Physiological and pharmacological regulation of 20-kDa growth hormone

KIN-CHUEN LEUNG,1 CHRIS HOWE,2 LILY Y.-Y. GUI,1,2 GRAHAM TROUT,2 JOHANNES D. VELDHUIS,3 AND KEN K. Y. HO1

1Pituitary Research Unit, Garvan Institute of Medical Research, St. Vincent’s Hospital, Sydney, New South Wales 2010; 2Australian Sports Drug Testing Laboratory, Australian Government Analytical Laboratories, Sydney, New South Wales 2073, Australia; and 3Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

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GROWTH HORMONE (GH) exists as a mixture of multiple molecular forms arising from both posttranscriptional and posttranslational modifications (1, 23). The major isof orm of molecular size 22 kDa comprises 191 amino acid residues, whereas the second-most abundant isof orm is 20 kDa in size and accounts for ~10% of total GH in the pituitary (19). This isof orm is generated by alternative splicing within exon 3 of the GH primary transcript, resulting in the deletion of residues 32–46 of 22-kDa GH (1, 6). The 20-kDa GH has growth-promoting and lipolytic activities similar to those of 22-kDa GH (18, 25) but has reduced antinatriuretic activity (21). Although these observations suggest that it has a slightly different metabolic profile, the physiological role of 20-kDa GH is not known.

Recently, studies using a highly specific enzyme-linked immunosorbent assay (ELISA) for 20-kDa GH have revealed that it circulates at a constant proportion to 22-kDa GH under a variety of physiological and pathophysiological conditions (12). However, it remains to be determined whether 20-kDa GH is regulated differently from 22-kDa GH. To gain more insight in the regulation of 20-kDa GH in blood, we have compared the serum levels of 20- and 22-kDa GH in normal subjects (14–17, 20, 33). The clinical data of some of these studies have been previously published in part. All study protocols were approved by the Research Ethics Committee of St. Vincent’s Hospital (Sydney, Australia), and informed written consent was obtained from each subject.

Spontaneous secretion in normal, acromegalic, and GH-deficient subjects (study 1). This study compared the 24-h integrated concentrations of 20- and 22-kDa GH in 39 normal subjects, 14 patients with acromegaly, and 23 with organic GH deficiency. The normal subjects comprised 25 men and 14 women aged 43.4 ± 3.1 yr (mean ± SE; range: 14–78 yr). The acromegalic subjects comprised 7 men and 7 women aged 40.7 ± 3.0 yr (range: 33–60 yr). The GH-deficient subjects comprised 15 men and 8 women aged 42.3 ± 4.3 yr (range: 16–77 yr). The mean age was not significantly different between the three groups. Data reporting GH concentrations obtained from a polyclonal RIA from some of these subjects

SUBJECTS AND METHODS

Subjects and study design. Five separate studies were performed to investigate the physiological and pharmacological regulation of 20-kDa GH secretion (14–17, 20, 33). The clinical data of some of these studies have been previously published in part. All study protocols were approved by the Research Ethics Committee of St. Vincent’s Hospital (Sydney, Australia), and informed written consent was obtained from each subject.

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have been published previously (14, 17). The diagnosis of acromegaly was based on symptoms and signs, elevated insulin-like growth factor I (IGF-I) levels, and failure of GH suppression in response to a 75-g oral glucose challenge. GH deficiency was established by the insulin-induced hypoglycemia test with the peak GH response <3 ng/ml (17). All subjects underwent a 24-h study with blood taken every 20 min, from which equal aliquots were obtained to form an integrated pool sample. Levels of 20- and 22-kDa GH in the pool samples were measured. To characterize spontaneous secretion of the GH isoforms, the 24-h profiles in a subset of eight normal subjects (4 men and 4 women) were studied by deconvolution analysis.

**Acute stimulation test (study 2).** We examined the effect of an insulin-induced hypoglycemia test (ITT) on 20-kDa GH secretion in 12 normal subjects (5 men and 7 women; aged 59.9 ± 6.4 yr, range: 34–77 yr) and 12 patients with organic hypopituitarism (5 men and 7 women; aged 52.2 ± 3.9 yr, range: 40–65 yr). Briefly, these patients had a history of a structural lesion of the pituitary or hypothalamus treated with surgery or radiotherapy requiring hormone replacement therapy for any combination of secondary adrenal, gonadal, or thyroid failure or diabetes insipidus. In the ITT study, an insulin bolus (0.1 U/kg) was given intravenously at time 0, and venous blood was collected at 0, 30, 60, 90, and 120 min for GH measurement. All subjects achieved a nadir blood glucose of 2.2 mmol/l or less in response to ITT.

