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IN VIVO BIOLOGICAL POTENCY OF RAT AND HUMAN GROWTH HORMONE-RELEASING FACTOR AND FRAGMENTS OF HUMAN GROWTH HORMONE-RELEASING FACTOR

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ABSTRACT: The biological potency of the synthetic replicates of three peptides isolated from a human pancreatic tumor with growth hormone releasing activity and rat hypothalamic growth hormone-releasing factor was evaluated in conscious freely-moving rats and anesthetized rats. All 4 peptides are equipotent on a molar basis in their ability to stimulate GH secretion. Studies with synthetic fragments of the human derived material indicated that the amino-terminal amino acid is required for activity. Deletion of as many as 13 amino acids from the carboxy-terminal failed to decrease GH-releasing activity; however, deletion of 16 amino acids resulted in a significant decrease and deletion of 20 amino acids resulted in complete loss of bioactivity.

A potent and specific growth hormone-releasing factor was recently isolated from a human islet cell tumor of the pancreas (1). This 44-amino acid peptide is amidated at the carboxy-terminus and has been designated hpGRF-44 or somatocrinin. Two probable proteolytic degradation products of somatocrinin containing the amino-terminal 40 (hpGRF-40) and 37 (hpGRF-37) amino acid sequence of hpGRF-44 were also characterized from this same tissue (1) while only hpGRF-40 was isolated from another source (2,3). Spiess et al. (4) have recently reported the characterization of rat hypothalamic growth hormone-releasing factor (rGRF). We report herein the in vivo biological potency of hpGRF-44, -40 and -37 to secrete growth hormone (GH) in conscious freely-moving rats which had been passively immunized against both endogenous somatostatin and rat hypothalamic growth hormone-releasing factor, a model which has been shown to be idealy suited for the study of GRF regulation of GH secretion(5). In addition, we report a comparison of the in vivo biological potency in anesthetized rats of hpGRF-44 and rGRF as well as that of various shortened

Abbreviations: GH: growth hormone; hpGRF-44, hpGRF-40 and hpGRF-37: human pancreas growth hormone-releasing factor composed of 44, 40 and 37 amino acids, respectively; rGRF: rat hypothalamic growth hormone-releasing factor.

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fragments of hpGRF-44 and PHI-27, a intestinal peptide with considerable sequence homology to hpGRF-44 (6).

MATERIALS AND METHODS

Male Sprague-Dawley rats were used in these experiments. Animals were housed in a temperature- (21-22 C) and humidity-controlled vivarium under a 14h light: 10h dark lighting schedule (lights on at 0600h). Food and water were available ad libitum.

Biopotency of hpGRF-44, -40 and -37 in conscious freely-moving rats: Rats weighing 475 ± 13 g were prepared with chronic indwelling venous catheters as described (5,7). On the day of the experiment animals were left completely undisturbed in their isolation chambers. Two hours prior to the onset of blood sampling, rats received a 1 ml infusion of antiserum (5.8 mg protein) raised against somatostatin and a 2 ml infusion of a monoclonal antibody (3.2 mg protein) raised against rat hypothalamic growth hormone-releasing factor (8) which does not recognize hpGRF. At 1000h a 0.12 nmol/kg dose of either hpGRF-44, -40 or -37 was injected i.v. Four minutes later a 0.15 ml blood sample was drawn. Two hours later, each rat was administered the same peptide but now at a 0.24 nmol/kg dose and a blood sample was drawn 4 minutes later. This procedure was repeated again two hours later with a 0.48 nmol/kg dose. A total of 19 rats were used in this study; n = 6 or 7 for each peptide.

Biopotency of hpGRF-44 and rGRF in anesthetized rats: Rats weighing 270 ± 2 g were anesthetized with sodium pentobarbital (NaPb, 50 mg/kg ip). Five to ten minutes later the animals were fitted with a catheter placed in an external jugular vein and advanced to the superior vena cava. Two hundred units of sodium heparin were then injected i.v. An initial blood sample (0.15 ml) was drawn 15 min after NaPb administration. This was immediately followed by the administration of 0.02 nmol/rat of either hpGRF-44 or rGRF with subsequent blood samples drawn as indicated in Fig 2. A total of 23 rats were used in this study; n = 11 or 12 for each peptide.

