Central and Peripheral Administration of Growth Hormone Secretagogue L-692-585, Somatostatin, Neuropeptide Y and Galanin in Pig: Dose-dependent Effects on Growth Hormone Secretion

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Summary and Implications

A dose-dependent response of GH plasma concentration to icv injection of 3, 10 and 30 µg/kg BW of the GH-secretagogue, L-692,585 (585) was established in Yorkshire barrows (40-45 kg BW). Icv administration of CRH at 150 µg/kg BW did not raise basal GH concentration compared with saline treatment, and icv injection of 585 at three dosages did not significantly alter cortisol plasma concentration. Icv injection of SRIF dose-dependently decreased GH plasma concentration, whereas icv treatment of SRIF + 585 dose-dependently increased GH concentration but at peak levels less than seen with 585 alone. Icv injection of pGAL modestly increased GH peak concentration, whereas when given in combination with 585 acutely raised GH plasma concentration. GH plasma concentration remained at basal levels after icv injection of pNPY, whereas when given in combination with 585 modestly elevated GH concentration at peak levels less than seen with 585 alone. The administration of 585 icv elicited a slower and less robust GH response than that following iv 585; desensitization of the GH response occurred following repeated icv 585 but not repeated iv 585. These findings demonstrate that 585, administered iv or icv, is a potent GH-secretagogue and confirm its central role in the pig.

Introduction

Growth hormone (GH) is released in a circadian, pulsatile fashion controlled by two hypothalamic hormones, GH-releasing hormone (GHRH) induces whereas somatostatin (SRIF) inhibits GH release. The development of the novel nonpeptidyl growth hormone secretagogue, L-692,429 and analogs, with potent GH-releasing activity both *in vivo* and *in vitro* and the natural ligand, ghrelin, provides a means to understand biological mechanisms of GH release.

L-692,429, a substituted benzolactam, acts in a manner identical to the peptidyl GH secretagogue, GHRP-6, but distinct from that of the endogenous GHRH. L-692,585 (585) is approximately 5- to 30-fold more potent based on *in vitro* and *in vivo* studies, and with no detectable change in receptor specificity. Our preliminary experiment showed that 585 has a direct but limited action at the level of the pituitary gland, and that an intact hypophyseal stalk is required for a maximal GH and cortisol response.

We selected pigs as the species for this study because their responsiveness and specificity to this class of secretagogues closely resembles that of the human. Intracerebroventricular (icv) stainless steel cannulas were placed surgically to elucidate the nonpeptidyl GH secretagogues' effect on central regulation of GH secretion in immature male castrates. The pharmacological actions on central mechanisms by which 585 controls GH release was determined by a) dose-dependent effects of icv administered 585, b) by a switchback design of icv and iv administered drug and c) by icv administration of somatostatin, porcine neuropeptide Y (pNPY) and porcine galanin (pGAL) alone or in combination with 585.

Materials and Methods

Animals

Fourteen Yorkshire castrated males, weighing 40-45 kg, were used in this study. Animals were housed in individual stainless steel metabolism crates for the duration of the study, and a corn and soybean meal-based diet was available *ad libitum*.

Surgical Procedures

Pigs were anesthetized with an ear vein injection of sodium thiopental (0.14 mg/kg BW, Pentothal, Abbott Laboratories, North Chicago, IL, USA) for endotracheal intubation and maintained on a closed-circuit system of halothane (2-5 %; Ayerst Laboratories, Rouses Point, NY, USA) and oxygen (300 cc/min) for the duration of surgery. An external jugular vein was surgically exposed, and a catheter (id. 1.27 mm; od 2.29 mm; Tygon microbore tubing, Fisher Scientific, Pittsburgh, PA, USA) directed toward the cranial vena cava was inserted and sutured in place. The catheter was directed sc to the dorsal neck region and exteriorized between the scapulae for repeated blood sample collections. After 3 days, the animal's head was oriented in a large animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA) so that the plane formed by the frontal and parietal bones was parallel to the instrument tabletop. An 18-gauge stainless steel cannula was placed in the left lateral-cerebral ventricle according to predetermined coordinates (anterior-posterior, +14.0 mm; lateral, 6.0 mm to the bregma;

horizontal, 18.0 mm to the dura mater). Two stainless steel screws and cranioplastic cement (Plastics One, Inc., Roanoke, VA, USA) secured the cannula. A backflow of cerebrospinal fluid indicated that the cannula was icv.