**Sex steroids (study 3).** We investigated the effects of androgen replacement in six hypogonadal men and 2 oral estrogen replacement in nine postmenopausal women on 20- and 22-kDa GH concentrations. Some of these GH data quantified by a polyclonal RIA have been published previously (20, 33). The hypogonadal subjects, aged 18–26 yr, comprised five men with Klinefelter’s Syndrome and one with isolated hypogonadotrophic hypogonadism. Hypogonadism was established by the finding of a subnormal testosterone level of <7 mmol/l (normal: 12–30 mmol/l). All subjects were studied during testosterone therapy (250 mg testosterone enanate every 4 wk; Primoteston; Schering, Sydney, Australia) for at least 6 wk in a random order. The study during testosterone therapy was undertaken after at least 2 mo of interrupted therapy, 7–12 days after the last injection.

In the estrogen study, the postmenopausal women, aged 55–72 yr, were at least 1 yr postmenopausal. Studies were undertaken before and 3 mo after starting oral conjugated estrogen (1.25 mg; Premarin; Wyeth-Ayerst, Philadelphia, PA). Blood samples were taken every 20 min over a 24-h period. A pool sample was formed from obtaining equal aliquots of the study samples, from which assays for 20- and 22-kDa GH were performed to obtain 24-h integrated concentrations of the isoforms.

**Acute suppression (study 4).** The effects of octreotide on 20- and 22-kDa GH concentrations in eight patients with active acromegaly aged 25–65 yr in a controlled study were examined (15). Each of the patients participated in two studies involving the administration of either saline or 100 μg octreotide (SMS 201–995; Sandoz Australia, Sydney, Australia) subcutaneously after an overnight fast. Blood samples were collected hourly for 8 h, and serum GH was measured in all samples.

**Exogenous 22-kDa GH (study 5).** We studied the effect of administration of recombinant 22-kDa GH on circulating 20-kDa GH levels in six healthy men aged 21–29 yr (16). All subjects were nonsmokers, not taking medications, and were required to abstain from alcohol for 1 wk before the study. Recombinant 22-kDa GH (16) was administered subcutaneously at a dose of 0.1 mg/kg. Blood samples were obtained immediately before and at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 24 h after administration for GH measurement. IGF-I concentrations were measured at 0, 4, 8, 12, and 24 h.

**GH assays.** Serum 20- and 22-kDa GH were measured by isoform-specific ELISAs, using the corresponding recombinant GH isoforms as standards (16, 25). An anti-20-kDa GH monoclonal antibody (D05) and the horseradish peroxidase-conjugated anti-GH monoclonal antibody (D14/HRP; see Ref. 12) were generous gifts from Dr. Yukio Schimazaki (Niho Schering KK, Osaka, Japan). D05 has cross-reactivities <0.1% with other GH isoforms and prolactin (Ref. 24 and Leung, unpublished observations), whereas D14/HRP recognizes both 20- and 22-kDa GH. The 22-kDa GH-specific monoclonal antibody (A36020047P) with cross-reactivity of <0.1% with 20-kDa GH was purchased from BioPacific (Emeryville, CA).

In the 20-kDa GH ELISA, 2.5 μg/well D05 were used to coat a 96-well multidish at 4°C for 18 h and blocked with SuperBlock buffer (Pierce, Rockford, IL) at 23°C for 2 h. Next, 100-μl samples were added in duplicate to the wells with 100 μl of 100 mmol/l NaH2PO4, pH 7.0, containing 2 mmol/l EDTA, 100 mmol/l NaCl, 2% BSA, 10 μg/ml heterophile blocking reagent 1 (Scantibodies Laboratory, Santee, CA), and 0.1 g/l thimerosal followed by incubation at 4°C for 18 h. After four washes with 10 mmol/l Tris-Cl (pH 8.0), 150 mmol/l NaCl, 0.05% Tween 20, and 0.1 g/l thimerosal (TBS buffer), 0.5 μg/well D14/HRP was added, and incubation continued at 23°C for 2 h. After washing with TBS buffer, signal was developed with hydrogen peroxide and 3,3'-5,5'-tetramethylbenzidine (Pierce) at 23°C for 20 min. The incubation was terminated with the addition of 2 mol/l H2SO4, and absorbance was measured at 405 nm. The detection limit of this assay was 7.5 ng/l. The intra-assay coefficients of variation (CVs) at 23,96, and 517 ng/l were 6.6, 5.6, and 3.4% (n = 6), respectively, whereas the interassay CVs at 64 and 517 ng/l were 10.9% (n = 17) and 9.4% (n = 14), respectively.