Biopotency of fragments and an analogue of hpGRF-44 and PHI-27 in anesthetized rats: Using hpGRF-44 as a reference standard, we evaluated the biopotency of 0.4 nmol/kg of the carboxy-terminal deleted fragments of hpGRF-44 which consisted of the first 24, 28, 31 and 34 amino acids of hpGRF-44. This large dose was chosen since we expected a decrease in potency based on preliminary observations. The experimental protocol was performed as described for anesthetized rats. A total of 30 rats were used in this experiment; n = 6 for each peptide. In addition, we evaluated the biopotency of a 500 ng/rat dose of the amino-terminal deleted fragment of hpGRF-40 corresponding to hpGRF(2-40), a 0.2 nmol/kg dose of the amino-terminal substituted analogue of hpGRF-40 corresponding to [His^1] hpGRF-40 and a 0.4 nmol/kg dose of PHI-27. A total of 12 rats were used in this study; n = 4 for each experiment.

Hormone assays: Plasma concentrations of GH were determined by radioimmunoassay using a double antibody method with reagents provided by National Pituitary Agency of the NIH with the exception of the first antisera which was provided by Dr. Y. Sinha (9). Samples were first diluted 1/5 and then assayed in triplicate using 20-100 l.

Peptide preparations: Peptides were synthesized by solid-phase techniques using a Beckman 990 peptide synthesizer. The peptides were dissolved in water and diluted with saline to attain working concentrations.

RESULTS

As illustrated in Fig. 1, hpGRF-44, -40 and -37 are equipotent in their ability to stimulate GH secretion in conscious freely-moving rats which had

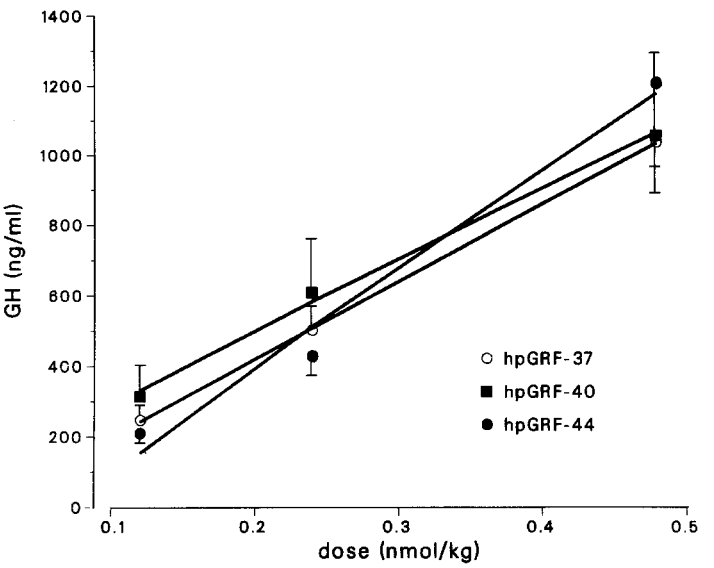

Figure 1 The biopotency of increasing doses of hpGRF-37, -40 & -44 in conscious freely-moving rats pretreated with antiserum raised against somatostatin and a monoclonal antibody raised against rat hypothalamic growth hormone-releasing factor which does not recognized hpGRF. The peptides were administered (iv) at two hour intervals in 0.5 ml saline. Data points represent the mean of 6 or 7 rats in each group, vertical bars represent the SEM.

been previously immunized against endogenous somatostatin and rat hypothalamic growth hormone-releasing factor. Using a three point multiple assay design (10) and assigning a potency of 1 to hpGRF-44, hpGRF-40 had a potency of 1.08 (95% confidence limits of 0.70 and 1.69) and hpGRF-37 had a potency of 0.97 (95% confidence limit of 0.62 and 1.50). As is evident, these peptides all showed a dose-response relationship. The biopotency of hpGRF-44 and rGRF were virtually identical when studied in anesthetized male rats (Fig. 2). As illustrated, both the magnitude as well as the time-course relationship of the pituitary GH response for these two peptides were not different. The GH response observed between experiments appears to vary somewhat as has been previously reported (11).