Test Compound and Method of Administration

L-692,585 was dissolved in saline for iv and icv administration. Solutions were prepared at the beginning of each trial, so that solubilized 585 was not stored longer than 1 day. L-692,585 or vehicle was administered icv in 150 µl solution and followed by 120 µl vehicle to fill cannula dead space. For iv injection 300 µl of 585 or saline solution was followed by 2 ml vehicle (saline). To evaluate the integrity of the pituitary gland and as a positive control, the animals were challenged with corticotropin-releasing hormone (CRH; 150 µg) and assayed for GH and cortisol. pCRH (American Peptide Co., Inc. Sunnyvale, CA, USA) was dissolved in sterile saline (0.9% NaCl) and stored frozen (-80 C) in 150-µl aliquots. Peptide hormone solutions were prepared at the beginning of each trial, so that solubilized CRH was not stored longer than 1 week. CRH or vehicle was administered icv in 150 µl solution. This was followed by 120 µl saline to account for dead space in the catheter.

Somatostatin 1-14 (SRIF), porcine galanin (pGAL) and porcine neuropeptide Y (pNPY) (American Peptide Co., Inc.) were dissolved in sterile saline (0.9% NaCl) and stored frozen (–80° C). L-692,585 was dissolved in saline for icv administration. Peptide hormone and 585 solutions were prepared at the beginning of each trial so that solubilized peptide hormone and 585 were not stored longer than 1 day. Peptide hormone solution (SRIF, pGAL, pNPY) with or without 585 or vehicle was administered icv in 150 µl solution and followed by 120 µl vehicle to fill cannula dead space.

Blood Samples

Blood (5 ml) was collected via an indwelling jugular cannula into heparinized tubes at each sampling. Samples were centrifuged, 1,500 × *g* within 2 h, and plasma was harvested and stored at –20° C until hormone assay.

Hormone Assays

A homologous porcine GH RIA was developed utilizing reagents supplied by Dr. A F Parlow, Pituitary Hormone and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA (porcine GH [pGH] for iodination and standards, USDA-I-1; anti-pGH, AFP10318545). Sensitivity of the assay was 0.9 ng/ml. Intra- and inter-assay variations were 2.9 and 15.6%, respectively.

Plasma cortisol concentrations were determined by a double-antibody RIA similar to that described previously. Plasma cortisol concentrations were quantified in duplicate 50-µl aliquots through the use of a solid-phase ^{125}I-RIA nonextraction technique. To validate the assay, known amounts of cortisol were added to charcoal-stripped cortisol-free plasma as follows: 0.25, 0.5, 1.0, 2.5, and 5.0 ng/tube in quadruplicate. The plasma blank values were assayed and values were subtracted from those in tubes with known amounts of cortisol. Sensitivity of the assay was 0.92 ng/ml, whereas intra- and inter-assay coefficients of variation were 11.2% and 2.5%, respectively.

Experimental Protocol

Dose-response effect of L-692,585 icv injection on GH and cortisol secretion.

Four pigs were used to determine the central dose dependent effects of 585 and CRH on GH and cortisol secretion. Saline or 585 at three dose levels (3, 10, 30 µg/kg BW) were administered by icv injections. Blood was collected at - 40, -20 (pretreatment), 0, 10, 20, 30, 40, 60, 80, 100, 120, and 140 min (post-treatment).

Switchback study of L-692,585 iv and icv injection on GH secretion.

L-692,585 at 30 µg/kg BW was administered to animals according to a switchback design outlined in Figure 1. Blood was collected as described previously.

