Administration of a Nonpeptidyl Growth Hormone Secretagogue, L-163,255, Changes Somatostatin Pattern, But Has No Effect on Patterns of Growth Hormone-Releasing Factor in the Hypophyseal-Portal Circulation of the Conscious Pig (44430)

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Abstract. **The activity of the growth hormone secretagog, L-163,255, on growth hormone (GH), growth hormone-releasing factor (GRF), and somatostatin (SRIF) levels was evaluated in a porcine model of hypophyseal portal blood (HPB) collection. Young, castrated pigs had HPB and jugular blood collected for ≈ 300 min. The blood collection was divided into discrete periods: baseline (BL) ≈ 180 min; GH response period (RSP) ≈ 90 min; and positive control period following a GRF bolus, 30 min. RSP was divided into a dominant response period (DOM) and a tail (TL). The spontaneous relationship between HPB GRF and SRIF and peripheral GH during BL has been reported (Proc Soc Exp Biol Med 217:188–196, 1998). The apex of the GH pulse resulting from L-163,255 administration was nonrandomly associated ($P < 0.05$) with descending periods of SRIF troughs. Frequency and amplitude of GRF and SRIF pulses, and frequency and depth of SRIF troughs were not different between BL and the beginning of DOM (the 20–30 min of GH increase). GH AUC was significantly greater ($P < 0.05$) for DOM compared to BL and TL, and for TL compared to BL. GRF AUC tended to be greater ($P < 0.1$) for RSP compared to BL, but the majority of the increase was in the TL period. There were no significant differences in the SRIF AUCs between the sampling periods. Furthermore, in a separate experiment, *fos* activity (a marker of neuronal activation) in the hypothalamus of pigs was examined after either L-163,255 (1x or 4x), isotonic saline (control), or hypertonic saline (positive control) administration. There were no differences in *fos* activity in the GRF, SRIF, or CRH immunopositive neurons between L-163,255 treatment and control. The pituitaries of the L-163,255-treated pigs showed marked *fos* activation compared to the controls. In conclusion, L-163,255 in pigs has its primary effect at the level of the anterior pituitary.**

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The endogenous secretion of growth hormone (GH) is believed to be caused by the interplay of the opposing actions of the hypothalamic hormones growth hormone–releasing factor (GRF, stimulatory) and somatostatin (SRIF, inhibitory) (1, 2). In anesthetized rats, increased GH levels have been associated with an increase in GRF coupled with a decrease in SRIF (3). However, recent studies have alluded to a control mechanism more complex than simple antagonism. We have previously described (4) the sponta-

neous secretion of GRF and SRIF into hypophyseal portal blood (HPB); no inverse relationship was shown between GRF/SRIF and GH. Of the associations examined in that report, the only significant finding was that GH pulse maxima occurred nonrandomly within periods of SRIF descent ($P = 0.005$). Furthermore, in conscious sheep, clonidine (α-2-adrenergic agonist), neostigmine (cholinesterase inhibitor), and tianeptine (5-HT uptake enhancer) all increase GRF in portal blood resulting in a GH release, but cause no change in portal SRIF (5–8). In the same model, a peptidyl growth hormone secretagogue (GHS) hexarelin (GHRP-1) injection resulted in an increase in portal GRF and peripheral GH with no change in SRIF, as well (9). Therefore, it appears that GH release does not have to be associated with coincidental opposition of GRF and SRIF in pigs and sheep.

The mechanism of action of the peptidyl GHS GHRP-6 and the similarly acting nonpeptidyl GHSs, L-692,429 and L-692,585, have been equally elusive. It is generally accepted that these secretagogues have an effect at both the pituitary (10, 11) and the hypothalamus (12–16). To test this assumption in pigs, the HPB collections previously reported (4) were extended to measure the GRF and SRIF concentrations coincident with GHS-stimulated GH secretion. In addition, a separate experiment was conducted to assess the effect of L-163,255 on the activation in the pig hypothalamus and pituitary of *fos*, the protein product of the immediate early gene c-*fos* that has been used extensively as a marker of neuronal activation in neuroendocrine systems (17).

