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Proteolytic processing of human growth hormone (GH) by rat tissues *in vitro*: Influence of sex and age

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ABSTRACT. Although a wealth of evidence exists indicating that proteolytic cleavage can enhance the biological activity of the growth hormone (GH) molecule, the mechanisms responsible for the generation of GH fragments are not completely understood. In the present work we investigated the ability of different rat tissues to cleave 22 kDa GH, as well as the influence of sex and age, the two major physiological regulators of GH secretion on this process. Our results show that tissue homogenates obtained from rat liver, skeletal muscle or adipose tissue (three well-documented target organs for the hormone) are able to cleave 22K-GH, while the hormone is resistant to cleavage by rat brain homogenates. This process is rather selective for 22K-GH, since the 20 kDa GH variant exhibits stability to degradation by all tissue homogenates investigated. Moreover, only a minor fraction of 22 kDa GH is cleaved under our experimental conditions, suggesting that GH microheterogeneity within the 22 kDa range may also determine hormone susceptibility. Finally, we also found that 22K-GH processing shows important age-related changes (the greatest intensity observed in 4-day-old pups), while no gender-related differences exist in any of the tissues investigated.

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INTRODUCTION

Human growth hormone (GH) is among the more heterogeneous polypeptide hormones. Several mechanisms contribute to this heterogeneity, including genetic, pretranslational, post-translational and post-secretory events (revised in 1). Genetic heterogeneity stems from the existence of five closely-related genes (GH-N, GH-V, CS-A, CS-B and CS-L) that constitute the GH gene family (2). Among them, only the GH-N gene is expressed after birth, giving rise to pituitary GH (2), although expression of the GH-N gene outside the pituitary also exists (3-5).

Pretranslational heterogeneity is originated by alternative splicing of the pre-mRNA. In the case of pituitary GH, this mechanism produces two major variants, 22K-GH and 20K-GH, with mol wt of about 22,000 and 20,000 dalton respectively. 22K-GH is the most abundant form of pituitary GH, comprising almost 75% of GH immunoreactivity in this organ. 20K-GH accounts for 5-10% of pituitary GH and, except for longer half-life in plasma and diminished insulin-like activity, its biological role remains unknown (1). Other less abundant forms of pituitary GH include post-translationally modified variants such as acetylated, deamidated and glycosilated forms, as well as aggregates and fragments of varying sizes. The physiological role of these post-translational variants is also poorly understood (1).

Finally, GH heterogeneity can also originate as a postsecretory event. Although GH is very stable in plasma, it undergoes receptor-mediated internalisation in many organs, followed, in some instances, by intracellular proteolysis. Although these processes have usually been considered a mechanism of hormone degradation and/or receptor down-regulation (1), evidence also exists suggesting that proteolytic cleavage of GH in peripheral tissues may constitute a mechanism of hormone activation. In this context, GH has been proposed to be a prohormone that would require proteolytic processing to exert its full range of biological activity (6, 7).

In order to gain further insight in the role of peripheral tissues in GH activation, in the present work we investigated the ability of different rat tissue ho-

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mogenates to generate low mol wt fragments of GH *in vitro*, as well as the influence of age and sex, two major regulators of GH secretion (8), in this process.

MATERIALS AND METHODS

Animals

Male and female Wistar rats, bred in our facilities, were used in this study. The animals were housed in an isolated room, under a controlled 14-hour light/10 hour dark cycle (lights on at 07:00 h). Dry food (Panlab, Spain) and tap water were available *ad libitum*. Three-month-old animals were used, except for studies in infant rats in which experiments were also performed in 4-, 12- and 20-day-old pups.

Reagents

Recombinant human GH (22K-GH, Saizen) was obtained from Serono (Madrid, Spain). 20K-GH was kindly provided by the National Hormone and Pituitary Program (NHPP, Torrance, CA). Polyclonal rabbit anti-GH serum was a gift from Prof. J.A.F. Tresguerres (Universidad Complutense, Madrid, Spain). Phenylmethylsulfonylfluoride (PMSF) and Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Sigma Chemical Co (St. Louis, MO). Protein-A-horseradish peroxidase conjugate and the ECL kit were purchased from Amersham-Pharmacia Biotech (Stockholm, Sweden).