SRIF with and without L-692,585 icv injection on GH secretion.

Doses of SRIF (2, 8 µg/kg BW) with and without 585 (30 µg/kg BW), 585 alone or saline was administered by icv injections.

pNPY and pGAL with and without L-692,585 icv injection on GH secretion.

pNPY or pGAL (4 µg/kg BW) with and without 585 (30 µg/kg BW) was administered by icv injections.

Statistical Analysis

Mean pretreatment GH and cortisol concentration as well as post treatment peak GH and cortisol concentration were obtained for each animal. Peak hormone level and peak occurring time were used to characterize hormone response profile of individual pigs. Data are expressed as the mean ± SE. All data were subjected to analysis of variance (ANOVA) using the general linear model of to establish whether significant differences ($p<0.05$) were present, in which case *P* values for pair-wise differences between groups were calculated by Fisher's least square difference test or Satterthwaite approximate F-test of models appropriate for the experimental designs.

Results

Icv dose response effects of L-692,585 and CRH on GH and cortisol secretion.

After icv administration of 585, plasma GH concentration increased in a dose-related manner, with a return to baseline by 60 min (Figure 1a and 1b). GH peak response occurred at 10 min after 3 and 10 µg/kg BW 585 icv injection and at 20 min after 30 µg/kg BW 585 icv injection. GH concentration after icv administration of CRH was similar to the saline

control (p>0.05). GH peak concentrations were 4 ± 0.9 ng/ml (± SE), 3.5 ± 0.3 ng/ml, 24 ± 9 ng/ml, 35 ± 10 ng/ml, and 50 ± 9 ng/ml for the saline control, CRH (150 μg) and 585 (3, 10, and 30 μg/kg BW doses), respectively.

There was no significant difference between dose-dependent groups in cortisol levels (Figure 1c and 1d). Administration of 585 resulted in cortisol levels of similar magnitude in all groups compared with saline injected controls throughout 140 min. In contrast, cortisol levels abruptly increased after CRH icv administration (p<0.05) within 10 min and remained high compared with saline and 585 treated groups. A modest increase to peak cortisol levels occurred at 10 min after 585 administration with levels returning toward baseline by 40 min. Cortisol peak concentrations were 41 ± 8, 75 ± 9, 52 ± 23, 52 ± 15, and 63 ± 18 ng/ml for the saline control, CRH (150 μg), and 585 (3, 10, and 30 μg/kg BW doses), respectively.

Interaction of L-692,585 iv and icv injection on GH secretion.

In the switchback study, pigs were injected iv or icv with saline or 30 μg/kg BW 585 to determine peripheral and central action of the GH secretagogue. Average of peak GH response to the iv and icv 585 injection was 80 ± 10 ng/ml at 10 min and 53 ± 10 ng/ml at 20 min, respectively (Figure 2a). GH secretion was attenuated with the increase in numbers of icv injection in the switchback study (p<0.05). Peak GH responses from the 1st through 4th icv 585 injection were 67 ± 18 ng/ml at 20 min, 39 ± 22 ng/ml at 10 min, 20 ± 10 ng/ml at 30 min, and 27 ± 9 ng/ml at 20 min, respectively (Figure 2b). The data also showed that the 585 icv injection significantly attenuated the response to the 585 iv injection (p<0.001). The average peak GH response to iv 585 injection before and after icv 585 injection was 97 ± 14 and 62 ± 11 ng/ml, respectively (Figure 2c). In contrast, 585 iv injection did not significantly affect the GH response to icv 585 injection (p>0.05). The average peak GH response to icv 585 injection before and after iv 585 injection was 58 ± 17 and 49 ± 10 ng/ml, respectively (Figure 2d).

SRIF with and without L-692,585 icv injection on GH secretion.

