Ghrelin: a hypothalamic GH-releasing factor in domestic fowl (*Gallus domesticus*)

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Abstract

Ghrelin, a recently discovered peptide in the mammalian hypothalamus and gastrointestinal tract is thought to be the endogenous ligand for the GH secretagogue (GHS) receptor and it stimulates GH release in rats and humans. The possibility that ghrelin is present in birds was therefore assessed, since a GHS receptor is present in the chicken pituitary gland.

Although immunoreactive ghrelin is readily detectable in the rat stomach and ileum, ghrelin immunoreactivity could not be detected in the chicken proventriculus, stomach, ileum or colon, whereas somatostatin immunoreactivity, in contrast and as expected, was readily detectable in the chicken gastrointestinal tract. Ghrelin immunoreactivity was, however, present in the chicken hypothalamus, although not in the arcuate (infundibular) nucleus, as in rats. Discrete parvocellular cells and neuronal fibers with ghrelin immunoreactivity were present in the anterior medial hypothalamus. This immunoreactivity was specific and completely abolished following the pre-absorption of the antibody with an excess of human

ghrelin. Ghrelin immunoreactivity was also present in clusters of large ovoid magnocellular cells in the nucleus magnocellularis preopticus pars medialis, nucleus magnocellularis preopticus supraopticus and in the chiasm-opticus. Immunoreactivity for ghrelin was restricted to the cytoplasm of the perikarya and their axonal sprouts. Immunoreactivity for ghrelin was not seen in any other hypothalamic nuclei.

In a preliminary experiment, circulating GH concentrations in conscious immature chicks were promptly increased following bolus i.v. administration of human ghrelin. The increase in GH concentration (approximately three times that in the controls) was comparable with that induced by the same dose (10 µg/kg) of human GH-releasing hormone, although less than that (approximately sixfold) induced by thyrotropin-releasing hormone.

These results demonstrate the presence of a ghrelin-like protein in the chicken hypothalamus and suggest that it participates in the regulation of GH secretion in birds.

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Introduction

Growth hormone (GH) secretion is regulated by a myriad of interacting, multihierarchical factors that dynamically modify somatotroph function at neural and humeral interfaces in neurocrine, neuroendocrine, endocrine and paracrine ways (Harvey 1995). The hypothalamus is, however, thought to primarily regulate GH release in mammals through antagonistic interactions between stimulatory (GH-releasing hormone; GHRH) and inhibitory (somatostatin; SRIF) hypophysiotropic-releasing hormones. A third hypothalamic factor, ghrelin, may also be involved (Dieguez & Casanueva 2000, Kojima *et al.* 2001), since it is present in the arcuate nucleus of the mouse, rat and human hypothalamus (Kojima *et al.* 1999, Hosoda *et al.* 2000a, Korbonsits *et al.* 2001) and is three to five times more potent than GHRH in stimulating GH release in these species (Kojima *et al.* 1999, Arvat *et al.* 2000, Seoane *et al.* 2000). It is, however, not known if ghrelin is involved in GH regulation in other species.

In birds, the control of GH secretion differs from that in mammals, since chicken GHRH has minimal GH-releasing activity in chickens (Harvey 1999) and because thyrotropin-releasing hormone (TRH) is a potent GH-releasing factor (Harvey 1990). The possibility that ghrelin may participate in GH release in birds was therefore assessed, especially as ghrelin is thought to be the endogenous ligand for a GH secretagogue (GHS) receptor identified in chicken pituitary glands (Toogood *et al.* 1999, Gaylinn *et al.* 2000).

Materials and Methods

Immunocytochemistry

White Leghorns at 4 weeks of age were killed by cervical dislocation (with approval from the University of Alberta Health Sciences Animal Welfare Committee) and hypothalami were rapidly dissected from the heads, washed in

ice-cold phosphate-buffered saline (PBS; pH 7.4) and collected into freshly prepared paraformaldehyde (4% w/v) (Sigma, Mississauga, Ontario, Canada). The hypothalami were removed in one piece, with two parallel cuts 2.0–2.5 mm lateral to the midline, a transverse cut in front of the preoptic area and the roots of the oculomotor nerves and a cut 4–5 mm deep parallel to the base of the brain (MacNamee & Sharp 1989). After overnight fixation, at 4 °C, the tissues were dehydrated in a graded series of alcohol (50% v/v, 15–30 min; 70%, 30–60 min; 95%, 30–60 min; 100%, 30–60 min) and cleared with hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax for 24 h at 60 °C, under normal atmospheric pressure. Serial transverse sections (4–8 µm) were cut with a microtome and mounted onto treated slides (Fisher Scientific). The sections were cut anterior–dorsally, using co-ordinates A10.0 to A5.6, according to the histological atlas of Kuenzel & Masson (1988). Magnocellular nuclei were identified according to Yasuda (1980) and Mikami (1985).

Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlington, CA, USA and Sigma), using an avidin–biotin–peroxidase (ABC) (Hsu *et al.* 1981) method. Sections were incubated with a specific commercial antibody raised in rabbits against rat ghrelin (RAB-031–31; Phoenix Pharmaceuticals, Belmont, CA, USA), with which human ghrelin has 100% cross-reactivity. The primary antibody was diluted 1:500 in 1–5% normal goat serum, overnight, at 4 °C. After incubation, the slides were washed three times for 15 min in PBS. Sections were then incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (Sigma; 1:200). The slides were washed in PBS and incubated in ABC reagent for 1 h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate diaminobenzidine tetrahydrochloride (Sigma), which resulted in a brown coloration. The specificity of staining was determined by preabsorbing the primary antibody with synthetic human ghrelin (Phoenix Pharmaceuticals; 1 mg/ml) for 1 h prior to section incubation. Non-specific staining was determined by replacing the primary antibody with pre-immune rabbit serum or with PBS or by omission of the secondary antibody. As a positive control (Kojima *et al.* 1999), sections of the rat stomach were similarly stained for ghrelin immunoreactivity. Sections of the chick stomach and gastrointestinal tract were similarly stained for SRIF immunoreactivity, using an antibody raised in rabbits against SRIF₁₄ (Di Scala-Guenot *et al.* 1984) at a dilution of 1:200, since SRIF immunoreactivity has been found throughout the chicken gastrointestinal tract (Denbow 2000).

GH-releasing activity

The GH-releasing activity of human ghrelin (Phoenix Pharmaceuticals) was assessed in conscious 4-week-old

White Leghorn chicks, in comparison with human GHRH_{1–44} and TRH (Bachem, Torrance, CA, USA). Groups of birds ($n=8–16$) were given a bolus i.v. injection of peptide or the 0.9% (w/v) NaCl vehicle (1 ml/kg body weight). Each peptide was administered at a dose of 10 µg/kg, since this is the maximally effective dose of TRH and GHRH in chickens (Harvey & Scanes 1984) and is comparable to doses of ghrelin that stimulate GH release in rats (4–20 µg/kg in rats, Hosoda *et al.* 2000b) and humans (5 µg/kg, Takaya *et al.* 2000; 33–66 µg/kg, Peino *et al.* 2000). Venous blood samples were collected from each bird 10 min after injection, at the time of the maximal GH response to TRH and GHRH (Harvey & Scanes 1984).

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). Statistical differences in the results were determined by analysis of variance.

Results

Immunohistochemistry

As expected (Kojima *et al.* 1999, Date *et al.* 2000a), ghrelin immunoreactivity was abundantly present within the X/A-like endocrine cells of the rat stomach (Fig. 1A and C). Staining was completely abolished following the preabsorption of the antiserum with human ghrelin (Fig. 1B). In contrast, sections of the chicken stomach (and the proventriculus, ileum and colon) had no ghrelin immunoreactivity (data not shown), but were stained for SRIF immunoreactivity (Fig. 1D and F). In the hypothalamus, ghrelin immunoreactivity was present in discrete cells in the nucleus anterior medialis hypothalami (AM) (Fig. 2A–C and Fig. 4), and was also present in clusters of large ovoid cells in the nucleus magnocellularis preopticus pars medialis (PRM) (Fig. 2A, E and F and Fig. 4), nucleus magnocellularis preopticus pars supraopticus (PRS) (Fig. 3A, E and F and Fig. 4) and in the chiasma opticus (CO) (Fig. 3A–C and Fig. 4). Immunoreactivity for ghrelin was restricted to the cytoplasm of the perikarya and their axonal sprouts. Ghrelin staining was lost following the preabsorption of the antibody with human ghrelin (Fig. 2D and Fig. 3D) and not seen when the ghrelin antibody was replaced by normal rabbit serum or PBS (data not shown). Immunoreactivity for ghrelin was not present in any other hypothalamic nuclei.