Tissue preparation and S9 fraction isolation

The rats were killed and tissue samples from liver, skeletal muscle, adipose tissue, and brain were processed as described elsewhere (9). Briefly, a 2.5% (wt/vol) homogenate of each tissue sample was placed in an ice-cold buffer (50 mM PBS, pH 7.2; 1.15% KCl) and homogenised with a polytron (Kinematica, Basel, Switzerland). The crude homogenates were sedimented at 1000 g for 2 min at 4 C, and the resulting supernatant was centrifuged again at 9000 g for 30 min at 4 C to prepare S9 pellet and S9 supernatant fractions. S9 pellet fractions (which contain large cellular organelles such as lysosomes and mitochondria) were finally resuspended in ice-cold buffer, and their protein concentration was determined with a commercial kit (BioRad protein assay, Bio-Rad Laboratories, Hercules, CA).

In vitro *generation of human GH fragments*

Different amounts of S9 fractions from each tissue were incubated with 22K-GH or with 20K-GH in a total volume of 500 µL, under gentle agitation. Reactions were carried out at 37 C for 45 min, and then resolved in a 15% SDS-PAGE, and electrotransferred onto nitro-cellulose paper (Trans-blot Transfer Medium, BioRad). Membranes were then incubated for 1 h with the anti-GH serum (dilution 1:1000), extensively washed, and incubated for 45 min with the protein A-horseradish peroxidase conjugate. Protein bands were visualised with the ECL Western Blotting kit and photographed (Hyperfilm-ECL, Amersham-Pharmacia Biotech).

Analysis of results

Immunoreactive GH bands were scanned with an imaging densitometer (GS-670, BioRad) and optical density (OD) was determined with the provided software (Molecular Analyst, v 3.0.1, BioRad). Results are presented in arbitrary OD units.

RESULTS

No differences in GH breakdown were found between male and female rat homogenates in any of the tissues investigated (see below). Therefore, pooled data are presented.

Incubation of 22K-GH with S9 fraction isolated from rat liver, skeletal muscle or adipose tissue resulted in the formation of low-weight GH-immunoreactive products. In the three tissues, the major product detected showed a Mr of about 17,000 (Fig. 1A). Peptides of lower mol wt (about 8 kDa) were also found, though they were less evident (Fig. 1A). In contrast, the hormone was resistant to degradation by brain homogenates (Fig. 1A), at least under our experimental conditions, despite higher amounts of S9 fraction (up to 625 µg) were tested. In all cases, the hormone was resistant to degradation by the supernatant obtained during the isolation of the S9 fraction (Fig. 1B), indicating the specific intracellular localisation of the enzymes involved in GH processing. When 22K-GH was denatured (by heating at 95 C for 10 min) before incubation, a clearly different cleavage pattern was observed (Fig. 1C). This finding may indicate that denaturation of the molecule allows the action of other types of enzymatic activity, thus suggesting that preservation of the native structure of the 22K-GH molecule is necessary for specific proteolytic cleavage.

At odds with the results obtained with 22K-GH, we found that when tissue homogenates were incubated with 20K-GH the generation of low mol wt products was absent (Fig. 1D). This finding indicates that the enzymes involved in the breakdown of the 22K-GH are selective for this variant.