Materials and Methods

Experiment 1: Effect of L-163,255 on GH, GRF, and SRIF Secretions in Conscious Pigs. *Experimental protocol.* Animals used were the same six Yorkshire male castrates used in the previous report (4). The pigs were 4–5 months of age and weighed ≈ 63 kg. Jugular and portal blood samples were collected continuously and simultaneously in 5-min increments from each pig over a 300-min period. Treatments within that time included a 5-min saline infusion at ≈ 120 min and a 5-min infusion of L-163,255 HCl at 1 mg/kg (Fig. 1) given at ≈ 180 min. A pGRF(1–44)NH_2 (Bachem California, Torrance, CA) bolus was administered as a positive assay control (5 μg/kg) at ≈ 270 min. Both the L-163,255 and the GRF were dissolved in 10 ml saline for i.v. administration.

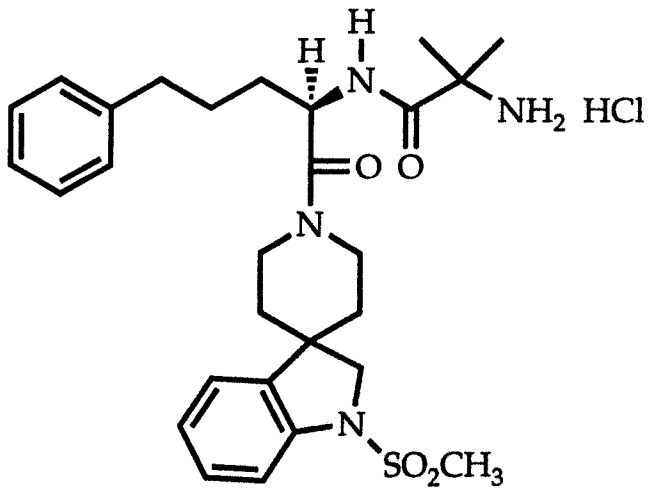

Figure 1. Structure of L-163,255.

Animal/surgical/blood collection procedures. Details of animal care, surgical procedures, and sample collection for the HPB portion of the study were reported previously (4). Animals were housed in AAALAC-accredited facilities in compliance with all applicable federal, state, and local regulations and in accordance with the *Guide for the Care and Use of Laboratory Animals*, NIH Publication no. 86–23, revised in 1985. All animals were subjected to a 12-hr light: 12-hr dark cycle beginning at 0700. A commercially available swine diet (14% crude protein-Hunterdon Hog Mash, Agway, Flemington, NJ) was fed twice daily at 15 g/kg body weight. Water was provided *ad libitum*.

Pituitary exposure was as previously described (18). Briefly, a transpalpebral eye exenteration was performed, and a burr hole was drilled caudal and slightly dorsal to the optic canal to expose the junction of the pituitary stalk and anterior pituitary.

On the day of collection, the pig was placed in a Panepinto sling (Charles River Laboratories, Wilmington, MA). Whole animal heparinization began between 0830 hr and 1030 hr, and experimental blood collection began when portal blood flow was established and stabilized, between 1130 hr and 1230 hr. HPB was removed at a rate of 0.06–0.08 ml/min.

Jugular and portal blood were collected continually and simultaneously into polypropylene tubes placed in a refrigerated fraction collector. Samples were pooled into 5-min portions. Aprotinin (Trasylol, Boehringer Mannheim, Indianapolis, IN) was added (500 KIU/tube) to chilled portal collection tubes prior to blood collection. Blood samples were maintained on ice and centrifuged at 30-min intervals. Harvested plasma was frozen immediately and stored at –70°C. Hematocrits were determined for jugular and portal blood samples at various time points during each experiment and used to correct portal samples for cerebral spinal fluid contamination, if present. Pituitary stalk lesions were verified on postmortem examination performed immediately at the conclusion of each experiment.

Hormone assays. Assays used have been previously described (4). Briefly, porcine GH (pGH) was measured in jugular plasma by a homologous assay system developed using reagents supplied by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, Harbor-University of California-Los Angeles Medical Center, Torrance, CA; (pGH for iodination and standards, USDA I-1; antipGH, AFP10318545). Intra- and interassay variations were 4.5% and 2.6%, respectively. The minimum level of detection of the GH assay was 0.45 ng/ml.

Porcine GRF (1–44)NH_2 was measured in unextracted portal plasma by a double antibody radioimmunoassay. Intra- and interassay coefficients of variation were 2.0% and 9.6%, respectively. The threshold of sensitivity for the GRF assay was 50 pg/ml.

