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Synthetic Growth Hormone Secretagogues Control Growth Hormone Secretion in the Chicken at Pituitary and Hypothalamic Levels

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In the chicken growth hormone (GH) secretion is predominantly controlled by two hormones, thyrotropin-releasing hormone (TRH) and somatostatin (SRIH), respectively stimulating and inhibiting GH release. In view of the hypothesis of a novel GH secretagogue (GHS) in mammals, this specific species was used to further assess the exact function of two nonpeptidyl GHSs—L-692,429 and L-163,255. Both synthetic products stimulate GH secretion directly at the level of the pituitary as shown in in vitro perifusion studies. Plasma GH levels increase within 10–15 min after a single challenge of L-692,429 or L-163,255. A SRIH pretreatment dimishes this GH response. Both GH-releasing peptide mimetics decrease hypothalamic TRH concentrations, whereas SRIH levels are not affected. The novel GHS may therefore control GH secretion both at the level of the pituitary and the hypothalamus. The present article shows that nonpeptidyl mimetics also control GH secretion in nonmammalian species suggesting that the endogenous hormone may be a conserved GH stimulator in several vertebrates. The GH response to GHS in birds may be regulated both directly at the level of the pituitary and by releasing another endogenous GH stimulator (TRH) from the hypothalamus.

Key Words: GH; chicken; L-692,429; L-163,255; TRH

Introduction

The control of growth hormone (GH) secretion has been investigated in avian species for more than 20 yr. The first hormone tested for its GH-releasing activity was mammalian GH-releasing hormone (GHRH) *(1)*. Several in vivo and in vitro studies showed, however, that thyrotropin-releasing hormone (TRH) is the main GH stimulator in birds *(2,3)*. This important role of TRH within the somatotropic axis is also suggested by the high abundancy of TRH receptor binding *(4)* and mRNA *(5)* in the caudal lobe of the chicken pituitary, which is devoid of thyrotrophs and filled with somatotrophs, and the positive correlation between hypothalamic TRH content and plasma GH levels during pre- and postembryonic development *(6)*.

Recently, increasing evidence is available that another GH secretagogue (GHS) may be originating from the hypothalamus. Both peptidyl (e.g., GH-releasing peptide-6 [GHRP-6]) and nonpeptidyl agonists (e.g., L-692,429) release GH in mammals *(7–10)*. These GHSs act directly on the pituitary gland and release GH via a mechanism independent of GHRH *(11,12)*. The identification and cloning of an endogenous G-protein-coupled receptor, specifically activated by GHSs, suggested that an extra endogenous GHS exists in mammals *(13,14)*. A 28 amino acid peptide was recently isolated from rat stomach and shown to specifically increase GH secretion by binding to the GHS receptor (GHS-R). It was called ghrelin *(15)*.

The in vitro and in vivo specificity and efficacy of the synthetic nonpeptidyl GHSs to release GH has been studied extensively in mammals *(10,16–18)*. Data on their potency in nonmammalian species, however, are scarce. Because GHRP-6 is also active as a GHS in the chicken *(7)*, we recently analyzed the in vitro and in vivo actions of L-692,429 on GH secretion *(19)*. In this article, we present our latest data, using both L-692,429 and L-163,255, a more potent derivative, as synthetic GH stimulators.

Results

In Vitro Experiments

Figure 1 shows the in vitro response of somatotrophs to the two GHSs. A clear increase in GH release is induced by L-692,429 at a concentration of 100 n*M*, known to evoke a maximal response in this species *(19)*. Although the increase in GH is small in the 100 n*M* L-163,255 group (Fig. 1), calculation of the different parameters (Table 1) indicates an approximately twofold stimulation. L-163,255 at a concentration of 10 n*M* did not alter GH release (Fig. 1, Table 1). The in vitro GH secretory activity of L-692,429 was significantly higher than that of L-163,255: 100 n*M* L-