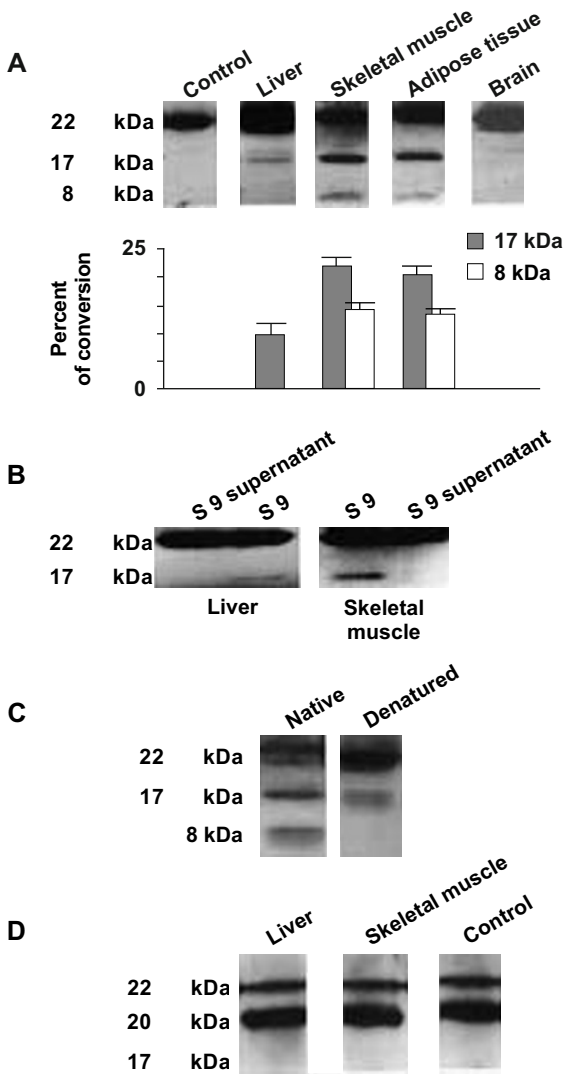

Fig. 1 - *Western blot analysis of hGH fragments generated* in vitro. *Panel A: 22K-GH (100 µg) was incubated with 160 µg of S9 fraction isolated from rat liver, skeletal muscle, adipose tissue or brain. Reactions were resolved by SDS-PAGE, electrotransferred onto nitrocellulose and incubated with anti-22K-GH serum. 22K-GH not exposed to tissue extracts was used as control. In the densitometric evaluation, the percent of conversion (calculated as the 22K-GH/17K-GH ratio or 22K-GH/8K-GH ratio) is shown. Each bar represents the mean±SE of 3 separate experiments. Panel B: Effect of incubation of 22K-GH (100 µg) with the supernatant obtained during the isolation of the S9 pellet fraction from liver or muscle samples. Panel C: One hundred micrograms of 22K-GH were denatured by heating at 95 C for 10 min. prior to incubation with 160 µg of S9 fraction isolated from rat skeletal muscle samples. Although 22K-GH breakdown can be observed under these experimental conditions, the cleavage pattern is different to that observed with the native (control) hormone. Panel D: 20K-GH (100 µg) was incubated with 160 µg of S9 pellet fraction isolated from rat liver or skeletal muscle homogenates. In contrast to the results obtained with the 22 kDa variant, 20K-GH did not undergo proteolytic cleavage in any of the tissues investigated. 20K-GH not exposed to tissue extracts was used as control.*

Although the amount of 17K-GH generated was similar in skeletal muscle and adipose tissue homogenates, it was sharply decreased (about 2-fold) in liver homogenates (see Fig. 1A). To investigate whether this finding could be due to the involvement of different enzymatic activities, we analyzed the effect of two protease inhibitors on the generation of GH fragments. Presence of 0.5 mM TLCK (an inhibitor of tripsin-like proteases) in the incubation did not modify the pattern of generation of low-weight variants in any tissue investigated (data not shown). In contrast, incubation in presence of 1 mM PMSF (a serine-protease inhibitor) impeded 22K-GH cleavage in both skeletal muscle and adipose tissue homogenates, while did not modify it in liver homogenates (Fig. 2).

