), (I) and (L) show individual somatotrophs (arrows) surrounded by 'ghosts' (arrowheads) of lysed oRBCs. Some pituitary cells (chevrons) did not secrete GH under basal and stimulated conditions.

Figure 5 Plaques produced by GH-secreting pituitary cells following a 60-min incubation in 10^{-6} to 10^{-10} M human ghrelin. (a) The relative number of plaque-forming cells and (b) the size of the plaques produced. Values are means $\pm$ S.E.M. ($n=4$). Asterisks indicate values significantly different from the corresponding controls (number of plaque-forming cells in the controls, $8.15 \pm 0.33\%$ ($n=5$); area of plaques formed in controls, $7360 \pm 390 \mu\text{m}^2$ ($n=5$) ($P < 0.05$, analysis of variance)).

20–100 $\mu\text{g/kg}$), although the GH response was not dose-related (Kaiya *et al.* 2002). Human ghrelin was also found to be an effective GH secretagogue in 4-week-old Leghorn chicks at a dose of 10 $\mu\text{g/kg}$ (Ahmed & Harvey 2002). The plasma GH concentration in these birds was increased to the levels seen in birds injected with the same dose (10 $\mu\text{g/kg}$) of GHRH, although the GH response was less than that of GHRH on a molar basis and less than that produced by TRH (Ahmed & Harvey 2002).

The results of the present *in vivo* studies show that ghrelin-induced GH secretion in chicks is dose-related, although the maximum *in vivo* response (approximately threefold increase in the plasma GH concentration) was induced by the 10 $\mu\text{g/kg}$ dose, whilst the minimally effective dose was 1 $\mu\text{g/kg}$. A similarly narrow dose-response range has also been seen for ghrelin-induced *in vivo* GH secretion in rats (Seoane *et al.* 2000, Tolle *et al.* 2001). The *in vivo* GH response of chicks to GHRH is, like ghrelin, almost 'all-or-none' over the same dose range, whereas the *in vivo* GH response of chickens to TRH is demonstrable over four orders of magnitude (Harvey & Scanes 1984).

The ED_{50} for human ghrelin-induced *in vivo* GH secretion in chicks (approximately 3.0 $\mu\text{g/kg}$) is below the effective dose range of rat ghrelin in rats (3–60 nmol/kg (10–200 $\mu\text{g/kg}$); with a maximally effective dose of 12 nmol/kg (40 $\mu\text{g/kg}$), Seoane *et al.* 2000), but it is within the effective dose range of human ghrelin in humans (0.2–6.6 $\mu\text{g/kg}$; Peino *et al.* 2000, Takaya *et al.* 2000). The minimally effective dose of ghrelin in chicks *in vivo* (1 $\mu\text{g/kg}$) is also less than that in rats (3 nmol/kg; 10 $\mu\text{g/kg}$; Seoane *et al.* 2000), although human ghrelin is an effective GH secretagogue in man at doses of 0.8 and 0.2 $\mu\text{g/kg}$ (Peino *et al.* 2000, Takaya *et al.* 2000, Hataya *et al.* 2001). *In vivo* GH secretion in humans, therefore, appears to be more sensitive to ghrelin stimulation than in chickens and rats. The reduced GH responsiveness of chickens to human ghrelin is, however, unlikely to be due to the use of a heterologous secretagogue, since rat and chicken ghrelin are equally effective in rats at a dose of 20 $\mu\text{g/kg}$ (Kaiya *et al.* 2002) and human and chicken ghrelin are equally effective in chicks at doses of 2–10 $\mu\text{g/chick}$ (Kaiya *et al.* 2002). The *in vivo* GH response of Leghorn chicks to a maximally effective intravenous dose of ghrelin

is also of shorter duration than that in mammals, since it only lasted for 20 min in chickens, whereas it lasts for at least 90 min in man (Takaya *et al.* 2000, Arvat *et al.* 2001) and for at least 40 min (Tolle *et al.* 2001) or 45 min in rats (Seoane *et al.* 2000).

It is also of interest that the *in vivo* GH-releasing activity of ghrelin in Leghorn chicks appears to be greater than that observed in broiler chicks by Kaiya *et al.* (2002), since human and chicken ghrelin were ineffective in broilers at a dose of 0.4 µg/bird (a dose of at least 4.0 µg/kg). This is, however, likely to reflect a strain difference, since TRH is similarly more effective in inducing GH release in Leghorns than in broiler fowls (Harvey 1993b).