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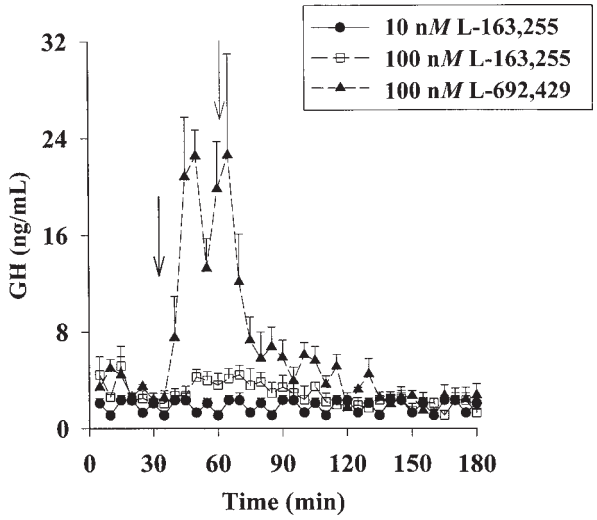

Fig. 1. In vitro GH release from pituitaries of 1-d-old chickens (C1) stimulated for 30 min with L-692,429 (100 n*M*) or L-163,255 (10 or 100 n*M*) after a 30-min baseline registration. Values shown are mean ± SEM ($n = 4$). Arrows indicate the stimulation period (taken into account the time needed for the medium to reach the fraction collector).

692,429 induced a fourfold higher stimulation of GH release compared to 100 n*M* L-163,255. This product-specific action at the level of the pituitary has been confirmed in a similar perifusion experiment. Both mimetics did not alter α-subunit levels in the different samples.

In Vivo Experiments

Because in the preliminary experiment only 50 µg of L-692,429 induced a significant increase in plasma GH (125%), this concentration was used to elucidate further the action of this mimetic. The high level of dimethyl sulfoxide (DMSO) (5%) did not affect plasma GH concentrations.

An acute challenge with 50 µg of L-692,429 induced higher circulating GH levels within 5 min (Fig. 2). This increase lasted at least until 15 min after treatment. No changes were observed in plasma α-subunit levels. Because TRH is a potent GH-releasing factor in the chicken *(2–4)*, hypothalamic TRH measurements were included in the study to link GH changes to possibly altered hypothalamic TRH release. A clear, transient decrease in hypothalamic TRH was recorded within 5 min (26% reduction), suggesting a rapid increase in the TRH secretion in response to the L-692,429 treatment (Fig. 2). Hypothalamic TRH concentrations returned to normal within 10 min and no further changes were observed. Hypothalamic SRIH content was not altered by the L-692,429 treatment.

L-163,255 treatment also resulted in increased plasma GH levels. Contrary to L-692,429, low doses of L-163,255 (0.5 and 5 µg) also provoked this response (Table 2). Regarding magnitude, no clear dose response was observed indicating that 0.5 µg of L-163,255 already induces the maximal GH response. The increase in GH was, however,

Table 1
Influence of L-163,255 (10 and 100 n*M*) and L-692,429 (100 n*M*) on In Vitro GH Secretion in 1-d-old Chickens (C1)[a]

Group	Basal secretion	Stimulation factor	Net increase
10 n*M* L-163,255	2.23 ± 0.12	1.05 ± 0.08	–4.58 ± 4.05
100 n*M* L-163,255	2.14 ± 0.49	1.98 ± 0.29[a]	16.97 ± 3.13[a]
100 n*M* L-692,429	3.07 ± 0.18[a]	7.33 ± 0.71[b,c]	114.3 ± 20.6[b,c]

[a,b]Values shown are mean ± SEM ($n = 4$). Significant differences with the 10 n*M* L-163,255 group and the other two experimental conditions ([a]$p < 0.05$; [b]$p < 0.01$).

[c]Significant differences between both 100 n*M* conditions ($P < 0.01$).