In the three tissues in which we found 22K-GH breakdown, increasing the amount of S9 fraction present in the reaction induced an initial increase in the generation of low mol wt fragments, followed by a plateau phase. Interestingly, when this maximum of conversion was reached, there was still uncleaved 22K-GH present in the reaction (Fig. 3A). To rule out the possibility of limited capacity of the proteolytic enzymes, we incubated the highest amount of homogenate tested (160 µg) with decreasing amounts of 22K-GH. Interestingly, reducing the amount of 22K-GH present in the reaction did not induce an increase in the intensity of 22K-GH breakdown (despite the enzymatic activity was kept constant). In all cases, only a minor fraction of 22K-GH (always lower than 20%) was converted to the 17K-GH variant (Fig. 3B).

As stated above, results obtained with tissue homogenates from male or female rats were identical in all cases (data not shown). In contrast, 22K-GH breakdown showed important age-related changes (Fig. 4). In both liver and muscle homogenates, the greatest amount of conversion was observed in 4-day-old pups and progressively decreased thereafter. Also in infant rats, 22K-GH was resistant to breakdown by brain homogenates.

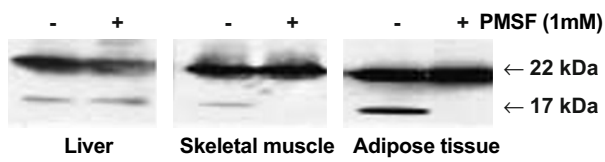

Fig. 2 - *Effect of class-selective protease inhibitors on 22K-GH cleavage. 22K-GH (100 µg) was incubated with 160 µg of S9 fraction isolated from rat liver, skeletal muscle or adipose tissue in presence of 1 mM PMSF (a serine-protease inhibitor), and analyzed by western blot. While PMSF completely blocked 22K-GH breakdown in both skeletal muscle and adipose tissue, it did not induce any effect in liver homogenates.*

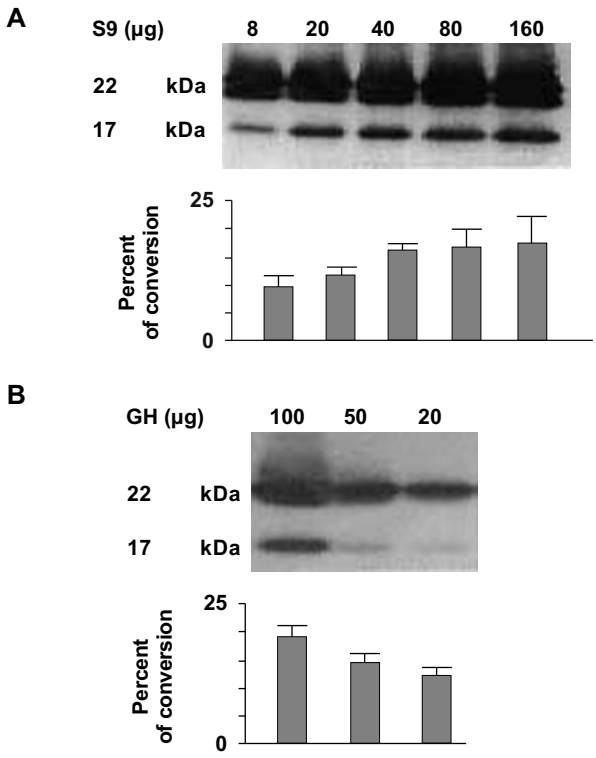

Fig. 3 - *Panel A: Effect of increasing the amount of S9 pellet fraction on 22K-GH breakdown. One hundred micrograms of 22K-GH were incubated with increasing amounts (8, 20, 40, 80 and 160 µg) of rat skeletal muscle homogenates. Increasing the amount of S9 fraction augmented the generation of 17K-GH immunoreactivity until a plateau was reached. Densitometry depicts the percent of conversion (calculated as the 22K-GH/17K-GH ratio). Results are the mean±SE of 3 separate experiments. Panel B: A fixed amount of S9 pellet fraction (160 µg) was incubated with decreasing amounts of 22K-GH. Notice that reducing the amount of 22K-GH present in the reaction (and thus increasing the enzyme/substrate ratio) did not produce an increase in the percent of conversion. At all 22K-GH concentrations tested, only a minor fraction (less than 20%) was cleaved. Densitometry depicts the percent of conversion (calculated as the 22K-GH/17K-GH ratio). Results are the mean±SE of 3 separate experiments, and are expressed in arbitrary OD units.*

