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Growth hormone (GH)-releasing activity of chicken GH-releasing hormone (GHRH) in chickens

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ABSTRACT

Two peptides with sequence similarities to growth hormone releasing hormone (GHRH) have been identified by analysis of the chicken genome. One of these peptides, chicken (c) GHRH-LP (like peptide) was previously found to poorly bind to chicken pituitary membranes or to cloned and expressed chicken GHRH receptors and had little, if any, growth hormone (GH)-releasing activity *in vivo* or *in vitro*. In contrast, a second more recently discovered peptide, cGHRH, does bind to cloned and expressed cGHRH receptors and increases cAMP activity in transfected cells. The possibility that this peptide may have *in vivo* GH-releasing activity was therefore assessed. The intravenous (i.v.) administration of cGHRH to immature chickens, at doses of 3–100 μg/kg, significantly increased circulating GH concentrations within 10 min of injection and the plasma GH levels remained elevated for at least 30 min after the injection of maximally effective doses. The plasma GH responses to cGHRH were comparable with those induced by human (h) or porcine (p) GHRH preparations and to that induced by thyrotropin releasing hormone (TRH). In marked contrast, the i.v. injection of cGHRH-LP had no significant effect on circulating GH concentrations in immature chicks. GH release was also increased from slaughterhouse chicken pituitary glands perifused for 5 min with cGHRH at doses of 0.1 μg/ml or 1.0 μg/ml, comparable with GH responses to $hGHRH_{1-44}$. In contrast, the perifusion of chicken pituitary glands with cGHRH-LP had no significant effect on GH release. In summary, these results demonstrate that cGHRH has GH-releasing activity in chickens and support the possibility that it is the endogenous ligand of the cGHRH receptor.

1. Introduction

Growth hormone releasing hormone (GHRH) peptides based on human, pig and rat sequences increase circulating growth hormone (GH) concentrations when systemically administered to chickens, turkeys and ducks (Harvey, 1999). The maximal GH response to these peptides occurs with doses between 1 μg/kg and 10 μg/kg and occurs within 5–10 min of intravenous (i.v.) GHRH injection. The *in vivo* GH response to these peptides largely reflects direct pituitary action and the presence of specific GHRH receptors (Porter et al., 2006; Toogood et al., 2006; Wang et al., 2006, 2007) and results in the degranulation of somatotrophs (Hull et al., 2000; Baudet and Harvey, 2003). However in marked contrast, the originally described chicken GHRH-like peptide (McRory et al., 1997), was found to have little (if any) GH-releasing activity in chicks (Harvey, 1999) or at the chicken GHRH receptor (Toogood et al., 2006).

A gene for a chicken (c) GHRH-like peptide was cloned and sequenced almost two decades ago (McRory et al., 1997). This gene could be alternatively spliced to produce mRNAs coding for several peptides, the most abundant of which had only 42% sequence identity with human GHRH and 47% identity with rat GHRH. This 46 amino acid GHRH-like peptide ($cGHRH\text{-}LP_{1-46}$) and a synthetic fragment of its first 29 amino acids ($cGHRH\text{-}LP_{1-29}$) had very little effect on plasma GH concentrations in chicks 10 min after i.v. administration, at doses of 1, 10, 30 and 100 μg/kg (Harvey, 1999). These peptides also failed to stimulate GH release from chicken pituitary glands *in vitro* (Harvey, 1999), and the full-length peptide was unable to displace the binding of labeled human (h) GHRH to the cloned chicken GHRH receptor even at very high doses (Toogood et al., 2006). This total lack of GH-releasing activity

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likely reflects an error in the original sequencing of the peptide cDNA, which resulted in an asparagine at residue 21 (McRory et al., 1997) rather than lysine, as observed in all other GHRH peptides (Sherwood et al., 2000). This possibility was supported by the re-cloning of the chicken GHRH gene and recognition of the correct K^{21} cGHRH sequence (Lys^{21}-cGHRH-LP) (Toogood et al., 2006; Wang et al., 2007). This possibility is further supported by the finding that K^{21} cGHRH-LP_{1-33} displaced labeled hGHRH binding to the cloned cGHRH receptor whereas the original N^{21} cGHRH-LP_{1-46} did not (Toogood et al., 2006). K^{21}-cGHRH-LP_{1-33} could, moreover, displace the binding of labeled hGHRH to chicken pituitary membranes, albeit at high dose levels (Toogood et al., 2006). K^{21} cGHRH-LP_{1-33} and K^{21} cGHRH-LP_{1-44} similarly increased the accumulation of cAMP in HEK 293 cells transfected with the cGHRH receptor, although they were approximately 100 fold less potent than $hGHRH_{1-32}$ (Toogood et al., 2006).

The relatively poor activity of K^{21} cGHRH-LP at the cGHRH receptor is comparable to the activity seen for related peptides like PACAP and VIP (Toogood et al., 2006) and suggests that cGHRH-LP may not be its true endogenous ligand. The similar finding of two GHRH-like peptides in the goldfish, only one of which was active at the goldfish GHRH receptor (Kee et al., 2005), is consistent with this view.

In a previous report (Toogood et al., 2006) we were the first to suggest that a previously unrecognized GHRH-like peptide (cGHRH) that was present in the sequence found by the chicken genome project (GenBank BX929984.1) was the true chicken GHRH. In mammals, two copies of the ancestral GHRH/PACAP gene diverged phylogenetically, such that one copy encodes PACAP and PACAP-related peptide (PRP) and the other encodes GHRH and GHRH-related peptide (Sherwood et al., 2000). The previously studied chicken GHRH-like peptide is similar to mammalian PRP, since it is on the same gene with PACAP and contains a C-terminal sequence motif found in mammalian PRP (Toogood et al., 2006). The new cGHRH examined here is more like mammalian GHRH, in that it is located on a separate gene from PACAP, lacks the PRP C-terminal motif, and has slightly higher sequence homology to mammalian GHRH (10 vs 12 amino acid differences compared to human in the crucial 1–29 region of the peptide).