The 22-kDa GH ELISA was performed in a similar manner as described above, except that the 22-kDa GH-specific antibody (A36020047P) was used in place of D05, and 20 μl of sample was assayed. The detection limit of the assay was 40 ng/l. The intra-assay CVs at 123 and 1,780 ng/l were 8.8 and 4.6% (n = 6), respectively, and the interassay CVs at 128 and 1,954 ng/l were 8.6 (n = 12) and 10.0% (n = 14), respectively. Both the 20- and 22-kDa ELISA were unaffected by GH-binding protein (GHBP) at physiological concentrations (data not shown).

**Deconvolution analysis.** To determine the basal and pulsatile secretion of 20-kDa GH, we studied the 24-h concentration profiles obtained from 20-min sampling in eight normal subjects (study 1) using multiple-parameter deconvolution analysis (26). This algorithm calculates the following specific measures of GH secretion: basal secretion rate, number of secretory bursts, interburst interval, amplitude (maximal level of secretion) and mass (total amount of secretion) per burst, half-duration of burst (duration of the secretory burst at half-maximal amplitude), pulse and total production rates, and circulating half-life. Values of 20- and 22-kDa GH below the limit of detection were entered as those corresponding to the detection limit (7.4 ng/l for 20 kDa and 40 ng/l for 22 kDa). This analysis has specificity and sensitivity of ~90% for pulse secretion (27).

**Statistical analysis.** The GH isoform ELISA standard curves were fitted using a four-parameter logistic equation (AssayZap; BioSoft, Cambridge, UK). Results were expressed as means ± SE. The degree of significance of differences between groups was calculated using Student’s t-test or
ANOVA (StatView 4.5; Abacus Concepts, Berkeley, CA) where appropriate and was set at $P < 0.05$.

RESULTS

Spontaneous secretion in normal, acromegalic, and GH-deficient subjects. Figure 1A shows the mean 24-h concentrations of 20-kDa GH in normal ($n = 39$), acromegalic ($n = 14$), and GH-deficient ($n = 23$) subjects. The mean values were significantly higher in acromegaly (682 ± 276 ng/l; $P < 0.0005$) and lower in GH deficiency (9.6 ± 4.0 ng/l; $P < 0.0001$) than in normal (47.4 ± 5.9 ng/l) subjects. Similarly, acromegalic patients had higher and GH-deficient patients had lower 22-kDa GH concentrations than normal subjects (10,145 ± 3,338, 111 ± 38, and 665 ± 68 ng/l, respectively; $P < 0.0001$; Fig. 1B). However, the ratios of 20- to 22-kDa were not significantly different, with values of 5.9 ± 0.8, 7.1 ± 2.4, and 6.9 ± 0.8% for acromegalic, GH-deficient, and normal subjects, respectively (Fig. 1C).

The levels of 22-kDa GH in the normal group were significantly higher in women ($n = 14$; 844 ± 114 ng/l) than in men ($n = 25$; 565 ± 79 ng/l; $P < 0.05$). The 20-kDa GH levels were higher in women (60.8 ± 9.6 and 39.9 ± 7.1 ng/l, respectively), although this did not reach statistical significance ($P = 0.087$). The 20-to-22-kDa ratios (7.1 ± 1.2 and 6.8 ± 1.0%, respectively) were not different.