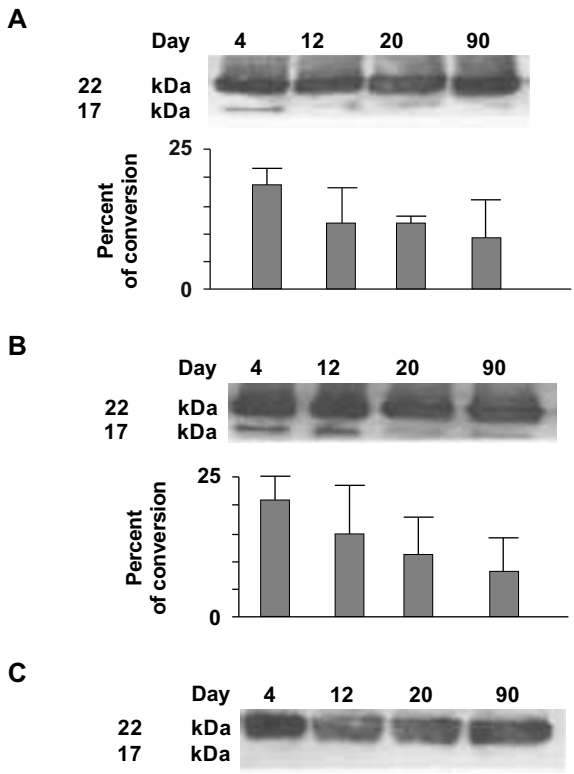

Fig. 4 - *Western blot analysis of age-related changes in the pattern of 22K-GH cleavage. Tissue homogenates (160 µg) were obtained from 4-, 12-, 20- and 90-day old rats. In both liver (panel A) and skeletal muscle (panel B) generation of 17K-GH was highest in 4-day-old rats and decreased thereafter. In the densitometric evaluation, the percent of conversion (calculated as the 22K-GH/17K-GH ratio) in each tissue is shown. Each bar represents the mean±SE of 2 separate experiments. At all ages investigated, 22K-GH was resistant to degradation by rat brain homogenates (panel C).*

DISCUSSION

GH actions are exerted after binding to specific membrane-bound receptors. It is presently known that binding of GH to its receptor induces receptor homodimerization which, in turn, promotes the association and phosphorylation of the receptor-associated tyrosine-kinase JAK-2. Once activated, JAK-2 promotes the recruitment and phosphorylation of a cascade of target molecules, that are responsible for most of the biological actions of the hormone (10, 11).

In addition, GH binding to its receptor also promotes the internalization of the GH-GHR complex, and its subsequent translocation to different cellular compartments, including the cell nucleus. The biological significance of this process is not completely understood, although it has been suggested that GH would regulate the transcription of target genes by acting directly within the cell nucleus (12-14), a mechanism of action also proposed for other peptide hormones (15, 16). Alternatively, internalisation of the GH-GHR complex may provide a mean for proteolytic breakdown of GH, resulting in the generation of low mol wt variants. Although GH proteolytic breakdown of GH in target tissues has been usually considered as part of a mechanism of hormone degradation, it may also constitute a way for hormone activation. This latter possibility is in keeping with the consideration of GH

as a prohormone that would need to be cleaved to low mol wt variants in order to be able to exert its full range of biological actions (6, 7).

Over the years, numerous GH fragments have been synthesized or generated by proteolytic digestion of the hormone in the laboratory, and experimental evidence exists suggesting that the multiple actions of GH are produced by different parts of the molecule (6, 7, 17-19). Moreover, GH fragments can be isolated from the pituitary, although at fairly abundant concentrations; and from plasma, where the 22 kDa form may even not be the predominant GH variant (20, 21). The greater concentration of GH fragments in plasma than in the pituitary suggests that they originate by proteolytic breakdown of the hormone in the periphery. The existence of proteolytic activity toward GH has been reported for several tissues *in vitro*, including thyroid gland, skeletal muscle, liver and adipose tissue (9, 22-24 and the present study). These GH fragments would recirculate and exert their specific actions through binding to the GH receptors or to separate receptors, not identified yet (25); or, alternatively, they could exert their actions by acting directly within the cell nucleus as proposed for the intact hormone.