Peak GH responses following icv administration of 2 or 8 μg/kg SRIF were 3 ± 0.6 and 2 ± 0.3 ng/ml, respectively, which were decreased significantly compared with saline alone (4 ± 0.9) at 10 min (p<0.05). GH levels after 2 or 8 μg/kg SRIF treatment continued to drop until 30 min. Peak GH response following 2 or 8 μg/kg SRIF + 585 were 31 ± 5 and 31 ± 7 ng/ml at 20 min, respectively. GH response after SRIF + 585 was significantly lower than 50 ± 9 ng/ml 585 alone at 20 min (p<0.05, Figure 3a). There were no differences between two different doses (2 or 8 μg/kg) of SRIF alone or together with 585 in GH response.

pNPY and pGAL with and without L-692,585 icv injection on GH secretion.

GH response following icv administration of pNPY was 3 ± 1 ng/ml, which was similar to saline injection. pNPY + 585 increased GH release (25 ± 3) at 20 min significantly higher (p<0.05) than saline control (Figure 3b). Peak GH response following icv administration of pGAL was 11 ± 3 ng/ml at 20 min and significantly higher than saline control (p<0.05). Peak GH response following icv administration of pGAL + 585 was 42 ± 4 ng/ml at 10 min, which was significantly higher than pGAL alone and saline control (p<0.05, Figure 3b). Peak GH response of 585 alone (50 ± 9) was significantly higher than pGAL + 585 (36 ± 3) at 20 min, but the GH response (AUC, ng min^{-1} ml^{-1}) was similar (p>0.05) following icv administration of pGAL + 585 (876 ± 106) and 585 alone (1284 ± 321) (Figure 3b). pGAL alone increased GH release significantly (11 ± 3, p<0.05) but pNPY neither increased or decreased GH response compared with saline control (Figure 3b). pNPY + 585 increased GH release compared with saline control; however, the response was less than pGAL + 585 or 585 alone (p<0.05, Figure 3b).

Discussion

We describe for the first time *in vivo* potency of centrally administered L-692,585, a more potent nonpeptidyl GH secretagogue analogue of L-692,429, in eliciting GH release in swine. Transection of the hypophyseal stalk (HST) in the pig resulted in a blunted but detectable GH response to 585 iv injection. Remarkably, the 2-fold and 16-fold increase in GH secretion to 585 and GHRH + 585 respectively in HST Yorkshire barrows was similar in magnitude to that seen with these treatments *in vitro* with dispersed pituitary cells. The minimal GH response in face of the absence of hypothalamic somatostatin (SRIF) tone in the HST animals also indicated that inhibition of SRIF tone, in itself, was not adequate to induce an intact response. A hypothalamic stimulus also seems to be required to elicit a peak GH response to the GH secretagogue.

The interactions of iv and icv 585 administration and the effects of continuous injection in this switchback design revealed that the GH response to icv 585 injection was delayed and was lower than that following iv-administered 585. Attenuated GH responses to the successive icv 585 injections were clearly illustrated (Figure 2), although the GH response to the fourth icv 585 injection was slightly higher than that to the 3rd icv 585 treatment, the difference was not significant. The GH responses to increasing numbers of iv 585 injections did not show any sign of attenuation. These findings may suggest different mechanisms of GH-secretagogue action regulating GH secretion by a direct pituitary stimulatory effect when 585 is injected iv, and a slower less robust response to icv 585 via GHRH and SRIF neurons within the porcine hypothalamus.

This switchback design also revealed that there was a significant difference in the GH response to iv 585 injection before and after icv administered 585. In contrast, 585 iv

injection did not influence the GH response to icv injection of the secretagogue. There might be a feedback action to GHRP that directly stimulated the brain. Specific binding sites in rat and porcine anterior pituitary membranes and in rat hypothalamic membranes with MK0677, another class of GH secretagogue.

