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Production of Antisera to Growth Hormone-Releasing Factor: Usefulness in Radioimmunoassay and Passive Immunization

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Abstract

We have produced two antisera (R-1 & R-2) to human growth hormone-releasing factor (GRF) [1-44] NH_2. Both antisera can be used for human GRF radioimmunoassay (RIA) at a final dilution of 1 : 50000. The antiserum R-2 was specific for the C-terminal amidated sequence of human GRF-44 and selectively recognized GRF [1-44] NH_2 but not GRF [1-44] OH or GRF [1-40] OH. The antiserum R-1 also significantly bound ^{125}I-rat GRF [1-43] OH at a final dilution of 1 : 5000 and enabled us to establish RIA for rat GRF. In both RIA systems, intra- and inter- assay coefficients of variation at 50% inhibition were 8 and 12%, respectively. A median effective dose was 90–120 pg in human GRF RIA and 250–300 pg in rat GRF RIA. Utilizing the RIA, we demonstrated that the hypothalamic GRF content in rats which received monosodium glutamate during the neonatal period was less than 20% of that of controls. However, the hypothalamic GRF content was not altered in rats made hypothyroid by methimazole administration, another condition known to greatly impair GH secretion. An iv administration of the antiserum R-1 significantly suppressed GH release following the injection of antisomatostatin serum. Thus, these antisera can be a useful tool in examining the physiological and/or pathophysiological roles of GRF in human and rat.

Growth hormone-releasing factor (GRF), a 44 residue peptide with an amidated C-terminus originally isolated from a pancreatic tumor that caused acromegaly (Guillemin *et al.*, 1982), has also been characterized from the human hypothalamus (Ling *et al.*, 1984). The peptide has the characteristics of the physiological GRF in human (Guillemin *et al.*, 1982; Ling *et al.*, 1984). To date, structurally similar peptides with high intrinsic GH releasing activity have been characterized from four different species (Guillemin *et al.*, 1982; Spiess *et al.*, 1983; Böhlen *et al.*, 1983; Esch *et al.*, 1983; Brazeau *et al.*, 1984).

The production of well characterized antiserum to GRFs could be a useful tool in examining the physiological and/or pathophysiological significance of GRF in human and experimental animals. This can be accomplished by establishing radioimmunoassay to measure GRF in biological materials, passively immunizing animals with the antiserum, and immunocytochemical studies on

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GRF.

We have produced and characterized two antisera to human GRF [1-44] NH_2. The present studies deal with the development of radioimmunoassay for human and rat GRFs utilizing the antisera. In addition, the effect of neonatal monosodium glutamate treatment and hypothyroidism on hypothalamic GRF content was examined in rats. These conditions are known to greatly impair GH secretion in rodents (Hervas *et al.*, 1975; Bakke *et al.*, 1978). Thus altered GH secretion may result from changes in hypothalamic GRF content. Also included in the present studies are the effect of passive immunization of rats with anti-GRF serum on GH release following the administration of anti-somatostatin serum in rats.

Materials and Methods

Peptides

Synthetic peptides: human GRF [1-44] NH_2, peptide histidine isoleucine 1-27 (PHI-27) and porcine vasoactive intestinal polypeptide (VIP-28) were purchased from Protein Research Foundation (Osaka, Japan). Synthetic human GRF fragments and rat GRF [1-43] OH were generously supplied by Dr. Nicholas C. Ling (The Salk Institute, San Diego, CA, U.S.A.).

Induction of antibody

Synthetic human GRF [1-44] NH_2 (2.28 mg) and BSA (8 mg) was dissolved in 5 ml of 0.1 M phosphate buffer pH 7.4. To this solution, 2 ml of 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 was added dropwise over 15 min. After stirring for 4 h at room temperature, the mixture was dialyzed against 2 L of distilled water at 4°C overnight. The water was changed once during the dialysis. The dialyzed solution was then distributed into 5 glass test tubes and lyophilized. For each immunization one tube of human GRF-BSA conjugate was used. The conjugate was reconstituted with 1 ml of 0.9% NaCl to which 1 ml Freund's complete adjuvant (Difco Laboratories, Detroit, MI) was added, and emulsified. The emulsion was injected into two mixed-breed rabbits at multiple intradermal sites. Booster injections prepared in the same manner were given every 2-3 weeks and the rabbits were bled 7 days after each immunization. Antisera obtained after the 5th immunization was used for studies.