The comparison of the growth hormone releasing potencies of various carboxy-terminal deleted fragments of hpGRF-44 is illustrated in Fig. 3. The increase in GH concentrations following iv administration of hpGRF(1-34) and hpGRF(1-31) were not different from the response observed with hpGRF-44. In contrast, hpGRF(1-28) showed a significant ($p<0.01$) decrease in bioactivity and hpGRF(1-24) demonstrated no bioactivity. There was no increase in plasma GH concentrations in rats receiving hpGRF(2-40), the amino-terminal deleted fragment of hpGRF-40, or PHI-27 (data not shown). When histidine was substi-

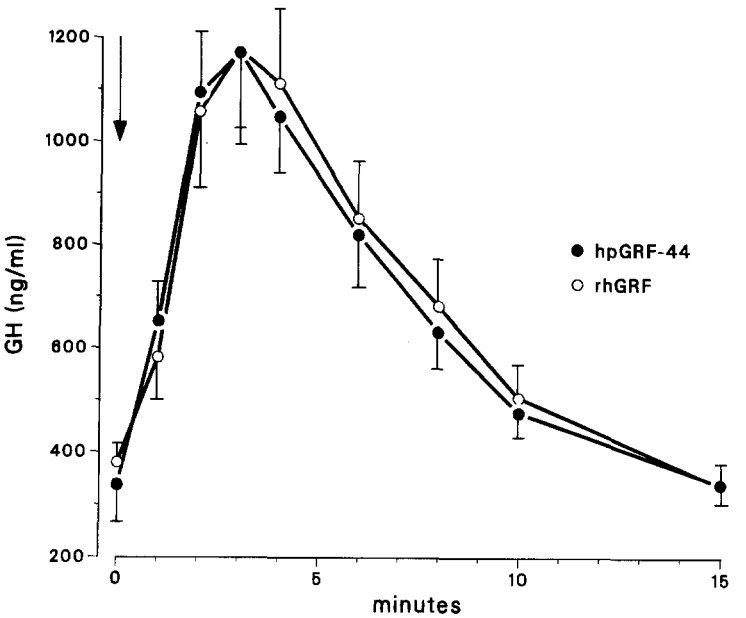

Figure 2 The biopotency of 0.02 nmol hpGRF-44 & rGRF in anesthetized male rats. Animals were injected with 50 mg/kg sodium pentobarbital (ip) 15 minutes prior to the initial blood sample. The peptides were administered (iv) immediately after time 0 in 0.5 ml saline. Data points represent the mean of 11 or 12 rats in each group; vertical bars represent the SEM.

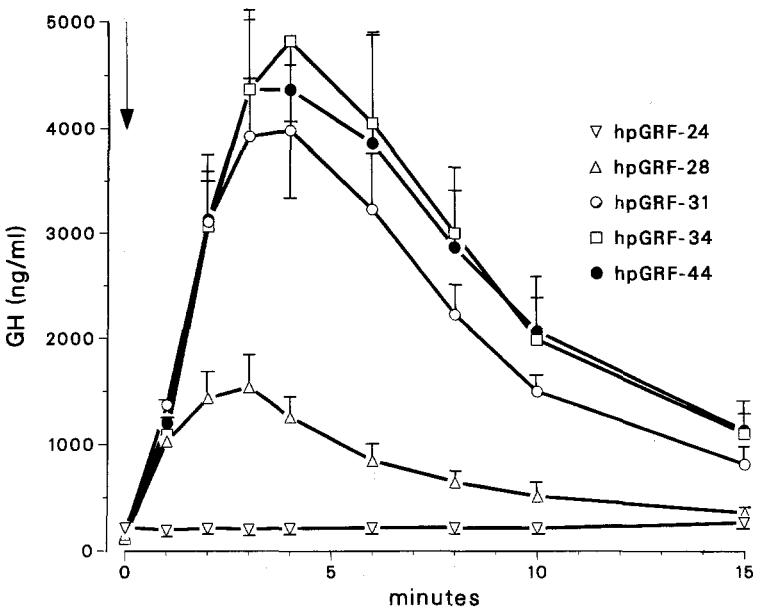

Figure 3 The biopotency of 0.4 nmol/kg hpGRF(1-24), (1-28), (1-31), (1-34) & (1-44) in anesthetized male rats. Animals were injected with 50 mg/kg sodium pentobarbital (ip) 15 minutes prior to the initial blood sample. The peptides were administered (iv) immediately after time 0 in 0.5 ml saline. Data points represent the mean of 6 rats in each group; vertical bars represent the SEM.

tuted for tyrosine in hpGRF-40 the peptide still possessed potent biological activity, as evidenced by the increase in plasma GH concentrations which rose to 1860 ± 434 ng/ml 3 min after iv administration.