NPY is widely distributed in the mammalian brain and is involved in numerous functions including control of feeding, growth and reproduction. Immunohistochemical localization of NPY was determined throughout prepubertal development in the Meishan pig, a Chinese breed known for its superior reproductive characteristics. NPY-like immunoreactivity (NPY-IR) in cell bodies and fibers is in many areas of the brain at gestational day (g)30, including the basal telencephalon, hypothalamus, mesencephalon, pons, and medulla. Throughout prenatal development, cell bodies containing NPY-IR generally increase in number and distribution in the porcine brain. The intensity of NPY-IR in fibers also increases throughout gestation. During postnatal development the number of cell bodies displaying NPY-IR decreases. The arcuate nucleus (ARC) of the hypothalamus shows a dramatic reduction in number of immunoreactive cell bodies between postnatal day (pn) 1 (day of birth) and pn 20, just before weaning. Some additional increases in immunoreactivity occur postnatally, especially in the periventricular hypothalamus and the hippocampus.

GAL is a neuropeptide found in the mammalian brain and is involved in numerous functions including the control of feeding, growth and reproduction. Immunohistochemical localization of GAL was examined throughout prepubertal and early postnatal development in the Chinese Meishan pig. GAL-like immunoreactivity (GAL-IR) in cell bodies and fibers was evident in the brain at g30, primarily in the hypothalamus. Throughout prenatal development, cell bodies and fibers containing GAL-IR generally increased in number and distribution in the brain. During postnatal development, the number of cell bodies displaying GAL-IR decreased, particularly in hypothalamic areas. Distribution of GAL-IR in fibers became more widespread throughout gestational development, showing a pattern by pn 1 that continued during later postnatal ages (pn 10, 20 and 50). The GAL-IR results support the hypothesis that GAL participates in the control of feeding, growth and reproduction in the pig.

Icv administration of somatostatin (SRIF) decreased ($p<0.05$) GH secretion compared with saline treated controls, and decreased ($p<0.05$) peak GH response when given in combination with 585 as compared with 585 treatment alone. pGAL modestly increased ($p<0.05$) GH levels compared with saline controls, but when given icv in combination with 585 peak GH response was lower ($p<0.05$) compared with 585 alone. pNPY administered icv was without effect on GH levels compared with saline controls and decreased ($p<0.05$) peak GH response when given in combination with 585 as compared with 585 alone. pNPY exerts a negative influence on GH secretion possibly through either stimulating SRIF neurons by inhibiting GHRH neurons. NPY-containing neurons have been reported to be located near GHRH neurons in the ARC and express GH receptor mRNA.

Figure. 1ABCD.