Radioimmunoassay (RIA) for human and rat GRFs

Synthetic human GRF [1-44] NH_2 and rat GRF [1-43] OH were iodinated respectively using the chloramine T method (Hunter and Greenwood, 1962) and purified on a Sephadex G-75 fine column (0.7×47 cm) with 0.5 M acetic acid as eluant. The buffer solution used for the RIA was 0.05 M Tris-HCl, pH 8.6, containing 0.01 M EDTA, 0.9% NaCl, 0.1% HSA, 0.02% Tween 20 and 0.02% sodium azide. To each disposable plastic tube (12×75 mm), 200 μl of the buffer, 100 μl of appropriately diluted antiserum and 100 μl of standard solution of synthetic GRF in a dose range from 3.9 to 2000 pg were added and the mixture was incubated at 4°C overnight. Tracer was added on the second day in a volume of 100 μl and incubated again at 4°C overnight. On the third day, goat anti-rabbit serum was added in a volume of 100 μl. After another overnight incubation, the mixture was centrifuged and the supernatant was aspirated and the pellet was counted in a γ-counter.

Gel chromatography of hypothalamic extract

Pituitary stalk-median eminence tissue (SME) was removed from normal adult male Wistar rats and human SME was obtained at autopsy from patients who died of hematological disorders within 2 to 6 h after death. Acid extract of SME was prepared as described below and was subjected to gel filtration on a Sephadex G-50 fine column (0.7×47 cm) with 1 M acetic acid as the eluant. Each fraction (0.5 ml) was lyophilized and reconstituted with 0.9% NaCl and assayed for GRF.

Animal studies

In the first study, neonatal Wistar rats received a sc injection of monosodium glutamate (MSG, 4 mg/g BW; in sterile water) or 10% NaCl as an isotonic control on days 1, 3, 5, 7 and 9 after birth. At 28 days of age, the pups were weaned, sexed and placed in group cages. They were maintained in an air conditioned animal quarter with a lighting schedule of 0800–2000 h and fed food and water ad libitum. Only female rats were used in the present study at 75 days after birth. In the second study, a group of

male Wistar rats weighing 180–200 g received methimazole (Mercazole, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) 20 mg/kg BW ip for 14 days and control rats received saline.

All rats were sacrificed by decapitation. Anterior pituitary tissue was removed, weighed and homogenized in 2 ml 0.01 M $NaHCO_3$. An aliquot of homogenates was taken for protein determination (Sano *et al.*, 1981) and the rest of the homogenate was centrifuged at 2000×g for 30 min under refrigeration. The supernatant was kept frozen at −20°C until assayed for GH. The brains of all the animals were also removed and the pituitary stalk-median eminence tissue (SME), measuring approximately 1.5 mm in diameter and 1 mm in thickness with the pituitary stalk as a reference point was dissected under a microscope using fine iris scissors. The tissue fragments were immediately frozen on dry ice and homogenized individually with 2 ml of 1 M acetic acid containing 20 mM hydrochloric acid, 0.0001% phenylmethylsulfonyl fluoride, 0.0001% pepstatin A, and 0.001% 2-mercaptoethanol, and lyophilized. They were reconstituted with 0.9% NaCl and assayed for rat GRF.

In the third study, male Wistar rats weighing 200–220 g were anesthetized with urethane (1.5 g/kg BW, ip). A group of rats were administered 1 ml of rabbit anti-GRF serum (R-1) iv and control rats received 1 ml of normal rabbit serum (NRS). Sixty min after the administration of anti-GRF serum or NRS, all rats received 0.5 ml of goat anti-somatostatin serum (ASS). The characteristics of ASS was previously reported (Wakabayashi *et al.*, 1980). Serial blood specimens were obtained through the jugular vein at the times indicated in Table 3.