SRIF was measured in unextracted portal plasma by radioimmunoassay. Intra- and interassay coefficients of variation were 2.7% and 11.2%, respectively. The threshold of sensitivity for the SRIF assay was 50 pg/ml.

Statistical analysis. The samples used in the analysis of the patterns of hormone secretion were partitioned into an average 180-min baseline period (BL, prior to administration of L-163 255) and an average 90-min GH response period (RSP) (Table I). RSP did not include the samples following GRF administration. RSP was further partitioned into a dominant response period (DOM) and a tail period (TL). The partition between the DOM and the TL was based on the change between two consecutive samples, as the GH concentration declined from the peak value. When the change was less than three times the GH coefficient of variation (CV), the second of the two samples was considered the beginning of the TL. Furthermore, the patterns of GRF and SRIF release were examined during the period of L-163,255-induced GH increase (initial 20–30 min of DOM).

Statistical methods used in analysis have been described previously (4). Briefly, cross-correlation coefficient analysis was first applied to evaluate if there was any general association between the concentration profiles (with n samples) of any two of the three hormones. Cross-correlation between the concentration of GH and the concentration of each of the other two hormones was calculated at zero lag ($k = 0$, comparisons of simultaneous measurements) and lags of 1 and 2 samples ($k = 1$ or 2, GH measurements were compared with measurements in GRF or SRIF that led by one or two samples). Cross-correlation coefficients within $\pm 2/\sqrt{n-k}$ were considered nonsignificant.

The GH, GRF, and SRIF pulses, and SRIF troughs presented in the profiles during the RSP period were determined by ULTRA (19) software with the threshold of three CVs.

In addition to the identification of pulse maxima, ULTRA also gives the durations of pulses/troughs including the ascending and descending periods. GH pulse maxima were then compared to periods of ascent in GRF (including pulse maxima) and to periods of ascent (including pulse maxima) and descent (including trough minima) in SRIF. The number of observed coincident events in two hormones (e.g., GH pulse maxima matched with ascent in GRF) were then tested to determine if the event was a random occurrence. Given m events in hormonal series A, n events in series B, and z samples in each series, the number of coincident events x_d has a hypergeometric distribution with probability distribution function denoted by $\Pr(x_d)$. To assess the probability that at least the observed number of coincident events x could be attributed to chance alone, the formula

$$\Pr(X_d > x_d) = \left(1 - \sum_{i=0}^{d} \Pr(x_d)\right)$$

was used.

To assess the effects of L-163,255 on GRF and SRIF, the paired t-test was applied to test the significance of dif-

Table I. Hypergeometric Probability Distribution Analysis of GH Pulse Maxima, Ascending GRF, and Descending SRIF Concentrations Measured in HPB Collected from Conscious Pigs During the GH Response Following L-163,255 Administration

GH pulse maxima coinciding with ascending periods of GRF pulses

Pig	Samples (z)	GH maxima (m)	GRF ascent[a] (n)	Matches (x_d)	P-value[b]
J	15	1	9	1	0.000*
L	21	2	10	0	0.738
M	19	2	8	1	0.164
N	17	1	7	1	0.000*
O	17	1	7	0	0.412
P	18	1	8	1	0.000*
total	107	8	49	4	0.268

GH pulse maxima coinciding with descending periods of SRIF troughs

Pig	Samples (z)	GH maxima (m)	SRIF descent[c] (n)	Matches (x_d)	P-value
J	15	1	4	1	0.000*
L	21	2	12	2	0.000*
M	19	2	9	2	0.000*
N	17	1	11	1	0.000*
O	17	1	6	1	0.000*
P	18	1	12	0	0.667
total	107	8	54	7	0.003*

Ascending GRF periods coinciding with descending SRIF periods

Pig	Samples (z)	GRF ascent (m)	SRIF descent (n)	Matches (x_d)	P-value
J	15	9	4	3	0.092†
L	21	10	12	5	0.575
M	19	8	9	4	0.255
N	17	7	11	5	0.160
O	17	7	6	2	0.484
P	18	8	12	2	0.999
total	107	49	54	21	0.895

[a] GRF ascent = number of samples in the ascending period of the GRF pulse maxima.
[b] P-value is calculated from $1 - \sum_{i=0}^{d} \Pr(x_d)$, where $\Pr(x_d)$ is the probability distribution function of hypergeometric distribution.
[c] SRIF descent = number of samples in the descending period of the SRIF troughs.
* Denotes significance at $\alpha = 0.05$.
† Denotes significance at $\alpha = 0.1$.

ferences in the following parameters between any two of the four periods: BL, RSP, DOM, TL:

(a) GRF or SRIF AUC;

(b) GRF and SRIF pulse amplitude, pulse duration and pulse frequency; and

(c) SRIF trough minima, duration and frequency.