Nevertheless, the existence of GH breakdown in peripheral tissues *in vitro* does not necessary reflect a mechanism of GH activation *in vivo*, since cellular integrity is not conserved in such experiments, and the hormone may interact *in vitro* with enzymes that would be taken apart *in vivo*. Therefore, one of the major concerns arising from this kind of experiments is whether the existence of GH cleavage in peripheral tissues represents a physiological mechanism of generation of active peptides, or is a first step in a general degradation pathway.

To our knowledge, evidence regarding the physiological importance of GH processing in target tissues is several fold. First, GH fragments with a mol wt similar to those isolated *in vitro* can be found in serum after im administration of the intact hormone (9). Since GH is very stable in plasma, these molecules are thought to be formed in the peripheral tissues following receptor-mediated endocytosis (1). Second, the enzymes involved in GH processing show a rather specific distribution. Inhibitor studies provided initial characterization of at least two different types of enzymatic activity. A chymotripsin-like serine protease activity, localized exclusively to the 9000 g pellet fraction, was found in skeletal muscle and adipose tissue homogenates. A similar enzymatic activity has been previously reported in muscle homogenates, but also in thyroid gland homogenates (9). On the other hand, liver enzymes showed identical subcellular localization, although cleavage pattern obtained was different, and their specific activity toward GH was 2-fold lower than that of muscle or adipose tissue homogenates and was not impeded by the same protease inhibitors. Interestingly, all these tissues (with the only exception of thyroid gland) are important target tissues for GH action. In contrast, GH processing activity was absent in brain, an organ in which GH actions are not so clearly documented to the present.

Thirdly, the specificity of GH cleavage also accounts for the substrate, since only 22K-GH was susceptible to undergo proteolytic cleavage, while the 20 kDa variant was resistant to it. The 20 kDa GH variant is originated in the pituitary by alternative splicing of the pre-mRNA, which results in the loss of part of exon 3. 20K-GH accounts for about 5-10% of pituitary GH, but its biological role remains unknown (1). Although the tertiary structure of 20K-GH has not been completely elucidated, the hormone has a reduced affinity for the GHR, thus suggesting that it has a different conformational folding than the 22 kDa variant. Although we do not have any readily explanation for the resistance to degradation showed by the 20K-GH variant, it is tempting to speculate that a specific protein folding is needed to allow the action of enzymes involved in the breakdown of the 22 kDa GH molecule. In this context, it is also interesting to point out that when the 22 kDa variant was denatured prior to incubation with the S9 pellet fraction, a different cleavage pattern was observed, thus suggesting the involvement of a different enzymatic activity.

This substrate specificity even exists within 22K-GH, since only a fraction of this variant was susceptible to proteolysis by peripheral tissue homogenates, at least under our experimental conditions. Microheterogeneity of pituitary-derived 22K-GH was recognized a long time ago, and to the present, two deamidated, one acetylated and two glycosylated forms have been reported within the 22 kDa range (1, 26, 27). Although the physiological significance of these variants is not yet understood (1), it has been shown that deamidation of pituitary-derived GH results in an increased susceptibility of the 22 kDa variant to metabolization by serine-proteases; a process that can be counteracted by the protease inhibitor PMSF (28). Hence, it is tempting to speculate that the presence of microheterogeneity in 22K-GH of recombinant origin may explain the partial breakdown observed in the present work.