Hormone assays and data analysis

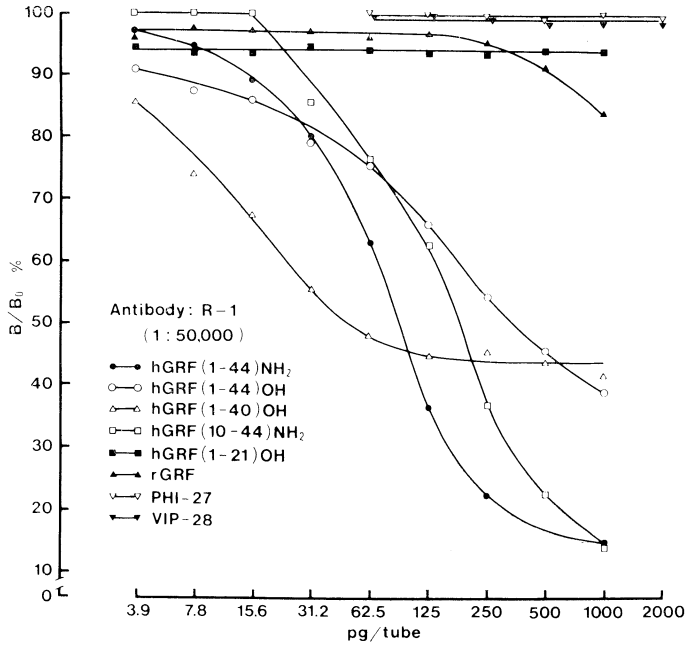
Rat GH and TSH contents were measured by double antibody RIA using materials supplied by NIADDK, NIH. Plasma T_4 concentrations were measured by RIA kit purchased (Daiichi Isotope Co., Ltd., Tokyo Japan). Results are expressed as mean±sem., and comparisons between data were performed by Duncan's multiple range test (Steel and Torrie, 1960) or Student's *t*-test.

Results

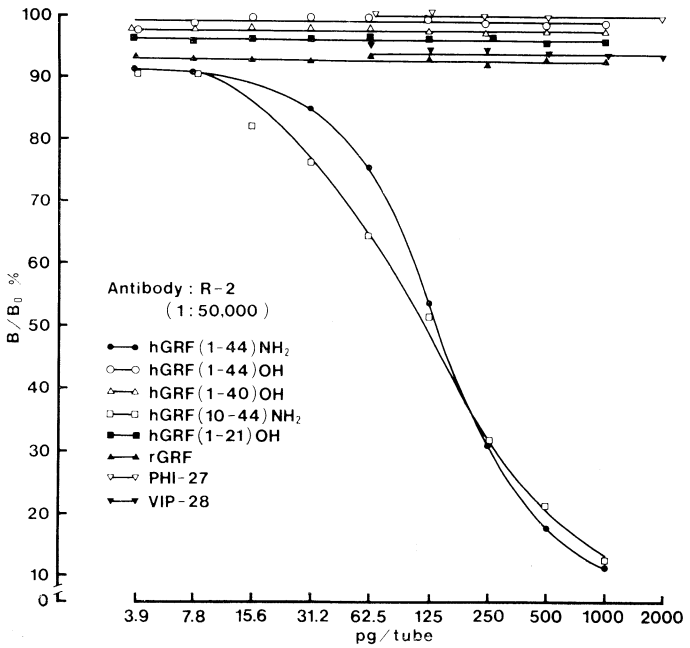
Sera from two rabbits (R-1 and R-2) immunized with human GRF-BSA conjugate showed significant binding with ^{125}I-human GRF [1-44] NH_2 after five immunizations. Typical standard curves of RIA for human GRF [1-44] NH_2 using the antisera R-1 and R-2 are shown in Figs. 1A and B, respectively. Included in the figure is the crossreactivity of the antisera with other structurally related peptides and various human GRF fragments. Both antisera were used at a final dilution of 1 : 50000 yielding maximum binding of approximately 50–60% of ^{125}I-human GRF [1-44] NH_2. Intra- and inter-assay coefficients of variation at 50% inhibition were 8 and 12%, respectively. The sensitivity of the RIA was 15.6–31.2 pg with the median effective dose 90–125 pg. Neither antiserum crossreacted with VIP, PHI or human GRF [1-21] OH. C-terminal amidated form of human GRF [10-44] NH_2 was completely recognized by both antisera. The antiserum R-2 was specific for the C-terminal amidated sequence of human GRF-44 and selectively recognized GRF-44 NH_2 but not GRF [1-44] OH or GRF [1-40] OH (Fig. 1B).