Except for pulse frequencies of the three hormones and SRIF trough frequency, the evaluations of the other parameters were done based on log scales to better meet the normality assumption required by the paired *t*-test.

The following definitions were used for statistical interpretation. A pulse was composed of an ascending period leading to a pulse maximum and a descending period after that maximum. A trough minimum was the single lowest point in a trough. A trough was the descending period leading to the trough minimum, the trough minimum, and the ascending period leading away from that minimum.

Experiment 2: Effect of L-163,255 on Hypothalamic and Anterior Pituitary Fos Activity. *Experimental protocol.* Nine, specific pathogen-free, Yorkshire male castrated pigs, weighing ≈ 20 kg were divided into four treatment groups: intravenous (IV) administration of isotonic saline ($n = 2$), intraperitoneal (IP) administration of hypertonic saline (1.5 *M* saline at 18 ml/kg bodyweight, $n = 1$), 1x L-163,255 (single IV dose administration of 1 mg/kg in 10 mls saline, $n = 3$), or 4x L-163,255 (single IV daily dose administration of 1 mg/kg in 10 mls saline for 4 days, $n = 3$). Forty-five min after treatment, all pigs were anesthetized and brains immediately perfused.

Brain harvesting and immunocytochemistry. Perfusions were conducted using a method previously published for sheep (20). Briefly, pigs were anesthetized with telazol (1 mg/lb, tiletamine HCL and zolazepam, Ft. Dodge Laboratories, Ft. Dodge, IA) at 45 min post-treatment and maintained with 4% isoflurane (Anaquest, Inc., Liberty Corner, NJ) delivered by mask. Both carotid arteries were cannulated and perfused with 1 liter $NaNO_3$ to prevent vasoconstriction starting at 60 min. The head was severed immediately after the beginning of perfusion, and the vertebral arteries were occluded by plugging the spinal cord to ensure full brain perfusion. Following the initial infusion, 1 liter of 25-g acrolein (Sigma) in 4% paraformaldehyde was perfused as a preservative. The brain was removed in one piece, and the hypothalamus and pituitary were isolated. Tissues were dehydrated in 30% sucrose solution for ≈ 3 days.

Immunocytochemical staining was accomplished using methods previously reported in detail (21). Briefly, cryoprotectant reagent was rinsed from tissues, and sections were treated with sodium borohydride (1% in potassium phosphate buffered saline) to remove any traces of the aldehydes from the sections. After rinsing, the tissue was placed into primary antisera as free- floating sections for 48 hr at 4°C. The primary antibodies were anti: human CRH and GRF (generously donated by Dr. Whylie Vale of the Salk Institute), SHRH 1–14 (INCSSTAR, Stillwater, MN; Cat # 20089), and *fos* (Oncogene Science, Cambridge, MA; Cat # PC38) used at final dilutions of 1:100,000; 50,000; 5000; and 300,000, respectively. Staining was accomplished using the avidin-biotin complex method (modified Elite Kits, Vector Labs, Burlingame, CA) with nickel-diaminobenzidine as chromogen. Concentrations for the reactants and the incubation times were as follows: primary antisera, 48 hr at 4°C; biotinylated goat anti-rabbit serum (1:600, Vector Labs, Burlingame, CA) 1 hr at room temperature; and avidin-biotinylated peroxidase-antiperoxidase complex (1:200) 1 hr at room temperature. Tissue was rinsed between the steps with KPBS containing 0.04% Triton X-100. When the reaction achieved the desired intensity of staining, the sections were mounted on slides and air dried overnight, counterstained with methyl green (0.2%), cleared in zylenes, and mounted with Permount (Fisher, Rochester, NY). Single immunolabelled tissues were counterstained with methyl green (0.2%). Immunoreactivity was detected as a blue-black reaction product. Sections of adult rat hypothalamus served as a positive control; substitution of normal rabbit serum for primary antibody failed to produce immunostaining (data not shown).