GH-releasing activity

Circulating GH concentrations in conscious, immature chicks were promptly increased ($P<0.001$) following the i.v. administration of human ghrelin (Fig. 5). The increase in GH concentration (approximately three times that in

Figure 1 (A) Ghrelin immunoreactivity in the rat stomach ($\times 100$). (B) Ghrelin immunoreactivity is lost following preabsorption of the primary antibody with excess human ghrelin ($\times 100$). (C) X/A-like endocrine cells are strongly stained for ghrelin immunoreactivity ($\times 1000$). (D) SRIF immunoreactivity in the chick stomach ($\times 100$). (E) SRIF immunoreactivity is lost following omission of the primary antibody ($\times 100$). (F) Higher magnification of the SRIF-immunoreactive cells in the chick stomach ($\times 1000$).

Figure 2 Distribution of ghrelin immunoreactivity in perikarya and fibers in the chick hypothalamus. (A) Ghrelin immunoreactivity in the AM and PRM (×100). (B and C) Magnification of the AM (×400 and ×1000 respectively). (D) Immunoreactive staining in the AM and PRM was lost following the preabsorption of the primary antibody with excess human ghrelin (×100). (E and F) Magnification of the PRM (×400 and ×1000 respectively). Solid arrows indicate stained perikarya whilst the dotted arrow indicates immunoreactive fibers. LSO, organum septi laterale; VIII, third ventricle.

Figure 3 Distribution of ghrelin immunoreactivity in perikarya and fibers in the chick hypothalamus. (A) Ghrelin immunoreactivity in the CO and PRS ($\times 100$). (B and C) Magnification of the CO ($\times 400$ and $\times 1000$ respectively). (D) Immunoreactive staining in the CO and PRS is lost following preabsorption of the primary antibody with excess human ghrelin ($\times 100$). (E and F) Magnification of the PRS ($\times 400$ and $\times 1000$ respectively). Solid arrows indicate stained perikarya whilst dotted arrows indicate immunoreactive fibers. RP, recessus preopticus.

Figure 4 Ghrelin immunoreactivity in the chick hypothalamus. (A) Schematic illustration of the location of immunostained cell bodies (solid and open circles) in the AM and in magnocellular cells in the PRM. (B) Schematic illustration of the location of immunostained cell bodies (solid and open circles) in magnocellular cells in the CO and PRS. SCNm, nucleus suprachiasmaticus, pars medialis; FPL, fasciculus prosencephali lateralis (lateral forebrain bundle); LSO, organum septi laterale (lateral septal organ). Based on the histological atlas of Kuenzel & Masson (1988).

Figure 5 Plasma GH in 4-week-old chicks 10 min after the i.v. injection of ghrelin, GHRH or TRH (all at 10 μ g/kg body weight), in comparison with controls injected with the 0.9% NaCl vehicle (1 ml/kg). * $P < 0.01$ compared with the controls. Numbers in each group are indicated in the bars. Values are means $\pm$ S.E.M.

the controls) was comparable with that induced by the same dose (10 μ g/kg) of human GHRH, although less ($P < 0.01$) than that (approximately sixfold) induced by TRH.

Discussion

These results demonstrate, for the first time, a stimulatory effect of human ghrelin on GH secretion in the chicken. The prompt GH-releasing activity of human ghrelin was comparable to that induced by the same dose (10 μ g/kg) of human GHRH, although of lesser magnitude than that induced by TRH (on a weight basis). Additional dose- and time-course studies, on a molar basis, are required to fully characterize its role as a GHS in birds, but it is of interest that activation of the GHS (ghrelin) receptor by two non-peptidyl mimics (L-692,429 and L-163,225) also promptly increased GH secretion in the chicken (Geris *et al.* 1998, 2001).

The stimulation of GH secretion by ghrelin could result from a direct pituitary action. Indeed, the presence of a GHS (ghrelin) receptor in the chick pituitary gland (Toogood *et al.* 1999, Gaylinn *et al.* 2000), the stimulatory actions of the peptidomimetics on GH release from perfused chicken pituitary glands (Geris *et al.* 2000, 2001) and the ability of ghrelin to induce GH release from rat pituitary glands *in vitro* (Kojima *et al.* 1999) support this possibility.