Utilizing a RIA with the antiserum R-2, a dilution curve of human SME paralleled that of the human GRF [1-44] NH_2 (Fig. 2) and on gel chromatography a major immunoreactive GRF of SME emerged in elution volumes concordant with ^{125}I-human GRF [1-44] NH_2 (Fig. 3).

Rat GRF differs from human GRF in about 30% of its amino acid residues and the main differences between the amino acid residues of human and rat GRFs are sited on the C-terminal portion (Guillemin *et al.*, 1982; Spiess *et al.*, 1983). ^{125}I-rat GRF [1-43] OH was bound approximately 55–67% by the antiserum R-1 at 1 : 5000 dilution. This enabled us to establish a RIA for rat GRF. The sensitivity of the RIA was 15.6–31.2 pg with the median effective dose approximately 250–300 pg (Fig. 4). Intra- and inter-assay coefficients of variation at 50% inhibition were 6 and 12%, respectively. A dilution curve of rat SME paralleled that



A: antiserum R-1.



B: antiserum R-2.

Fig. 1. Standard curve of RIA for human GRF [1-44] NH_2 and crossreactivities of the human GRF [1-44] NH_2 antiserum with other structurally related peptides and various human GRF fragments.

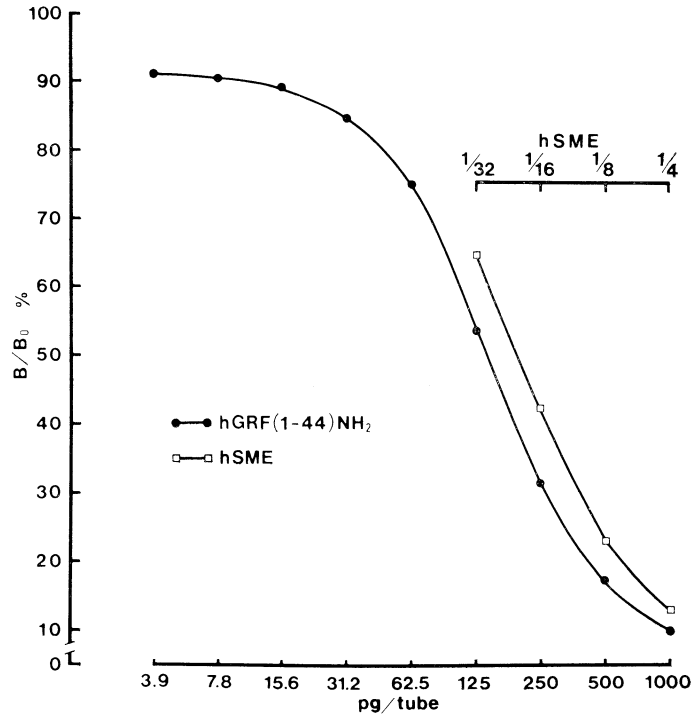


Fig. 2. Serial dilution curve of human SME. In this and Fig. 3, antiserum R-2 was used.