As shown in Fig. 2, both 20- and 22-kDa GH were secreted in a pulsatile manner, with peaks of 20-kDa GH coinciding with those of 22-kDa GH. The nadir levels for both isoforms fell below the detection limits of the respective assays. Deconvolution analysis was performed to determine the basal and pulsatile secretory and clearance events contributing to the secretion profiles (Table 1). This revealed similar numbers of burst, interburst intervals, and half-
duration of the two isoforms. The half-life of 20-kDa GH was ~30% longer than that of 22-kDa GH ($P < 0.02$). Both 20- and 22-kDa GH were secreted in a tonic and pulsatile pattern, with pulsatile secretion accounting for 76.4 ± 3.3% and 91.6 ± 1.6% of GH production, respectively. All secretory measures of 20-kDa GH, including basal secretion rate, mass and amplitude per burst, and pulse and total production rate, were significantly lower than those of 22-kDa GH. The pulsatile-to-basal ratio of 20-kDa GH was about threefold lower than that of 22-kDa GH, suggesting that a greater proportion of 20-kDa GH was secreted tonically.

**Acute stimulation test.** In normal subjects ($n = 12$), ITT rapidly induced parallel release of 20- and 22-kDa GH, with the levels increased maximally by 53 ± 20-fold ($P < 0.0001$) and 41 ± 13-fold ($P < 0.05$), respectively, at 60 min (Fig. 3A). The 20-to-22-kDa ratios did not change significantly at all sampling times (Fig. 3B). In patients with organic hypopituitarism ($n = 12$; Fig. 3C), there was a clear GH response in four subjects, with peak 20- and 22-kDa GH concentrations of 22.3–45.3 and 524–1131 ng/l, respectively. The 20-to-22-kDa ratios were unchanged throughout the test. Among the patients with no significant GH response, the concentrations of 22-kDa GH in four and 20-kDa GH in seven subjects were below the detection limit of the assays at all sampling times.

**Sex steroids.** Administration of testosterone to hypogonadal men ($n = 6$) significantly increased the mean 24-h concentration of 20-kDa GH from 31.1 ± 5.3 to 40.4 ± 3.7 ng/l (Fig. 4A; $P < 0.005$) and that of 22-kDa GH from 454 ± 39 to 641 ± 65 ng/l (Fig. 4B; $P < 0.005$). The 20-to-22-kDa ratios were unaltered, with values of 7.1 ± 1.7 and 6.9 ± 1.5% before and after testosterone treatment, respectively (Fig. 4C).

In postmenopausal women ($n = 9$), oral estrogen administration caused a parallel increase in both GH isoforms, with mean 24-h 20-kDa GH concentration increasing from 16.5 ± 1.9 to 48.2 ± 8.2 ng/l (Fig. 5A; $P < 0.005$) and that of 22-kDa GH from 395 ± 53 to 1,104 ± 144 ng/l (Fig. 5B; $P = 0.0001$). The 20-to-22-kDa ratios did not differ before and after estrogen treatment (4.4 ± 1.0 and 4.3 ± 1.5%, respectively; Fig. 5C).

**Acute suppression.** We examined the effects of octreotide on serum 20- and 22-kDa GH in patients with active acromegaly ($n = 8$). In the saline control study, both isoforms were secreted continuously at a constant 20-to-22-kDa ratio (Fig. 6, A and C). Octreotide administration rapidly reduced the levels of both isoforms, which remained suppressed for 4 h before rising gradually (Fig. 6B). Because the fall in 22-kDa GH was more rapid than 20-kDa GH, the treatment acutely increased the 20-to-22-kDa ratio from 8.6 ± 1.2% at time 0 to 11.0 ± 1.8% at 1 h and remained elevated for the first 4 h (Fig. 6C; $P < 0.05$) compared with that observed during the control study.

![Fig. 3. Effects of insulin-induced hypoglycemia on 20- and 22-kDa GH in normal (A and B) and hypopituitary (C) subjects.](http://ajpendo.physiology.org/)
Exogenous 22-kDa GH. To investigate the effect of exogenous 22-kDa GH on endogenous 20-kDa GH secretion, serum levels of both isoforms were measured in normal subjects \((n = 6)\) administered recombinant 22-kDa GH. After administration, the level of 22-kDa GH rose to a peak at 4 h and remained elevated for 12 h (Fig. 7A). In contrast, the level of 20-kDa GH fell progressively and rapidly and remained suppressed for up to 24 h. The 20-to-22-kDa ratio was reduced from 5.9 ± 3.1\% to below 0.1\% for 12 h after administration (Fig. 7B) and remained below the normal range at 24 h, which comprised mean ± SD values derived from the 24-h profile assessment in study 1 (Fig. 2). IGF-I concentration was not significantly different at 4 h (37 ± 8 nmol/l) compared with pretreatment (36 ± 7 nmol/l) but rose significantly to 45 ± 7 nmol/l at 8 h \((P < 0.002)\).