Finally, the fact that GH cleavage shows important age-related changes also supports the physiological relevance of this process. GH levels undergo important changes throughout life in both humans

and laboratory animals. GH levels are high during the intrauterine life, fall sharply after birth, and then remain low until puberty, when an increased GH secretion occurs. After this period GH levels progressively decline again, until old age (revised in 29). Interestingly, we found that the generation of low mol wt GH fragments increased during the early postnatal period, when growth is largely independent from the hormone (30-32). Whether this increased generation of low mol wt GH variants found in rat pups is a mandatory process for GH actions during this period remains to be elucidated. In summary, our data show the existence of an age-dependent and tissue-specific proteolytic activity towards human 22K-GH by rat tissue homogenates. While the exact physiological role of the low weight hGH fragments generated necessitates further investigation, our results suggest that GH breakdown in the target tissues may be one of the mechanisms underlying the actions of GH.

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REFERENCES

1. Baumann G.
Growth hormone heterogeneity: genes, isohormones, variants and binding proteins.
Endocr. Rev. 1991, *121:* 424-449.
2. Chen L.Y., Liao Y.C., Smith D.H., Barrera-Saldaña H.A., Gelinas R.E., Seeburg P.H.
The human growth hormone locus: nucleotide sequence, biology and evolution.
Genomics 1989, *4:* 479-497.
3. Weigent D.A., Baxer J.B., Wear W.E., Smith L.R., Bost K.L., Blalock J.E.
Production of immunoreactive growth hormone by monoclonal leukocytes.
FASEB J. 1988, *2:* 2812-2818
4. Mol J.A., Henzen-Logmans S.C., Hageman P.H., Misdrop W., Balnkenstein M.A., Rijnberk A.
Expression of the gene encoding human growth hormone in the human mammary gland.
J. Clin. Endocrinol. Metab. 1995, *80:* 3094-3096.
5. Costoya J.A., Vidal A., García-Barros M., Arce V., Devesa J.
Expression of the human growth hormone gene in proliferating and differentiated HL-60 cells.
Exp. Cell Res. 1996, *228:* 164-167.
6. Mittra I.
Somatomedins and proteolytic bioactivation of prolactin and growth hormone.
Cell 1984, *38:* 347-348.
7. Lewis U.J.
Variants of growth hormone and prolactin and their posttranslational modifications.
Annu. Rev. Physiol. 1984, *46:* 33-42.
8. Devesa J., Lima L., Tresguerres J.A.F.
Neuroendocrine control of growth hormone secretion in humans.
Trends Endocrinol. Metab. 1992, *3:* 175-183.
9. Wroblewski V.J., Masnyk M., Becker G.W.
Proteolytic cleavage of human growth hormone (hGH) by rat tissues *in vitro*: influence on the kinetics of exogenously adiministered GH.
Endocrinology 1991, *129:* 465-473.
10. Carter-Su C., Schwartz J., Smit L.S.
Molecular mechanism of growth hormone action.
Annu. Rev. Physiol. 1996, *58:* 187-207.
11. Costoya J.A., Finidori J., Moutoussamy S., Senaris R., Devesa J., Arce V.M.
Activation of growth hormone receptor delivers an antiapoptotic signal: evidence for a role of Akt in this pathway.
Endocrinology 1999, *140:* 5937-5943.
12. Lobie P.E., Wood T.J.J., Chen C.M., Waters M.J., Norstedt G.
Nuclear translocation and anchorage of the growth hormone receptor.
J. Biol. Chem. 1994, *269:* 31735-31746.
13. Mertani H.C., Waters M.J., Morel G.
Cellular trafficking of exogenous growth hormone in dwarf rat pituitary.
Neuroendocrinology 1996, *63:* 257-268.
14. Lobie P.E., Ronsin B., Silvennoinen O., Haldosen L.-A., Norstedt G., Morel G.
Constitutive nuclear localization of Janus kinases 1 and 2.
Endocrinology 1996, *137:* 4037-4045.
15. Henderson J.E.
Nuclear targeting of secretory proteins.
Mol. Cell. Endocrinol. 1997, *129:* 1-5.
16. Mertani H.C., Morel G., Lobie P.E.
Cytoplasmic and nuclear cytokine receptor complexes.
Vitam. Horm. 1999, *57:* 79-121
17. Lewis U.J., Lewis M.J., Salem M.A.M., Staten N.R., Galosy S.S., Krivi G.G.
A recombinant-DNA-derived modification of human growth hormone (hGH44-191) with enhanced diabetogenic activity.
Mol. Cell. Endocrinol. 1991, *78:* 45-54.
18. Jeoung D.-I., Allen D.L., Guller S., Yen V., Sonnenberg M.
Mitogenic and receptor activities of human growth hormone 108-129.
J. Biol. Chem. 1993, *268:* 22520-22524.
19. Sonnenberg M., Guller S., Wu K.-Y., Corin R.E., Allen D.L.
Activity of growth hormone peptides bGH 96-133 and hGH 95-133 in 3T3-F442A cells.
Mol. Cell. Endocrinol. 1994, *99:* 193-199.