Results

Effects of L-163,255 on Pulse Frequency, Duration, and Amplitude of GRF and SRIF. HPB concentrations of GRF and SRIF during baseline and L-163,255-induced GH response are shown in Figure 2. The maximal point of the prominent GH peak resulting from L-163,255 administration ranged from 26 to 352 ng/ml plasma and was nonrandomly associated with descending periods of SRIF troughs (Table I). Pulse frequencies during BL were 2.9 ± 0.7 pulse/ hr (mean ± standard deviation) and 2.9 ± 0.6 pulse/ hr, for GRF and SRIF, respectively. Duration of GRF and SRIF pulses during BL were 3.7 ± 0.6 min and 3.7 ± 1.2 min, respectively. Pulse amplitudes during BL were 551 ± 263 pg/ml and 1575 ± 405 pg/ml, for GRF and SRIF, respectively. L-163,255 had no significant effects on the frequency, duration, or amplitude of pulses of GRF (2.8 ± 0.4 pulse/ hr, 3.7 ± 0.6 min, and 484 ± 98 pg/ml, respectively) or SRIF (2.3 ± 0.2 pulse/ hr, 4.2 ± 0.5 min, and 1590 ± 486, respectively), as measured during RSP. SRIF pulse amplitude tended to be greater ($P = 0.07$) in DOM (1699 ± 611 pg/ml) than in TL (1437 ± 398), but was not significantly different from BL. Furthermore, frequency and amplitude of GRF and SRIF pulses were not different between BL and the 20–30-min period of GH increase following administration of L-163,255 (initial DOM period). During this period of GH increase, pulse frequencies were 2.9 ± 0.6 pulse/ hr and 2.5 ± 0.4 pulse/ hr, for GRF and SRIF, respectively. Pulse amplitudes during the period of GH increase were 480 ± 245 pg/ml and 1643 ± 585 pg/ml, for GRF and SRIF, respectively.

Effects of L-163,255 on SRIF troughs. Two trough minima were present on the boundary of BL and DOM (Pig J, Sample 38, and Pig O, Sample 37; Fig. 2). These values

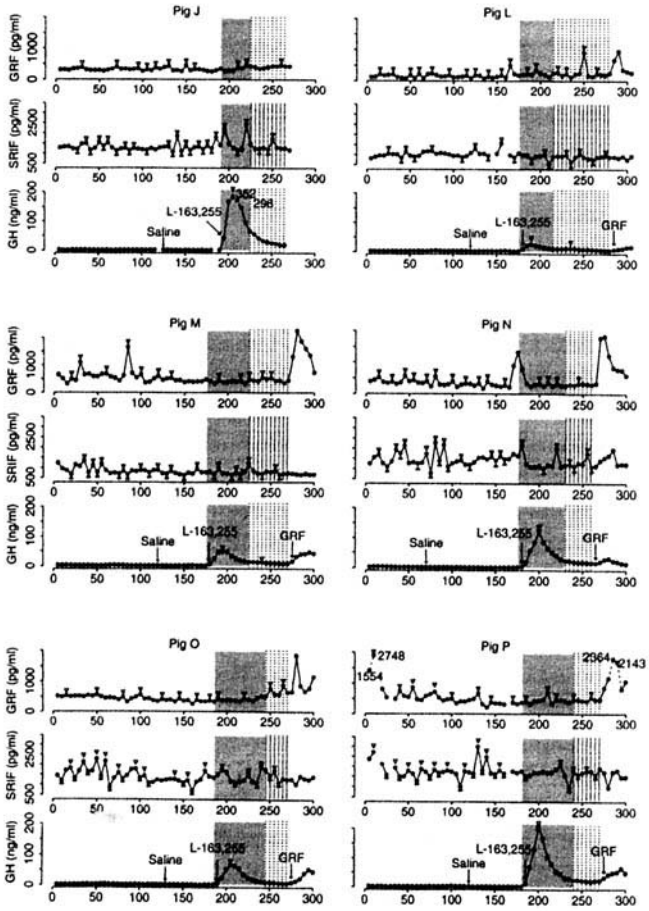

Figure 2. GRF and SRIF concentrations in HPB and GH concentrations in jugular blood of six conscious, castrated pigs. Each sample represents a 5-min continuous collection. The sampling period was ≈ 300 min for each pig. The baseline period, BL ≈ 0–180 min, was discussed previously (1). The GH response period discussed in this paper was partitioned into a dominant response period (DOM, ■ and a tail (TL, □). Significant pulses and SRIF troughs, detected using ULTRA, are indicated by triangles (δ,∇, respectively). Missing samples are shown as breaks in the splined data points. Values above the *Y*-axis scale are connected by dotted lines.