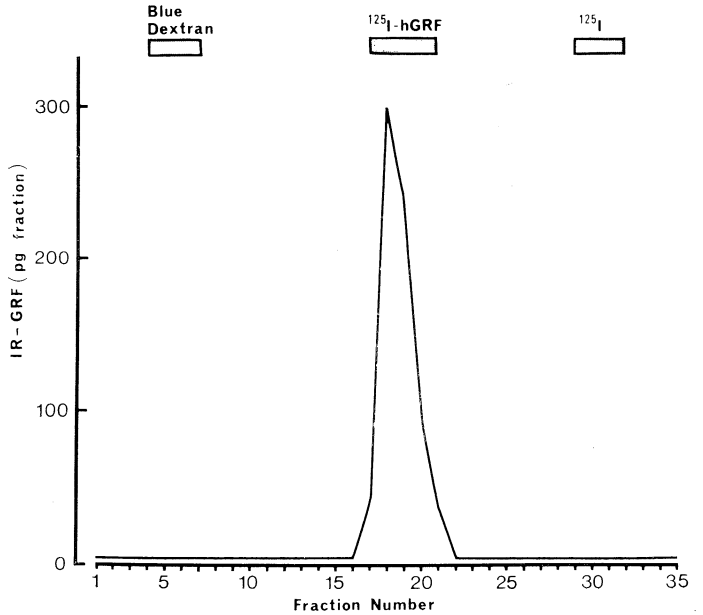


Fig. 3. Elution profile of immunoreactive GRF from extracts of human SME on Sephadex G-50 filtration chromatography.

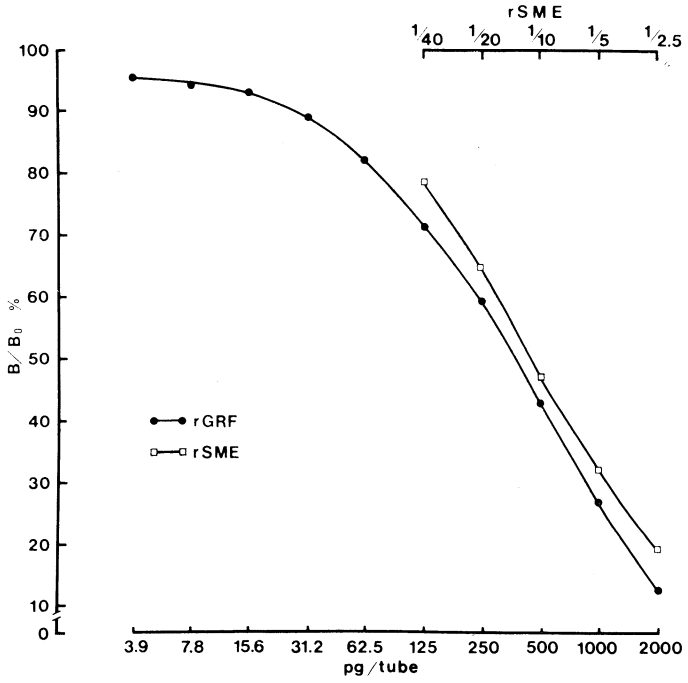


Fig. 4. Serial dilution curve of rat SME. In this and in Fig. 5, antiserum R-1 was used.