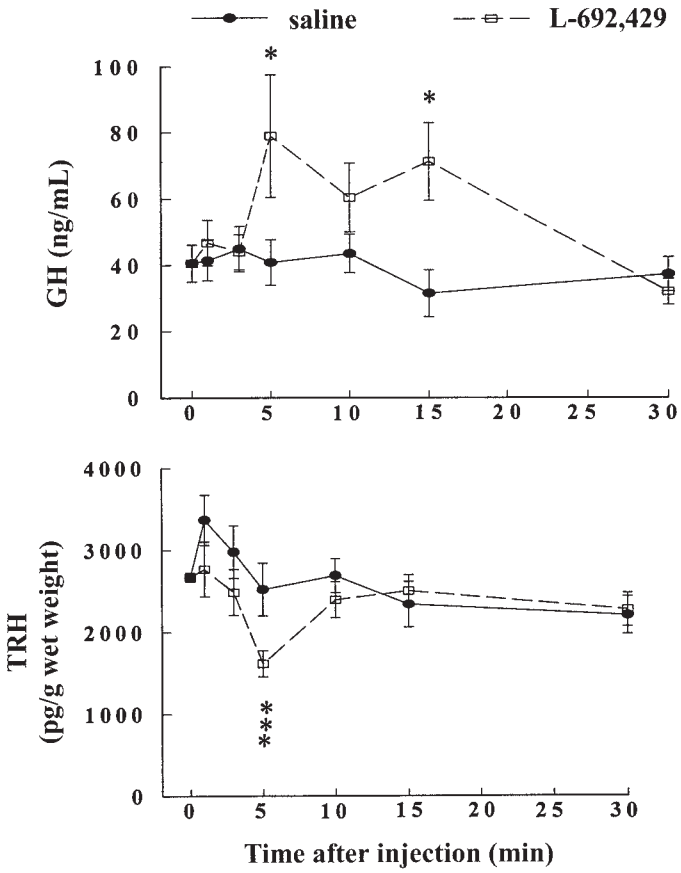

Fig. 2. Effect of a single injection of saline + 5% DMSO or 50 µg of L-692,429 (5% DMSO) on the circulating concentration of GH **(top)** or hypothalamic TRH content **(bottom)** in C1 chickens. Values represent mean ± SEM ($n = 10$/group). Asterisks indicate differences between the two experimental groups within one time point (*$p < 0.05$; ***$p < 0.001$).

more rapid when higher concentrations were administered. In addition, the drop in hypothalamic TRH content was observed earlier in animals injected with 50 µg of L-163,255. Although this decrease in TRH was not significant ($0.05 < p < 0.10$ at 5 min for 50 µg and at 10 min for the lower doses), it was observed consistently in the different groups injected with L-163,255 and was to a similar magnitude as

Table 2
Effect of Single Injection of Saline or L-163,255 (0.5-50 µg) on Plasma GH Levels and Hypothalamic TRH Concentrations in Newly Hatched Chickens (C1)[a]

	5 min		10 min	
Group	GH (ng/mL)	TRH (pg/g wet wt)	GH (ng/mL)	TRH (pg/g wet wt)
Saline	35.9 ± 5.7	1991 ± 278	45.6 ± 6.9	2158 ± 386
L-163,255 (0.5 µg)	63.2 ± 13.5	1749 ± 290	102.9 ± 28.3[b]	1529 ± 205
L-163,255 (5 µg)	58.4 ± 16.5	1679 ± 259	87.7 ± 16.8[b]	1458 ± 230
L-163,255 (50 µg)	71.7 ± 19.7[b]	1430 ± 177	70.0 ± 12.1	1888 ± 243

[a]Data shown are mean ± SEM (n = 10).
[b]Significant differences between experimental groups (L-163,255) and the control group (saline) ($p < 0.05$).

in the L-692,429 time study (28–32% reduction). Another experiment did show within 5 min a significant drop in hypothalamic TRH levels in chickens treated with 0.5 (1069 ± 330 pg/g of wet wt), 1 (1450 ± 402 pg/g of wet wt), or 2 µg of L-163,255 (1428 ± 358 pg/g of wet wt) vs control animals (2450 ± 406 pg/g of wet wt; each $p < 0.05$). Circulating α-subunit and hypothalamic somatostatin (SRIH) levels were never affected.