The EC₅₀ for human ghrelin-induced GH secretion in chicks *in vivo* is also comparable with that for human GHRH in chickens (Harvey & Scanes 1984), although it would appear to be less effective on a molar basis. Ahmed & Harvey (2002) similarly found that the GH-releasing activity of ghrelin in chicks *in vivo* was less than that induced by human GHRH. This contrasts with humans, in whom ghrelin is far more effective than GHRH *in vivo* (Arvat *et al.* 2000, 2001, Di Vito *et al.* 2002), as in rats (Seoane *et al.* 2000, Tolle *et al.* 2001). Ghrelin is also reported to be less effective than GHRH in stimulating *in vivo* GH secretion in dogs (Bhatti *et al.* 2002), although Cella *et al.* (1995) found that hexarelin (a GH secretagogue receptor agonist) was a more potent GH secretagogue than GHRH. The relative effectiveness of GHRH and ghrelin in stimulating *in vivo* GH secretion may, nevertheless, be species specific.

The *in vivo* GH response of chickens to ghrelin is likely to be, at least partly, due to direct actions on pituitary somatotrophs. The dose-related GH responses of individual and dispersed pituitary cells to *in vitro* ghrelin treatment support this view. Indeed, because the pituitary cells were dispersed and geographically separated in the RHPA amongst oRBCs, the somatotroph responses to ghrelin must be due to direct effects on the GH-secreting cells, rather than to actions mediated through other pituitary cell types. Dose-dependent GH responses to ghrelin have also been established with dispersed rat pituitary cells (Kojima *et al.* 1999, 2001, Tolle *et al.* 2001, Yamazaki *et al.* 2002), in which the EC₅₀ is also in the 10⁻⁸ M range. The minimally effective dose of ghrelin in chickens *in vitro* (10⁻⁹ M) is, however, higher than that (10⁻¹¹ M) required to provoke GH release in some studies with rat pituitary cells (Yamazaki *et al.* 2002), although it is comparable with that in other studies (Kojima *et al.* 1999). Other GH secretagogue receptor agonists also promote GH release from rat pituitary tissues (Locatelli *et al.* 1994) and a pituitary site of ghrelin action is undoubtedly involved, despite the finding by Pinilla *et al.* (2003) that ghrelin was ineffective in inducing GH release from the pituitaries of prepubertal rats at concentrations of 10⁻⁹ to 10⁻⁷ M, concentrations that were effective for GHRH.

The sensitivity of the *in vitro* GH response of dispersed chicken pituitary cells to ghrelin was comparable with the GH response to GHRH, although the responses were of lesser magnitude. Ghrelin-induced GH secretion from chicken pituitary cells is thus likely to be mediated by a rapid degranulation of the somatotrophs, as demonstrated for other GH secretagogues (Hull *et al.* 2000). The action of ghrelin is, however, accompanied by an increase in the number of somatotrophs induced to release GH and by an increase in the amount of GH released by individual somatotrophs. These somatotroph responses are very similar to the responses of chicken pituitary cells to GHRH stimulation, as revealed by RHPA analysis (Morpurgo & Porter 1995, Porter *et al.* 1995, Dean *et al.* 1997). These results are, however, the first to demonstrate ghrelin-induced GH secretion from individual somatotrophs for any species.

While ghrelin may have pituitary sites of action in mammals, the *in vivo* GH responses to ghrelin in mammals is thought to be largely mediated through the central nervous system (CNS) (Date *et al.* 2000, Wren *et al.* 2000, 2002, Tamura *et al.* 2002, Pinilla *et al.* 2003). Indeed, ghrelin inhibits somatostatin (SRIF) release (Tolle *et al.* 2001, Seoane *et al.* 2003) and increases GHRH tone (Tannenbaum & Bowers 2001, Tannenbaum *et al.* 2003), either of which would augment its pituitary action. Ghrelin may have similar actions in chickens, since synthetic ligands of the GH secretagogue/ghrelin receptor increase GH release at pituitary and extrapituitary sites (Geris *et al.* 1998, 2001). The possible contribution of SRIF disinhibition or GHRH stimulation or GHRH synergy (Takaya *et al.* 2000, Arvat *et al.* 2001, Hataya *et al.* 2001) to ghrelin-induced GH secretion in birds is, however, unknown. An extrapituitary action of ghrelin that suppresses the *in vivo* GH response of chicks to ghrelin is, however, suggested by the observation that the GH response to ghrelin *in vitro* (over four orders of magnitude) differed from the *in vivo* response (over two orders of magnitude). This contrasts with the *in vivo* GH-releasing activity of TRH in chickens, which is known to be partly due to stimulatory actions at CNS sites (Harvey *et al.* 1990). Although this also contrasts with the central actions of GH secretagogue receptor agonists in chickens (Geris *et al.* 2001), both stimulatory and inhibitory actions of GH secretagogues on SRIF release have been observed in rats (Bluet-Pajot *et al.* 2001).

In summary, these results demonstrated that ghrelin is a dose-related GH secretagogue in immature chickens, directly stimulating the number of somatotrophs secreting GH and the amount of GH released from individual somatotrophs.

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