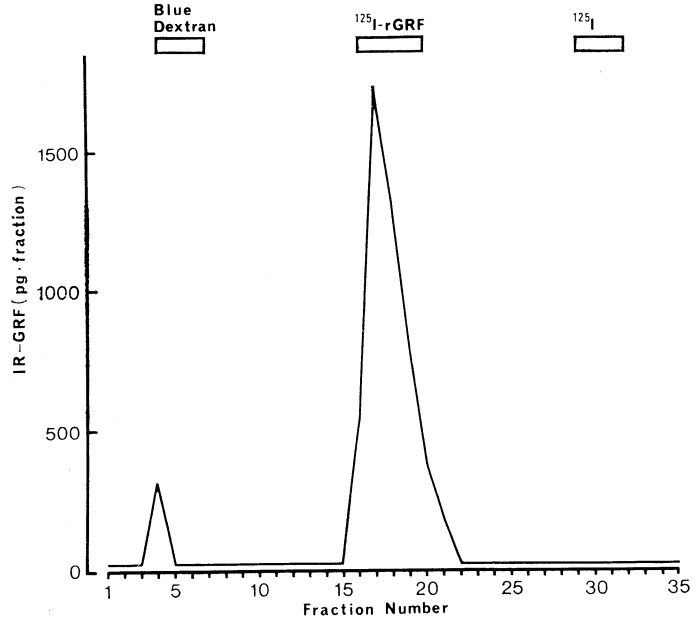


Fig. 5. Elution profile of immunoreactive GRF from extracts of rat SME on Sephadex G-50 gel filtration chromatography.

of rat GRF standard (Fig. 4) and on gel chromatography of SME, a major immunoreactive GRF was eluted in volumes corresponding to ^{125}I-rat GRF (Fig. 5). The antiserum R-2 did not bind ^{125}I-rat GRF appreciably even at a dilution of 1 : 500. In a separate study, synthetic rat GRF [1-43] OH was added to an aliquot of homogenates of rat cerebral cortex at doses of 0.125 and 0.25 ng in quadruplicates and lyophilized. After reconstitution with 0.9% NaCl, they were assayed for rat GRF. The recovery of immunoreactive GRF was 76–88% at both doses (data are not shown).

Adult rats which received MSG during the neonatal period showed reduced body growth as indicated by decreased body weight and reduced nasal-anal length at 75 day safter birth when compared to the controls (Table 1). Both the weight of anterior pituitary tissue and the concentration of pituitary GH were significantly decreased in MSG-treated rats when compared to the controls (Table 1). Immunoreactive GRF content in the SME of MSG-treated rats was reduced to less than 20% of that of the controls.

In rats which received methimazole, plasma T_4 concentrations were reduced significantly, while plasma TSH levels were significantly higher than those of the controls (Table 2). The weight of the anterior pituitary of methimazole-treated rats did not differ from that of the controls, but the pituitary GH concentration was significantly reduced in methimazole-treated rats when compared to the controls (Table 2). There observed no difference in immunoreactive GRF content in the SME between methimazole-treated and control rats (Table 2).

Basal plasma GH concentrations among

Table 1. Body growth, pituitary GH and hypothalamic GRF content of control and MSG-treated rats at 75 days of age.

	Control (4)	MSG-treated (5)
BW (g)	255.0±3.0	216.8±7.9*
Nasal-anal length (cm)	19.5±0.36	17.6±0.17*
Pituitary weight (mg)	9.96±0.16	2.90±0.24*
Pituitary GH (μg/mg protein)	945±102	617±94*
Immunoreactive GRF in SME (pg)	3905±961	431±88*

Number of rats in each group is shown in parentheses.

Values given are the mean±sem.

* $p<0.001$ vs. control (by Student's *t*-test).

Table 2. Plasma T_4, TSH, pituitary GH and hypothalamic GRF content of control and methimazole-treated rats.

	Control (5)	Methimazole-treated (5)
Plasma T_4 (μg/dl)	3.80±0.55	0.72±0.04*
Plasma TSH (ng/ml)	ND	992±94.1
Pituitary weight (mg)	6.44±0.63	6.56±0.48
Pituitary GH (μg/mg protein)	1800.0±125.1	842.6±121.3*
Immunoreactive GRF in SME (pg)	3266±221	3908±119

Number of rats in each group is shown in parentheses.

Values given are the mean±sem.

ND: not detectable.

* $p<0.001$ vs. control (by Student's *t*-test).

Table 3. Effect of iv administration of anti-GRF serum (R-1) on ASS-induced GH release in urethane-anesthetized rats.