were eliminated from analysis because it was unclear in which period to include them.

Somatostatin trough amplitudes were significantly lower ($P = 0.02$) during DOM (920 ± 261) than during BL (1150 ± 284 pg/ml); however, trough amplitude during the the 20–30-min period of GH increase following administration of L-163,255 (1176 ± 390 pg/ml) was not different than BL. SRIF trough duration was significantly longer during DOM (27.5 ± 7.1 min) than in BL (18.3 ± 4.9 min). The frequency of SRIF troughs, however, was not different among the periods, (BL = 3.1 ± 0.7 pulse/ hr and DOM = 2.0 ± 0.8 pulse/ hr).

Effects of L-163,255 on AUCs of GH, GRF, and SRIF. In the following interpretation, AUC refers to average AUC per sample interval.

GH AUC during DOM (62.3 ± 54.3 ng/ml, sample) was significantly greater ($P < 0.001$) than AUC during BL (2.2 ± 0.2 ng/ml, sample) and TL (17.3 ± 10.5 ng/ml, sample). GH AUC during TL was also greater ($P < 0.002$) than during BL.

GRF AUCs increased by an average of 17% ($P < 0.1$) during RSP (414.9 ± 87.2 pg/ml, sample) when compared to BL (357.6 ± 94.5 pg/ml, sample). The majority of this increase within RSP occurred during TL (448.8 ± 140.7 pg/ml, sample) as the AUC was 25% greater here compared to BL.

There were no significant differences in the SRIF AUCs between the sampling periods.

Cross-correlation coefficient analysis. Cross-correlation coefficients of the individual pigs showed significant positive or negative correlations at various comparisons, but there was no general trend in the data. During the post-treatment (RSP) period, for GRF vs. GH at zero sample lag, cross-correlation coefficients of the six pigs ranged in value between –0.52 and 0.17; only Pig O showed a significant negative correlation. At one sample lag, the correlation coefficients ranged between –0.72 and –0.04; Pigs J, M, and O showed significant negative correlations. At two sample lag, the correlation coefficients ranged between –0.80 and –0.11, with Pigs J and O showing significant negative correlations. For SRIF versus GH, correlation coefficients at zero, one and two sample lags ranged from –0.42–0.10, –0.43–0.13, and –0.14–0.38, respectively. No significant correlation was found.

During the Treatment-Dominated (DOM) period, for GRF versus GH, correlation coefficients at zero, one, and two sample lags ranged from –0.53–0.47, –0.66–0.39, and –0.82 to –0.09, respectively. Pig J at two sample lag was the only animal to show a significant negative correlation. For SRIF versus GH, correlation coefficients at zero, one, and two sample lags ranged from –0.56–0.17, –0.61–0.17, and –0.51–0.89, respectively. Pig N showed a significant negative correlation at one lag. Pigs J and O showed significant positive correlations at two sample lag.

Effects of GRF bolus dose on concentrations of GRF and GH. Following GRF administration, the average GRF concentration in portal plasma increased more than 2-fold (790–1910 pg/ml). The GRF peak occurred within two samples following GRF administration. GH concentration increased (15.9–26.3 ng/ml) during the same period, but peak GH concentrations (average 42.1 ng/ml) occurred 3–4 samples after GRF administration and 1–3 samples after the GRF peak.

Brain and pituitary immunocytochemistry. Fos activity was minimal in the arcuate (ARC), periventricular (PeVN), paraventricular (PVN), and supraoptic (SON) nuclei from L-162,255 and control pigs, but was markedly increased in the SON (only) of the hypertonic saline control pig (Fig. 3). There were no qualitative differences observed in the occurrence of GRF, SRIF, or CRH immunopositive neurons in the hypothalami between treatment groups and controls (data not shown). The anterior pituitaries of the 1x and 4x groups demonstrated marked *fos* immunoreactivity compared to the isotonic saline controls (Fig. 4).