Figure 3 shows that the maximal response after L-163,255 (0.5 µg) treatment was found at 15 min (275% increase). This is approximately twofold that induced by 50 µg of L-692,429 (125%) (Fig. 2). A single challenge with SRIH 1 h prior to the administration of secretagogue clearly lowered the responsiveness of somatotrophs to L-163,255.

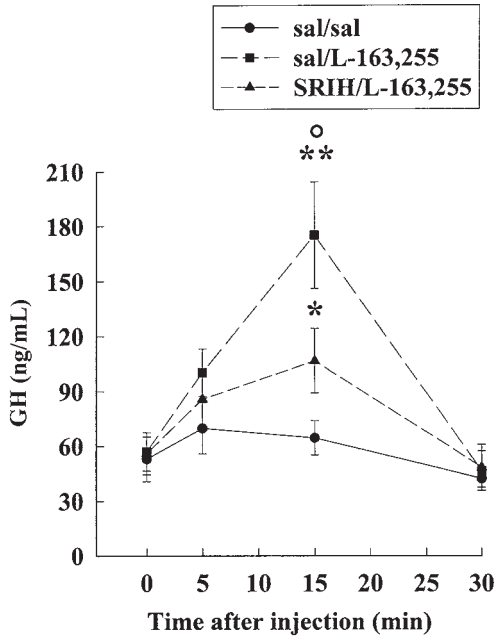

Fig. 3. Effect of SRIH (0.5 µg) pretreatment on the GH response to L-163,255 (0.5 µg) in 1-d-old chickens. The time interval between the two injections was 1 h. Data shown are mean ± SEM (n = 8). Asterisks indicate significant differences betweent he experimental groups and the control group (sal/sal) (*$p < 0.05$; **$p < 0.01$). Circle indicates significant differences within the two experimental groups ($p < 0.05$)

Discussion

After the discovery of the first nonpeptidyl GHS, L-692,429 *(9)*, many studies described the potency and action sites of this molecule and its derivatives. Although different species responded to nonpeptidyl GHSs with a higher secretion of GH, all animals studied were mammals. Because it is known that hypothalamic control of GH release may differ among different vertebrate classes, we recently looked at the GH-releasing activity of L-692,429 in an avian species, the chicken *(19)*. The present study further expands this study and shows that the novel endogenous GHS recently identified as ghrelin *(15)* is not only involved in the control of GH secretion in mammals but also in birds, suggesting that this hormone may be controlling GH release in several vertebrate species.

In vitro, the GH response to L-692,429 was more pronounced than to L-163,255. The latter GHS did not induce any GH response at 10 n*M* whereas a half-maximal increase has been observed with 10 n*M* L-692,429 *(19)*. Although both mimetics did not affect the release of thyrotropin or gonadotropins, it must be pointed out that measurements of prolactin (PRL) and adrenocorticotropic hormone (ACTH) have to be conducted to further confirm its specificity. In mammals, GHSs did not influence the in vitro ACTH release, and only small stimulatory effects on in vitro PRL secretion have been described in rats *(11,12,20)*. Because a clear corticosterone increase was previously reported in chickens injected with L-692,429 *(19)*, an ACTH stimulatory effect of these GHSs cannot be excluded.

Both mimetics induced a rapid and transient increase in plasma GH levels. As shown previously for GHRH and TRH *(21,22)*, SRIH inhibited this response. This action takes place directly at the level of the pituitary since the in vitro responsiveness of somatotrophs to L-692,429 drops after SRIH pretreatment (unpublished results).