**DISCUSSION**

We used an isoform-specific ELISA to study the regulation of 20-kDa GH over a range of pathophysiological conditions and in response to pharmacological tests. Mean 24-h concentrations of 20-kDa GH were higher in acromegalic and lower in GH-deficient than in normal subjects; however, the 20-to-22-kDa ratios did not differ between the three groups. We confirmed that 20-kDa GH was secreted in a pulsatile manner similar to 22-kDa GH, and its release was stimulated by hypoglycemia. We extended these observations by reporting that androgen and oral estrogen induced a parallel increase in 20- and 22-kDa GH without affecting the 20-to-22-kDa ratio. Using deconvolution analysis, we demonstrated for the first time that the half-life of 20-kDa was longer than that of 22-kDa GH.

Administration of exogenous 22-kDa GH completely suppressed endogenous release of 20-kDa GH. The concomitant increase in circulating 22-kDa GH levels resulted in a marked reduction in the 20-to-22-kDa ratio.

Although 20-kDa GH was identified more than 20 years ago (19), the regulation of this isoform is not fully understood, partly because of the lack of a specific assay. Using isoform-specific ELISAs for 20- and 22-kDa GH, we demonstrated that 20-kDa GH circulated at a constant ratio of 22-kDa GH in normal subjects. Women had significantly higher levels of 22-kDa GH than men, whereas the differences in 20-kDa GH levels showed a similar trend. Similar observations of sexual dimorphism of the two isoforms have been reported previously (24). Deconvolution analysis revealed cosecretion of 20- and 22-kDa GH, as indicated by similar burst num-

Fig. 4. Mean 24-h concentrations of 20-kDa (A) and 22-kDa (B) GH and the 20-to-22-kDa ratio (C) in hypogonadal men before (basal) and after testosterone (T) treatment.

Fig. 5. Mean 24-h concentrations of 20-kDa (A) and 22-kDa (B) GH and the 20-to-22-kDa ratio (C) in postmenopausal women before (basal) and after oral estrogen (EE) treatment.
bers, interburst intervals, and half-duration of burst. The levels of basal and pulsatile production of 20-kDa GH were lower than those of 22-kDa GH, consistent with the view that the latter is the major isoform. Basal secretion appeared to contribute to a larger portion of 20-kDa GH production compared with that of 22-kDa GH, as reflected by a threefold lower pulsatile-to-basal ratio, suggesting that different mechanisms may be involved in the basal and pulsatile secretion of GH isoforms. However, we cannot be certain that the lower pulsatile-to-basal ratio observed with 20-kDa GH is not an artifact of the analysis because values corresponding to the detection limit were entered for samples whose nadir values fell below this limit. Although this was also done for corresponding nadir values of 22-kDa GH samples, the proportion of 20-kDa GH basal secretion may be overestimated because of the lower pulse level of this isoform.

About 40–50% of basal samples revealed an unequivocally measurable level of both 20- and 22-kDa GH. The origin of basal GH secretion is unclear. There is evidence of constitutive GH release from somatotrophs (10, 22), which could account for the basal mode of GH production. It is conceivable that the tonic secretion of GH isoforms may be different from that of pulsatile release. The possibility of a nonpituitary source for basal GH production should also be considered. Cells from the immune system, including B cells, macrophages, and helper T cells, produce GH with biochemical and immunogenic properties similar to those of pituitary-derived GH (28, 31, 32). It is conceivable that 20- and 22-kDa GH may be produced by the white blood cells in a proportion different from that by somatotrophs, which could account for dissimilar 20-to-22-kDa ratios in basal (nonpituitary) and pulsatile (pituitary) production. However, there is no conclusive evidence for a detectable contribution of nonpituitary GH sources to circulating GH levels.

Fig. 6. The 20- and 22-kDa GH in acromegaly after saline (A; control) and octreotide administration (B). C: 20-to-22-kDa ratios for the control and treatment studies.