20. Lewis U.J., Ling N., Haro L.S.
Variants forms of human growth hormone in serum.
Acta Paediatr. 1994, *399* (Suppl.): 29-31.

21. Sinha Y.N., Jacobsen B.P.
Human growth hormone (hGH)-(44-191), a reportedly diabetogenic fragment of hGH, circulates in human blood: measurement by radioimmunoassay.
J Clin. Endocrinol. Metab. 1994, *78:* 1411-1418.

22. Schepper J.M., Hughes E.P., Postel-Vinay M.C., Hughes J.P.
Cleavage of growth hormone by rabbit liver plasmalemma enhances binding.
J. Biol. Chem. 1984, *259:* 12945-12948.

23. Husman B., Gustafsson J.-A., Anderson G.
Receptor-mediated endocytosis and degradation of bovine growth hormone in rat liver.
Mol. Cell. Endocrinol. 1988, *59:* 13-25.

24. Wroblewski V.J., Kaiser R.E., Becker G.W.
Proteolysis of human growth hormone by rat thyroid gland in vitro: application of electrospray mass spectrometry and N-terminal sequencing to elucidate a metabolic pathway.
Pharm. Res. 1993, *10:* 1106-1114.

25. Rowilson S.W., Waters M.J., Lewis U.J., Barnard R.
Human growth hormone fragments 1-43 and 44-191: *in vitro* somatogenic activity and receptor binding characteristics in human nonprimate systems.
Endocrinology 1996, *137:* 90-95.

26. Díaz M.J., Dominguez F., Haro L.S., Ling N., Devesa J.
A 12-kilodalton N-glycosylated growth hormone-related peptide is present in human pituitary extracts.
J. Clin. Endocrinol. Metab. 1993, *77:* 134-138.

27. Haro L.S., Lewis U.J., García M., Bustamante J., Martínez A.O., Ling N.C.
Glycosylated human growth hormone: a novel 24 kDa hGH-N variant.
Biochem. Biophys. Res. Commun. 1996, *228:* 549-556.

28. Lewis U.J., Singh R.N.P., Bonewald L.F., Seavey B.K.
Altered proteolytic cleavage of human growth hormone as a result of deamidation.
J. Biol. Chem. 1984, *259:* 11645-11650.

29. Arce V., Lima L., Devesa J.
Growth hormone and ageing.
Endocrinologia 1991, *38:* 254-260.

30. Glasscock G.F., Nicoll C.S.
Hormonal control of growth in the infant rat.
Endocrinology 1981, *109:* 176-180.

31. Cella S.G., Locatelli V., Mennini T., Zanini A., Bendotti C., Forloni G.L., Fumagalli G., Arce V.M., De Gennaro Colonna V., Wehrenberg W.B., Müller E.E.
Deprivation of growth hormone-releasing hormone early in the rat's neonatal life permanently affects somatotropic function.
Endocrinology 1990, *127:* 1625-1634.

32. Price W.A., Stiles A.D.
Determinants of fetal and neonatal growth.
Curr. Opin. Pediatr. 1994, *6:* 135-141.