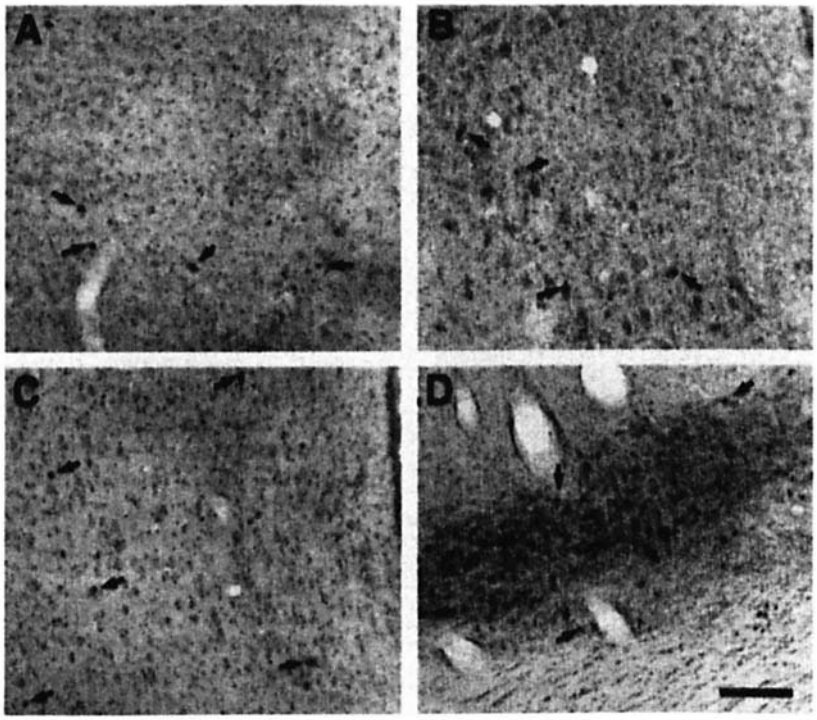

Figure 3. Arcuate nuclei (ARC) of treated pigs immunocytochemically stained (black) for c-*fos* protein (*fos*; arrows): (A) 1x dose (1 mg/kg iv L-163,255); (B) 4x dose (1 mg/kg iv L-163,255 for 4 consecutive days); and (C) isotonic saline control. A section through the supraoptic nucleus (SON) of a pig given hypertonic saline is shown in D. Note that in D a moderate amount of nuclear *fos* staining is present in neurons of the SON, but very little activity is seen in ARC (A, B, C). The counterstain is methyl green. Bar = 132 (A, B, C); 209 (D) micrometers.

Discussion

L-163,255 significantly increased secretion of GH in all animals in the current study. This response was similar to what has been reported previously (22). The increase in jugular plasma concentrations of GH was prolonged with levels remaining elevated for at least 90 min. No significant increase in GRF was seen in conjunction with the increasing GH secretion following L-163,255 administration. However, it is possible that there was a small increase in GRF caused by L-163,255 that was masked by our experimental conditions (i.e., 5-min pooled blood samples). Exogenous GRF administered at the end of each experiment was reflected in our assays within 10 min of intravenous administration and resulted in an increase in GH. The relationship between the GRF peak seen from exogenous administration of GRF and the resulting GH peak was variable with a 5–15-min lag period. This variability in response time and in peak magnitude is indicative of the complex regulation of GH secretion by the somatotroph.