Although the in vitro potency of L-163,255 was lower compared with L-692,429, 0.5 μg of L-163,255 already induced a clear increase in plasma GH concentrations. For L-692,429, on the other hand, a 100-fold higher amount is needed to increase circulating GH levels. Both in vivo concentrations used are comparable to those known to stimulate GH secretion in mammals *(10,23)*. To our knowledge, no data are available on the in vitro GH-releasing activity of L-163,255. This discrepancy in the in vivo and in vitro potency nevertheless indicates that in addition to a direct action at the level of the somatotroph, GHSs mediate GH secretion at an extrapituitary level. As recorded previously in mammals *(24,25)* and birds *(19)* GHSs modulate the hypothalamic pathway within the somatotrophic axis. Whereas benzolactams such as L-692,429 are considered to be stimulators of the release of GHRH from the median eminence *(25,26)*, L-163,255 does not alter GHRH levels in the portal blood of swine but suppresses SRIH secretion *(23)*. In the chicken, however, both benzolactam (L-692,429) and spiropiperidine (L-163,255) derivatives decreased hypothalamic TRH levels without affecting SRIH content. Both products can therefore alter GH release by stimulating TRH secretion from the hypothalamus.

Currently, we are developing an in vitro static culture of chicken hypothalami to relate the changes in hypothalamic TRH content effectively with an increased in vitro TRH release after GHS treatment. The lower GH response to L-692,429 after pretreatment with TRH antibodies further strengthens this hypothesis *(19)*.

In addition to hormone measurements, several techniques have been used to show the hypothalamic site of action for GHRP-6 and related secretagogues. Both the induction of c-fos *(26)* and the presence of GHS-R mRNA in GHRH neurons in the arcuate nucleus *(27)* are indicative of a central site of action of these GHSs. Because an antibody to chicken c-fos recently has been validated *(28)*, we plan to analyze the induction of this immediate early gene in GHS-treated chickens. This experiment together with the demonstration of chicken GHS-R mRNA in the paraventricular nucleus, the location of TRH neurons, will further address the proposed central action of GHSs in the chicken.

In conclusion, the present study clearly indicates that also in birds the analog to mammalian ghrelin, the putative endogenous ligand for the GHS-R *(15)*, functions as an extra stimulator of pituitary GH secretion. Although a direct action at the somatotrophs is taking place, the main site of action might be the hypothalamus where the release of TRH is modulated.

Materials and Methods

Animals

Chickens from a commercially developed layer strain (Hisex white) were used. Fertilized eggs or newly hatched cockerels were purchased from a local dealer (Euribrid, Aarschot, Belgium). Eggs were incubated in a forced-draft incubator, at 37.8°C, with increasing humidity and ventilation from d 14 onward (start of incubation = d 1). Posthatch chickens were kept in an acclimatized room for a 14-h light/10-h dark photoperiod. Water and food were available ad libitum. All experiments were performed on 1-d-old chickens (C1). All experiments were conducted in accordance with the highest standards of humane animal care.

In Vitro Experiments

Pituitaries of 1-d-old chickens (C1) were dissected and transferred into 1 of 12 individual perifusion chambers placed in a water bath at 37°C (one pituitary/chamber). M199 medium (Innogenetics, Belgium) was used as the perifusion medium at a flow rate of 12 mL/h. Each perifusion was divided into four periods: an equilibration period (1.5 h), a basal period (0.5 h), a stimulation period (0.5 h), and a poststimulation period (2 h). During the stimulation period, L-692,429 (100 n*M* [maximal GH response]) *(19)* or L-163,255 (10 and 100 n*M*) (Merck) was added to the medium (n = 4/group). Starting from the basal period onward, 1-mL samples from each chamber were collected over 5-min intervals and frozen prior to analysis of GH and α-subunit levels. The stimulation factor and the net GH increase above basal *(29)* were calculated for each individual chamber.