The results of the present study also demonstrate, for the first time, the presence of a ghrelin-like protein in a non-mammalian species, with cross-reactivity with rat and human ghrelin. Ghrelin-like immunoreactivity in the chick was, however, in dissimilar locations to its presence in mammals. In rats and humans, ghrelin is primarily found in the stomach and gastrointestinal tract (Kojima *et al.* 1999, 2001, Date *et al.* 2000a), although it is also present in the arcuate nucleus (Kojima *et al.* 1999, Korbonsits *et al.* 2001). In the chick, ghrelin immunoreactivity could not be detected in the stomach, duodenum, ileum or colon, even though SRIF immunoreactivity (as expected, Denbow 2000) was clearly present. This restriction of ghrelin immunoreactivity to the hypothalamus of the chick suggests that it evolved phylogenetically as a neuropeptide rather than as a gastrointestinal hormone.

Although ghrelin immunoreactivity was found in parvocellular cells in the hypothalamus, it was not in the arcuate nucleus (infundibular nucleus) as in rats and humans, but in discrete populations in the AM. Since parvocellular cells in the hypothalamus are thought to terminate on portal blood vessels in the median eminence, the presence of ghrelin in the AM suggests that it may be a hypophysiotropic releasing factor and stimulate pituitary GH release after secretion into the hypothalamo-hypophyseal circulation. Furthermore, as GH has recently been discovered in the AM (Ramesh *et al.* 2000), ghrelin may also have roles in the autocrine or paracrine regulation of GH synthesis or release within the brain.

In addition to the AM, ghrelin immunoreactivity was also found in magnocellular cells in the ventromedial hypothalamus (in the PRM and PRS (derived from the supraoptic nucleus) and in the CO). This is the first demonstration of ghrelin in magnocellular cells. The presence of ghrelin in these cells therefore resembles the presence of arginine vasotocin and mesotocin in the chicken hypothalamus (Tennyson *et al.* 1985, Robinson *et al.* 1988, Barth *et al.* 1997). Fibers from these cells have been shown to extend to the avian median eminence (Mikami 1985, Tennyson *et al.* 1985, Robinson *et al.* 1988) and it is therefore possible that ghrelin stimulates pituitary GH after release from these fibers into the hypophyseal portal circulation. Most magnocellular neurons do not, however, terminate in the median eminence but in the neurohypophysis. Ghrelin-induced GH secretion in the chick may thus be indirect and mediated by release into the short portal vessels connecting the pars nervosa with the pars distalis. Although indirect, this route is commonly utilized for transporting hypothalamic releasing factors to the adenohypophysis in non-primate species (Anthony *et al.* 1998).

In addition to a pituitary site of action, ghrelin-induced GH secretion could also be mediated centrally. Indeed, ghrelin has been shown to rapidly (within 10 min) stimulate GH secretion in rats through an inhibition of hypothalamic somatostatin release (Date *et al.* 2000b, Tolle

et al. 2001, Wren *et al.* 2000). Hypothalamic actions of ghrelin on *fos*-, *erg*-1, agouti-related protein and neuropeptide Y transcription (Hewson & Dickson 2000, Nakazato *et al.* 2001) and food intake (Kamegai *et al.* 2000, Masuda *et al.* 2000, Tschop *et al.* 2000, Nakazato *et al.* 2001, Shintani *et al.* 2001) are also well established, reflecting the widespread distribution of ghrelin receptors in the central nervous system (CNS) (Howard *et al.* 1996, Guan *et al.* 1997, McKee *et al.* 1997). The GH-releasing activity of human ghrelin in the chicken may, thus, be partially mediated by actions at CNS sites, especially as the non-peptidyl mimetics of GHS receptors partially stimulate GH release in chicks by rapidly increasing hypothalamic TRH release (Geris *et al.* 1998, 2001). The stimulatory effects of GHRH and TRH on GH secretion in the chick are similarly partially mediated by actions within the CNS (Harvey 1990, 1999). The possibility that ghrelin acts within the CNS is also supported by its ability to act at central sites to modulate food intake in the fowl (Furuse *et al.* 2001).

In summary, these results demonstrate the presence of ghrelin immunoreactivity in neurons of the chicken hypothalamus and stimulatory actions of ghrelin on GH secretion in the chick.

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