Fig. 7. A: effects of exogenous 22-kDa GH administration in normal subjects on 20- and 22-kDa GH. B: 20-to-22-kDa ratio plotted against the normal range (shaded area), which comprised mean ± SD values derived from the 24-h secretion profile study in Fig. 2. The fall in the 20-to-22-kDa ratio arises from suppression of 20-kDa GH and from the increase in circulating 22-kDa GH in blood after exogenous 22-kDa GH administration.
We observed from deconvolution analysis of spontaneous GH secretion and from the effects of octreotide that the clearance of 20-kDa GH was less than that of 22-kDa GH. Previous studies comparing metabolic clearance rates of the GH isoforms have given conflicting results. It was previously reported that pituitary-derived 20-kDa human GH (hGH) was cleared more slowly than 22-kDa hGH in the rat (4). However, this has not been confirmed by a recent study in humans using recombinant 20-kDa hGH (13) and has not been supported by studies in the guinea pig administered this isoform with human GHBP (9). These discrepancies may be because of the heterologous nature of the experimental models. Our observation from deconvolution analysis of a longer half-life of 20-kDa GH was supported by studies in acromegalic patients in whom 20-kDa GH levels fell less rapidly than 22-kDa GH after octreotide administration.

The clearance of GH is influenced by complexing to GHBP in blood, by receptor-mediated clearance through internalization, and by renal excretion. Because 20-kDa GH has a lower affinity for GHBP than 22-kDa GH (2) and fails to form a 1:1 complex (30), this reduced association may be predicted to enhance plasma clearance. That the converse was observed suggests that other mechanisms involving receptor-mediated cleavage may play a greater role. Wada et al. (29) have reported that the internalization rate of 20-kDa GH is less than that of 22-kDa GH (29). Because 20-kDa GH preferentially binds to somatogenic receptors (7), unlike 22-kDa GH, which binds both somatogenic and lactogenic receptors, it is conceivable that the reduced repertoire of receptor binding may also diminish receptor-mediated clearance. Finally, the clearance of 20-kDa GH could be reduced by a stronger tendency to aggregate than 22-kDa GH (5, 19).

The present studies confirm our previous findings that androgen and oral estrogen increase circulating GH (20, 33, 34) and extend these observations by demonstrating that sex steroids exert similar effects on both 20- and 22-kDa GH. The steroid stimulation may arise from a direct effect on GH expression in somatotrophs or from enhanced secretion by the pituitary. Animal studies show that androgen and estrogen augment pituitary GH content in the rat (8), but these effects have not been demonstrated in humans. Moreover, whether sex steroids regulate alternative splicing of the GH gene to generate the 20-kDa isoform is unknown. Our data showed that the relative proportion of 20- and 22-kDa GH was not changed by sex steroids, suggesting the alternative splicing is unlikely to be affected.

The effects of sex steroids on pituitary secretion of GH are complex. It is well recognized that GH release in response to the GH-releasing hormone arginine and ITT is greater in women than in men (11). Testosterone enhances both GH and IGF-I levels, suggesting that the effect of testosterone is exerted at the level of the pituitary (33). By contrast, oral estrogen increases GH but reduces IGF-I, strongly suggesting that the GH effect occurs indirectly through reduced feedback inhibition (34). Although the mechanisms by which testosterone and estrogen regulate GH secretion appear to be different, their impact on secretion of 20- and 22-kDa GH is the same. These observations support the proposal that the GH isoforms are stored in the same secretory granules in somatotrophs and released together in response to stimuli (3).

Administration of exogenous 22-kDa GH markedly suppressed endogenous secretion of 20-kDa GH and reduced the 20-to-22-kDa ratio. These results are consistent with previous findings of Wu et al. (35) that the ratio of 22-kDa to total GH increased when recombinant GH was administered. Hashimoto et al. (13) have also shown that administration of 20-kDa GH reduced the levels of 22-kDa GH. Taken together, both GH isoforms are potent negative regulators of their own secretion. Exogenous GH may exert the inhibitory effect directly on the hypothalamus-pituitary axis or indirectly by increasing circulating IGF-I, which feeds back to suppress pituitary release of GH. However, the latter mechanism is not supported by the finding that endogenous 20-kDa GH secretion was markedly reduced 2 h after 22-kDa GH administration, whereas a significant increase in the circulating IGF-I level was evident only at 8 h (16).

In conclusion, we provide further evidence that 20-kDa GH is cosecreted and circulates at a constant ratio with 22-kDa GH under various physiological and pharmacological conditions and strong evidence that its circulating half-life is prolonged. Administration of exogenous GH markedly and transiently alters the ratio of 20- to 22-kDa GH, suggesting that determination of the ratio may serve a useful marker for exogenous 22-kDa GH administration.

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