Time after ASS injection (min)	Plasma GH (ng/ml)	
	Control (5)	Anti-GRF serum treated (5)
0	12.5± 1.1	8.8± 2.5
5	170.9± 25.6	89.8± 8.4*
15	493.4±132.7	152.7±10.0*
30	224.3± 59.3	129.5±33.7*
45	154.0± 40.0	106.5±41.3*
60	101.9± 21.0	41.9± 9.8*

Number of rats in each group is shown in parentheses.

Rats received anti-GRF serum (R-1) or normal rabbit serum 60 min previously and ASS was injected at 0 min.

Values given are the mean±sem.

* $p<0.001$ vs. control (by Duncan's multiple range test).

rats which received the anti-GRF serum (R-1) did not differ from those administered NRS. An intravenous injection of 0.5 ml ASS resulted in a significant increase in plasma GH in both groups of rats (Table 3). The rise in the plasma GH level following ASS was significantly reduced among rats administered anti-GRF serum as compared to those injected with NRS (Table 3).

Discussion

We have developed antisera to human GRF-BSA conjugate in two rabbits. The antisera R-1 and R-2 were directed toward the [Leu^{22}-Leu^{44}] NH_2 region of human GRF [1-44] NH_2. There existed a number of differences in antigen recognition sites in the two antisera. The study of cloning and sequence analysis of cDNA in the human GRF have indicated that GRF [1-44] NH_2 is the mature peptide, because it was shown to be preceeded in the precursor molecule by a dibasic cleavage sequence (Arg-Arg) and terminated by cleavage and an amidation signal (Gly-Arg) (Gubler *et al.*, 1983; Mayo *et al.*, 1983). A RIA using the antiserum R-2 which is specific for the C-terminal amidated sequence of human GRF-44 can be useful in quantitating GRF in biological materials in human.

GH secretion is suppressed in rats treated with monosodium glutamate (MSG) during the neonatal period (Bakke *et al.*, 1978; Millard *et al.*, 1982) and those made hypothyroid (Hervas *et al.*, 1975). MSG administered sc during the neonatal period has been reported to destroy a vast majority of the perikarya in the hypothalamic arcuate nuclei while sparing the axons that passed through (Simson *et al.*, 1977). We have shown that immunoreactive GRF content in SME was severely depleted by neonatal MSG treatment. The data supports previous immunocytochemical and radioimmunoassay studies demonstrating the existence of a population of GRF containing nerve terminals in the median eminence derived from cell bodies in the arcuate nucleus (Bloch *et al.*, 1983; Merchenthaler *et al.*, 1984; Kita *et al.*, 1985). We have previously observed that a depletion of hypothalamic GRF either by the placement of electrolytic lesions in the bilateral hypothalamic ventromedial-arcuate nuclei (Wakabayashi *et al.*, 1985a) or by neonatal MSG treatment (submitted for publication) did not alter the magnitude of plasma GH response to GRF in rats, even though GH content in the pituitary was depleted to 50% of that of the controls. Impaired GH secretion in MSG-treated rats appears to result primarily from a deficiency of endogenous GRF as postulated by others (Millard *et al.*, 1982). We have also observed that in hypothyroid rats, the pituitary's sensitivity to GRF is not significantly altered, though the magnitude of the plasma GH response was reduced and tentatively concluded that a depletion of pituitary GH reserve is a primary cause of reduced GH secretion in hypothyroidism (Wakabayashi *et al.*, 1985b). The finding that immunoreactive GRF content in SME was not altered significantly in rats made hypothyroid by methimazole treatment is consistent with our previous postulation.

GH secretion from the pituitary is under a dual control mechanism: hypothalamic GRF being stimulatory and hypothalamic somatostatin being inhibitory. The removal of one of these factors is expected to shift the balance of control to favor the action of the other. Thus immunoneutralization of endogenous somatostatin resulted in a rise in plasma GH. The prior administration of the anti-GRF serum was able to suppress ASS-induced GH release but failed to abolish completely the GH response. The result is in accord with those presented by others (Thomas *et al.*, 1985). This may suggest that a substance(s) in addition to rat GRF [1-43] OH is involved in "rebound GH release" following ASS injection.

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