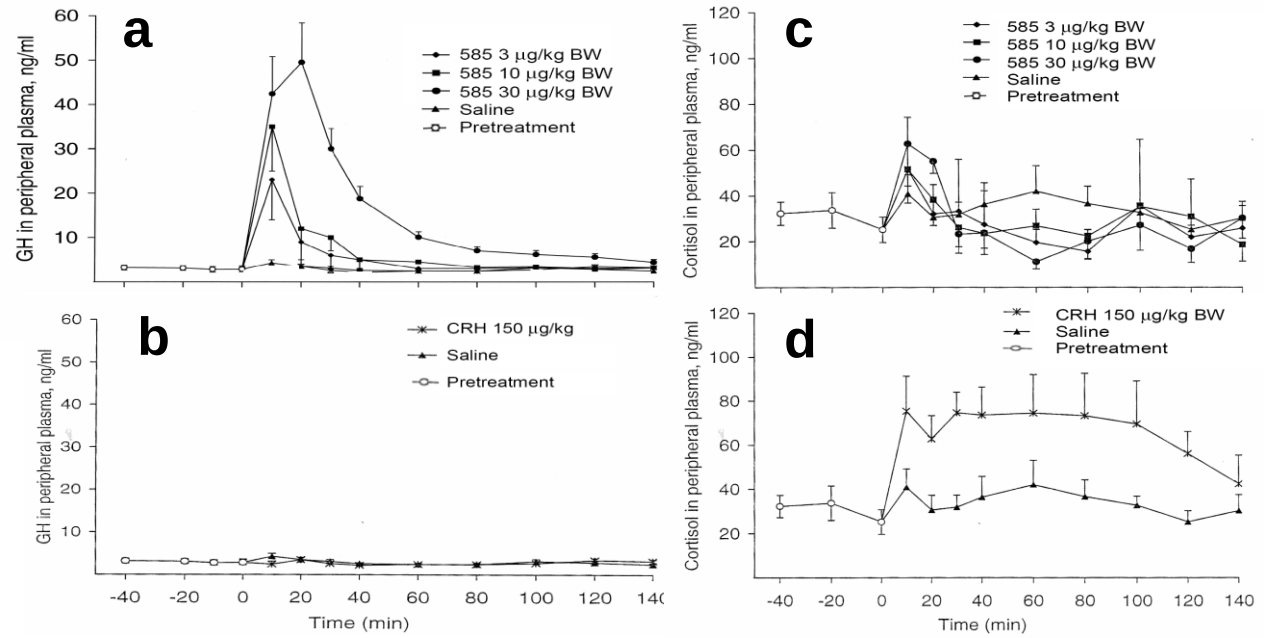

Figure. 1. L-692,585 and CRH icv dose response in male castrate pigs. **a** GH responses to 585 administered icv at 0 (saline vehicle), 3, 10, 30 µg/kg BW and **b** CRH at 150 µg/kg BW and **c** Cortisol responses to 585 administered icv at 0 (saline vehicle), 3, 10, 30 µg/kg BW and **d** saline vehicle and CRH at 150 µg/kg BW. Values for each dose level were means ± SE for each time point.

Figure. 2ABCD.