DISCUSSION

Guillemin et al (1) have reported that the in vitro growth hormone releasing activity of hpGRF-40 and hpGRF-37 are 30% and 12% that of hpGRF-44, respectively. Our present observations in vivo using conscious freely-moving rats and our earlier observations using anesthetized rats (12) have failed to indicate any difference in potency. Interestingly, there also appears to be no difference in the in vivo activity of either the human derived or the rat derived growth hormone-releasing factor in rats. Spiess et al. (4) have reported that in vitro rGRF is 2.44 times more potent than hpGRF-44 in stimulating GH release in a rat anterior pituitary monolayer culture system. The reason for these differences between the in vitro and in vivo observations is unclear. It may be due to the inherent variability of the in vivo system; however, the large number of animals tested in each experiment would argue against this possibility. Other possible explanations may be that in vivo there is proteolytic processing of these peptides to some common bioactive core; however, the absence of complete sequence homology between hpGRF-44 and rGRF argues against this possibility. The differences between the circulating half-life of each peptide may be such that differences in potency are confounded.

Further studies using synthetic fragments of hpGRF-44 have shown that deletion of as many as 13 amino acids (hpGRF(1-31)) from the carboxy-terminal failed to alter the in vivo growth hormone releasing activity. With further deletions from the carboxy-terminal we did observe a loss in bioactivity. Deletion of 16 amino acids from hpGRF-44, yielding hpGRF(1-28), resulted in a 64% decrease in the maximum GH increase and deletion of 20 amino acids, yielding hpGRF(1-24), resulted in complete loss of in vivo biological activity at the dose tested. Deletion of the amino-terminal amino acid, tyrosine, from hpGRF-40 resulted in complete loss of bioactivity in vivo. In addition, PHI-27, a natural intestinal peptide with 40% sequence homology with the corresponding amino-terminal of hpGRF-44, was devoid of any in vivo GH releasing activity. These observations confirm earlier in vitro results (1,3). The fact that rGRF which has histidine as its amino-terminal amino acid is active, as

well as our observation that [His]hpGRF-40 is active, suggests that either a tyrosine or histidine is required at the first position. These results indicate that *in vivo* the bioactive core of growth hormone-releasing factor is constituted by fragments consisting of residues 1-25 to 1-30.

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REFERENCES

1. Guillemin, R., Brazeau, P., Bohlen, P., Esch, F., Ling, N., & Wehrenberg, W.B. (1982) Science 218, 585-587.
2. Esch, F.S., Bohlen, P., Ling, N.C., Brazeau, P.E., Wehrenberg, W.B., Thorner, M.O., Cronin, M.J., & Guillemin, R. (1982) Biochem. Biophys. Res. Commun. 109, 152-158.
3. Rivier, J., Spiess, J., Thorner, M., & Vale, W. (1982) Nature 300, 276-278.
4. Spiess, J., Rivier, J., & Vale, W. (1983) Nature 303, 532-535.
5. Wehrenberg, W.B., Brazeau, P., Luben, R., Ling, N., & Guillemin, R. (1983) Neuroendocrinology 36, 489-491.
6. Tatemoto, K. & Mutt, V. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6603-6605.
7. Wehrenberg, W.B., Ling, N., Bohlen, P., Esch, F., Brazeau, P., & Guillemin, R. (1982) Biochem. Biophys. Res. Commun. 109, 562-567.
8. Luben, R.A., Brazeau, P., Bohlen, P., & Guillemin, R. (1982) Science 218, 887-889.
9. Sinha, Y.N., Selby, F.W., Lewis, U.J., & Vanderlaan, W.P. (1972) Endocrinology 91, 784-792.
10. Sakiz, E. (1964) Proc. 2nd Intern. Cong. of Endocrinology 83, 225-229.
11. Wehrenberg, W.B. & Ling N. (1983) Neuroendocrinology (in press).
12. Wehrenberg, W.B., Ling, N., Brazeau, P., Esch, F., Bohlen, P., Baird, A., Ying, S., & Guillemin, R. (1982) Biochem. Biophys. Res. Commun. 109, 382-387.