In Vivo Experiments

To identify an effective dose of L-692,429 and to test the effect of injecting DMSO, four treatments were evaluated in a preliminary experiment (C1): saline, saline + 5% DMSO, 5 μg of L-692,429 (0.125 μg/kg; 0.5% DMSO), and 50 μg L-692,429 (1.25 μg/kg; 5% DMSO). Injections were given into a leg vein. Animals were killed 15 min after injection and plasma was stored at –20°C (n = 8/group). A concentration of 1.25 mg/kg is used in mammalian studies *(10)*.

In a second experiment, chickens (C1) were divided into two groups: saline + 5% DMSO and 50 μg of L-692,429 (5% DMSO). Animals were killed by decapitation prior to (0 min) and 1, 3, 5, 10, 15, and 30 min after treatment (n = 10/group per time point studied). Blood and hypothalamic tissue were collected from each animal and stored at –20 or –80°C, respectively. Hypothalamic tissue was separated from the brain by removing consecutively the telencephalon, optic lobes, cerebellum, and brain stem. We are aware that the region studied contains more than the hypothalamus alone. However, because of the size and the fragile character of the neonatal brain, specific hypothalamic nuclei cannot be isolated with the punching method *(30)*. Furthermore, TRH and SRIH are predominantly found in this section *(31,32)* and a single GH (10 μg; unpublished results) or corticosterone (40 μg) *(33)* injection resulted in an increased hypothalamic TRH content within 1 h, indicative of the central feedback mechanism of peripheral hor-

mones. Therefore, the macrodissection of this region has been performed to study alterations in the hypothalamic TRH and SRIH content in the chicken.

A third series of C1 chickens was injected with saline (5% DMSO) or L-163,255 (three groups: 0.5, 5, or 50 µg). Blood and hypothalamic tissue were collected 5 and 10 min after treatment (n = 10/group per time point studied) and stored at –20 and –80°C, respectively.

Finally, the effect of SRIH pretreatment on the L-163,429-induced GH release was studied (C1 chickens). Two injections were administered 1 h apart. The first injection consisted of saline (groups 1 and 2) or SRIH (0.5 µg) (group 3). One hour later, saline (0.05% DMSO) (group 1: control) or 0.5 µg of L-163,255 (group 2 [positive control] and group 3) was injected. Blood was collected just prior to (0 min) or 5, 15, and 30 min after the second injection (n = 8/group per time point).

Extraction of SRIH and TRH

Extraction of SRIH and TRH was performed as described previously *(6)*. Extraction recovery of known amounts was 104.9 ± 5.3% (TRH) and 105.5 ± 13.2% (SRIH).

TRH and SRIH Radioimmunoassay

TRH and SRIH concentrations were measured as recorded previously *(6)*. The intraassay coefficients of variation (CVs) were 6.6 ± 0.4% (TRH) and 12.2 ± 2.1% (SRIH), and the interassay CVs were 15.6 ± 2.1% (TRH) and 16.1 ± 2.8% (SRIH). Displacement curves resulting from serial dilutions of hypothalamic or telencephalon homogenates were parallel to the standard curve.

Pituitary Hormone Measurements

GH levels were measured by radioimmunoassay (RIA) *(34)*. The sensitivity of the assay was 2 ng. The intra- and interassay CVs were 4.0 and 15.5%, respectively. Quantification of α-subunit concentration, which is the common glycoprotein subunit of thyrotropin (thyroid-stimulating hormone), luteinizing hormone, and follicle-stimulating hormone, was carried out according to Berghman et al. *(35)*. The sensitivity was 0.5 ng and the intra- and interassay CVs were 2.4 and 1.9%, respectively. Corticosterone concentrations were analyzed using a commercial kit (ICN, Belgium), validated for use in chickens *(36)* (sensitivity: 2 ng; intraassay CV: 7.3%; interassay CV: 6.9%). For all RIAs chicken plasma dilution and loading tests had good parallelism with the standard curve.

Statistics

Values represent the means ± SEM. Statistical analysis for both the in vitro and in vivo experiments was carried out by one-way analysis of variance.

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