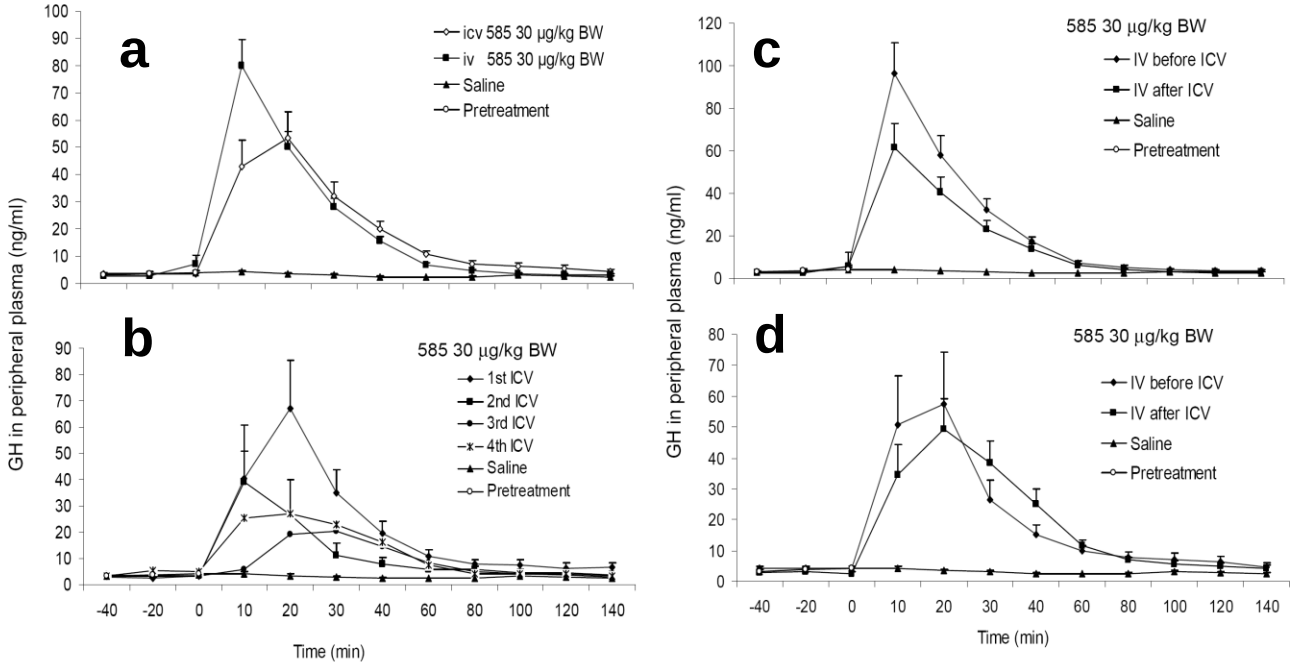

Figure. 2. L-692,585 iv and icv total response in male castrate pigs in switchback experiments. **a** GH responses to 585 administered iv or icv at 30 µg/kg BW. The baseline is saline-treated control. Values for each total response were means ± SE from 4 injections of 6 animals for each time point. **b** GH responses to 585 administered iv before icv at 30 µg/kg BW. The baseline is saline-treated control. Values for each iv or icv response were means ± SE from two injections for each time point. **c** GH orderly responses to 585 administered iv at 30 µg/kg BW. The baseline is saline-treated control. Values for each orderly responses were means ± SE from six animals for each time point. **d** GH responses to 585 administered icv before iv at 30 µg/kg BW. The baseline is saline treated control. Values for each iv or icv response were means ± SE from two injections for each time point.

Figure. 3AB.

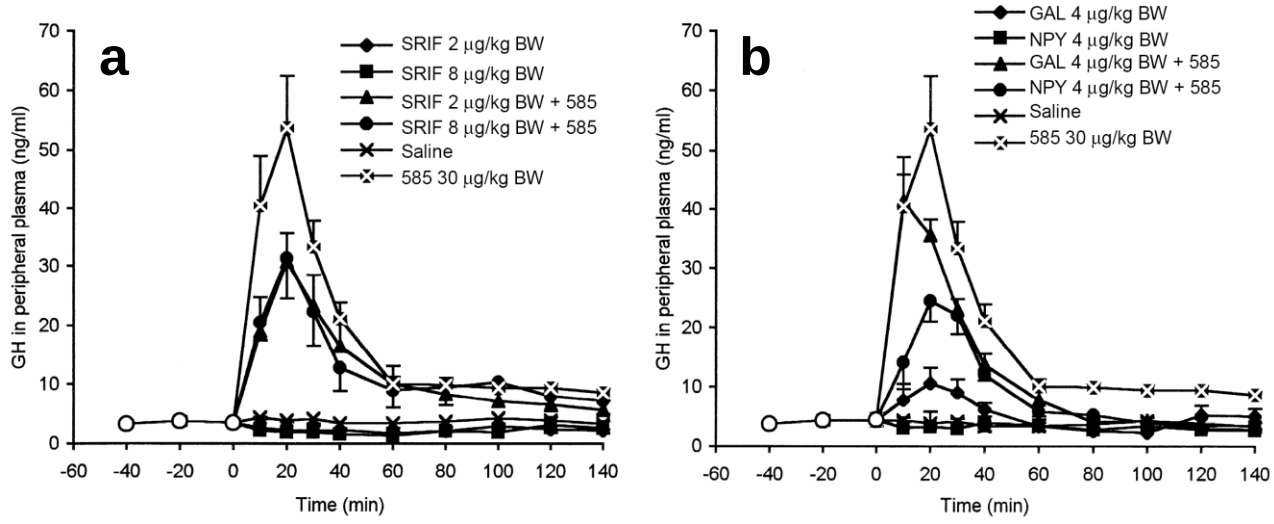

Figure. 3. a GH responses to icv administration of SRIF at 2 and 8 μg/kg BW alone and in combination with 585 at 30 μg/kg BW as compared with saline vehicle and 585 alone. **b** GH responses to icv administration of pGAL at 4 μg/kg BW, pNPY at 4 μg/kg BW and in combination with 585 at 30 μg/kg BW as compared with saline vehicle and 585 alone. Values are means ± SE from four and five animals at each time point.