L-163,255 administration did appear to influence SRIF secretion; the maximal point of the prominent GH peak resulting from L-163,255 administration was nonrandomly associated with descending periods of SRIF troughs. This same relationship was observed in our baseline evaluation (4) and may indicate that L-163,255 is mimicking the pathway of endogenous GH pulse generation. The significantly deeper and longer SRIF troughs after L-163,255 administration may indicate a direct/indirect effect of L-163,255 on SRIF secretion. However, the delay in the decrease in SRIF during the increasing period of the GH pulse is more suggestive of an indirect response of SRIF to L-163,255 or to pituitary feedback of GH. These differences, however, did not change the AUC or any of the other SRIF pulse parameters. Clark *et al.* (23) reported that GHRP in rats suppressed SRIF secretion; however, this was in contrast to reports by Yagi *et al.* (24) and Bowers *et al.* (25). Any suppression of SRIF would be additive to the functional antagonism of the secretagogues on SRIF (13). The GHS MK0677, a close analog of L-163,255, does not bind to SRIF receptors, and SRIF does not displace MK0677 from its receptor (13). Bennet *et al.* (26) did not find GHS receptor mRNA in the periventricular nucleus of rats where SRIF-containing nuclei are found; however, Argente *et al.* (27) found that GHRP-6 decreased SRIF mRNA in the periventricular nucleus of female dwarf rats treated continuously with GHRP-6 for 14 days. The later reference is consistent with the significant change in the pattern of SRIF release reported in the current study.

Evidence of a hypothalamic site of action for GHRP-6 and related secretagogues has been the topic of much recent research employing many techniques and species. Dickson *et al.* using brain immunocytochemistry techniques on rats showed that both c-*fos* and electrical activity of the arcuate nucleus were stimulated by GHRP-6 or L-692,585 administration *in vivo* (14–16). Pituitaries were not examined in Dickson's reports. Our data show that pig hypothalamus has only background levels of *fos* immunostaining in neurons in the ARC, PeVN, and PVN, respectively, before and after

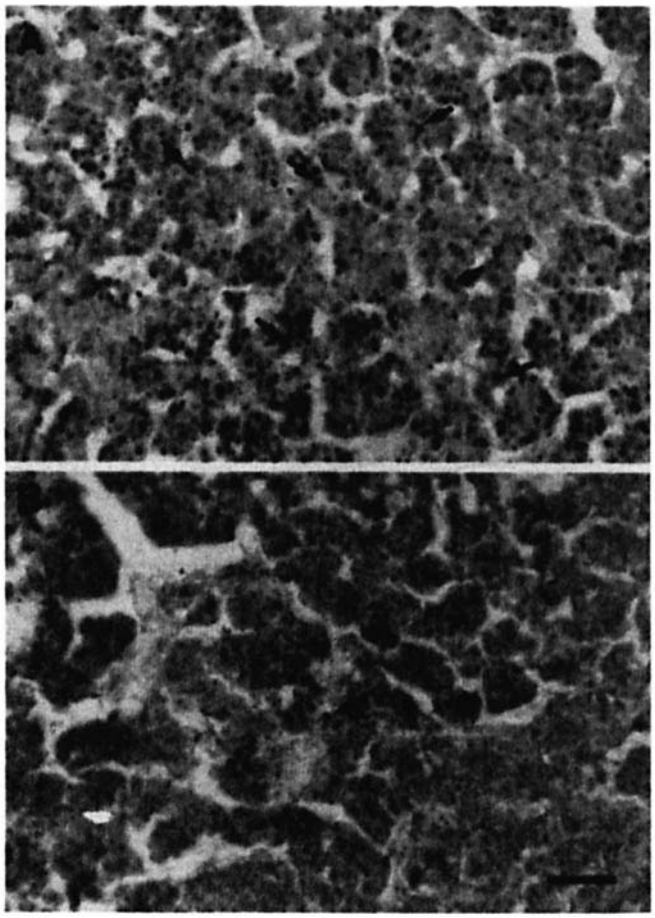

Figure 4. Anterior pituitary of treated pigs immunocytochemically stained (black) for c-*fos* protein: (A) 1x dose (1 mg/kg iv L-163,255); (B) saline control. The counterstain is methyl green. 200x magnification. Bar = 65 micrometers.

L-163,255 treatment. Furthermore, although L-163,255 does not change *fos* expression in the hypothalamus of pigs at 60 min post-treatment, it does increase expression in the anterior pituitary by 60 min. In comparison, intraperitoneal hypertonic saline treatment, which increases *fos* activation in the SON of adult rats (28), caused a similar *fos* response in the present study and served as an effective positive control for both the activation of neurons in the pig hypothalamus and our immunocytochemical technique.

In conclusion, the action of L-163,255 in the hypothalamus appears to be associated with a direct or indirect suppression of SRIF secretion; however, the enhanced *fos* expression following L-163,255 administration suggests that the anterior pituitary is the key site of action of L-163,255.

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