The possibility that this new peptide might be the true cGHRH with GH-releasing activity is supported by the subsequent demonstration that this peptide can act through the cloned and expressed cGHRH receptor to increase cAMP-protein kinase A activity in transfected CHO cells (Wang et al., 2007). The possibility that this peptide also stimulates GH release in chickens was therefore examined in the present study.

2. Materials and methods

2.1. Synthesis of cGHRH-like peptides

cGHRH-(1–29) HADAIFTDNYRKFLGQISARKFLQTIIGK and related GHRH-like peptides were synthesized using a 9-fluorenylmethoxycarbonyl strategy on tentagel resin (0.27 mmol/g) with a rink linker, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumh-exafluoro phosphage and N,N-diisopropylethylamine in dimethylformamide. If a double coupling was needed, N,N′-diisocarbodiimide and 1-hydroxybenzotriazole were used. Ninhydrin and chloranil tests were used to monitor the presence of primary and secondary amines. The peptides were cleaved from the resin with Reagent K (trifluoroacetic acid 82.5%, H_20 5% phenol 5%, thioanisole 5%, ethanedithiol 2.5%). The cleavage solutions were filtered, concentrated under pressure, and precipitated in cold ether. The resulting precipitate was dissolved in water and lyophilized. The product peptide was purified via HPLC and verified by mass spectrometry.

2.2. GH-releasing activity of cGHRH-LP and cGHRH

The GH-releasing activity of K^{21} GHRH-LP_{1-33}, K^{21} cGHRH-LP_{1-44} and $cGHRH_{1-29}$ was first assessed *in vivo* in immature male White Leghorns at an age (4–5 weeks) when they are maximally responsive to GH-secretagogues (Harvey and Scanes, 1984; Harvey et al., 1991; Harvey, 1999) In some cases, sodium pentobarbital anaesthetized birds were used [as detailed in Harvey and Scanes, 1984] to eliminate episodic GH secretion and maximize relative GH responsiveness. Peptides were freshly dissolved in 0.9% NaCl containing 0.01% (w/v) ascorbic acid (to prevent oxidation) and administered i.v. into a brachial vein in a volume of 1.0 ml/kg. Controls were similarly injected with vehicle. Blood samples were collected from the contralateral brachial vein before and/or 10 min after i.v. injection and in some case after 20 and 30 min. Samples were collected into heparinized tubes and kept on ice until centrifugation and separation of plasmas, which were stored at −20 °C prior to GH radioimmunoassay (Harvey and Scanes, 1984). For comparative purposes, groups of birds were similarly injected with $hGHRH_{1-44}$ (Bachem, Torrance, California, USA), porcine (p) $GHRH_{1-29}$ (a gift from Dr. Robert M. Campbell, Hoffman LaRoche, New Jersey) or thyrotrophin-releasing hormone (Bachem), since they are all established GH secretagogues in chickens (Harvey et al., 1991). Data were expressed as means ± SEM, and statistical differences in the results were determined by Students paired or unpaired *t* test, where appropriate.

K^{21} cGHRH-LP_{1-29} and $cGHRH_{1-29}$ GH-releasing activity was also determined *in vitro*, using 6–8 week old slaughterhouse (broiler fowl) pituitary glands perifused with Medium 199 (Sigma–Aldrich, Oakville, Ontario, Canada), as previously described (Harvey, 1990, 1999). Data were expressed as means ± SEM and statistical differences in the results were determined by Students *t* test or ANOVA, where appropriate.

3. Results

3.1. GH-releasing activity of cGHRH-LPs and cGHRH in vivo

As expected, $pGHRH_{1-29}$ and $hGHRH_{1-44}$ (both at 10 μg/kg) significantly ($P < 0.01$) increased plasma GH concentrations (approximately 3-fold) 10 min after i.v. administration in conscious 4 week-old chicks (Fig. 1A). In marked contrast K^{21} cGHRH-LP_{1-29} had no stimulatory action at 30 μg/kg and suppressed ($P < 0.05$) circulating GH concentration at higher (100 μg/kg and 300 μg/kg) dose levels. Basal GH concentrations in anesthetized chicks were far lower ($P < 0.001$) than those in their conscious counterparts but were increased ($P < 0.001$ approximately 5-fold) 10 min after $hGHRH_{1-44}$ administration, whereas K^{21} cGHRH-LP_{1-29} again lowered the GH concentration, at doses of 100 μg/kg and 300 μg/kg (Fig. 1B).

Similarly, whereas GH concentrations were greatly increased ($P < 0.01$) 10 min after $hGHRH_{1-44}$ and $pGHRH_{1-29}$ administration, K^{21} $cGHRH_{1-44}$ had no GH releasing activity at doses of 1, 10 or 50 μg/kg (Fig. 2). However, when injected i.v. into conscious birds, $cGHRH_{1-29}$ did increase circulating GH concentrations at doses of 10–100 μg/kg (Fig. 3). The GH response to the highest dose of the peptide (100 μg/kg) was, however, significantly less ($P < 0.05$) than that induced by the maximally effective doses (30 μg/kg and 10 μg/kg). The maximal GH response to cGHRH was comparable to that induced by TRH (at 10 μg/kg) (Fig. 3).

In a further *in vivo* study (Fig. 4) the GH-releasing activity of $cGHRH_{1-29}$, at 10 μg/kg, was also comparable with that induced