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Growth hormone, but not insulin-like growth factor I, induces a serum protease activity for insulin-like growth factor binding protein-3 in hypophysectomized rats in vivo

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Insulin-like growth factor binding proteins (IGFBPs) modulate IGF action. Proteolytic cleavage of IGFBPs yields lower molecular forms with reduced ability to bind IGFs, thereby increasing IGF bioavailability. In serum from normal adult rats, we found a proteolytic activity for IGFBP-3, presumably a cation-dependent serine protease. It is lacking in serum from hypophysectomized rats and restored by infusion of growth hormone (GH), but not IGF I. Thus, IGF I does not appear to mediate the GH effect on IGFBP-3 proteolysis. Rather, GH seems to modulate IGF action indirectly via alteration of IGFBP-3 structure.

Growth hormone; Insulin-like growth factor; Insulin-like growth factor binding protein; Proteolysis; Insulin-like growth factor bioavailability

1. INTRODUCTION

Insulin-like growth factor binding proteins comprise a family of 6 homologous proteins (IGFBP-1 to -6) found both in the circulation and in tissues [1,2]. IGFBP-3 is the most abundant binding protein in serum of adult rats and humans. After binding IGF, it aggregates with an acid-labile subunit (ALS) to form an M_r 150,000 ternary complex [3–5]. About 80% of serum IGF I and II are found in this complex. In the hypophysectomized rat, both IGF I and growth hormone are capable of increasing serum levels of IGFBP-3, whereas the formation of the ternary serum complex is propagated only by GH [6]. IGFBPs increase the half-life of IGFs and modulate IGF action in an inhibitory [7–9] or stimulatory [10–12] fashion. IGFBPs are substrates for proteases which appear in the serum under several physiological and pathological conditions: proteolytic degradation of IGFBP-3 (and other IGFBPs) was first observed in human and rat pregnancy [13–15]. IGFBP-3 degrading activity is also detected in serum from patients with prostatic cancer [16] and from patients after elective surgery [17]. Proteolysis of IGFBPs yields truncated molecules with reduced ability to bind IGFs, thereby possibly increasing IGF bioavailability. Recently, prostate-specific antigen [18] and plasmin [19] have been identified as IGFBP proteases. Recent studies also suggest that GH and/or IGF regulate IGFBP proteases: upon electrophoresis of serum from acromegalic patients, a band probably representing truncated IGFBP-3 can be detected [20], and IGFBP-4 proteolytic activity in fibroblast conditioned media depends on the presence of IGF [21,22]. We investigated the role of GH and IGF I in the proteolytic degradation of IGFBP-3. Hypox rats were used as an in vivo model.

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Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; rh, recombinant human; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; hypox, hypophysectomized.

2. MATERIALS AND METHODS

2.1. *Animals*

5- to 6-week-old male Tif RAI rats were hypophysectomized (courtesy of K. Müller and M. Cortesi, Ciba-Geigy, Basel). Alzet osmotic minipumps (model 2001, Alza, Palo Alto, CA) were implanted subcutaneously 2 weeks after hypophysectomy in 15 animals whose body weight had not increased more than 2 g/week. Five animals each were infused with vehicle (0.9% NaCl/0.1 M acetic acid), with 200 mU/rat × day of rhGH (Nordisk, Gentofte, Denmark) dissolved in 0.9% NaCl, or with 300 μg/rat × day of rhIGF I (gift of Drs. K. Müller and W. Märki, Ciba Geigy, Basel) dissolved in 0.9% NaCl/0.1 M acetic acid. After 6 days, the animals were anesthetized with 0.3 ml/animal of Innovar Vet (Pitman-Moore, Washington Crossing, NJ) and bled by aortic puncture. Aspirated blood was kept on ice for 30 min, centrifuged, and serum was collected. Serum from untreated normal age-matched animals was obtained by the same procedure. Sera from the treated and 3 untreated animals and from 5 healthy non-pregnant and 5 term pregnant women were pooled and stored at −20°C until used.

2.2. *Western ligand analysis*

IGFBPs were identified by the method of Hossenlopp et al. [23] with slight modifications [6]. Briefly, 2 μl of serum was subjected to 15% SDS-PAGE under non-reducing conditions. Separated proteins, including a molecular weight marker (Rainbow Marker, Amersham),

were electroblotted onto a nitrocellulose membrane. The membrane was incubated with approx. 4×10^6 cpm of [^{125}I]rhIGF II, washed, dried, and exposed to an X-ray film.

2.3. *IGFBP-3 proteolysis assays*

The method of Lamson et al. [24] was used with minor modifications. Glycosylated [^{125}I]rhIGFBP-3 (2 glycosylation variants with M_r of 42,000/45,000; approx. specific radioactivity 160 μCi/mg) was obtained from Anawa Co., Dübendorf, Switzerland. 50,000 cpm [^{125}I]rhIGFBP-3 (final concentration approx. 0.3 nM) was incubated with 5 μl of serum in a total volume of 20 μl of phosphate- buffered saline (PBS), pH 7.4, containing 1 mM of $CaCl_2$. Additional agents were added as indicated. rhIGFBP-3 was kindly provided by Drs. A. Sommer and C. Maack, Celtrix, Santa Clara, CA. rhIGFBP-4, -5, and -6 were expressed in yeast and purified as described [25]. After 8 h at 37°C, reactions were stopped by adding 20 μl of non-reducing sample buffer. Mixtures were subjected to 15% SDS-PAGE (constant voltage, 35–65 V overnight). Gels were dried in a vacuum drier (Bio- Rad, Richmond, CA), and bands were visualized by autoradiography.

2.4. *Densitometry*

The relative optical densities (OD) of autoradiographic bands were measured with a densitometer (Model 620, Bio-Rad). To estimate the extent of [^{125}I]rhIGFBP-3 degradation, the areas under the OD peaks from intact and degraded IGFBP-3 were integrated with a computer program (1 D Analyst II, Bio-Rad). The integrated peaks of the relative ODs from cleavage product bands were expressed as percentage of the total OD of all bands in each lane.

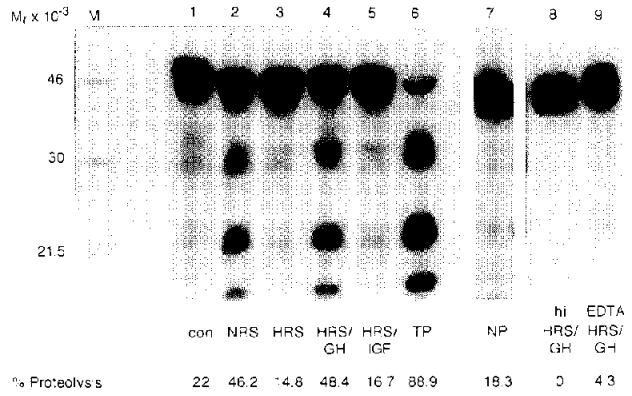

Fig. 2. SDS-PAGE of [^{125}I]rhIGFBP-3 after incubation for 8 h at 37°C with various serum pools. M, molecular weight marker; Lane 1, buffer control (con); lane 2, normal rat serum (NRS); lane 3, serum from hypox rats treated with vehicle (HRS); lane 4, serum from rhGH-treated hypox rats (HRS/GH); lane 5, serum from rhIGF I-treated hypox rats (HRS/IGF); lane 6, term pregnancy serum (TP); lane 7, non-pregnancy serum (NP); lane 8, heat-inactivated serum from rhGH-treated hypox rats (hi); lane 9, serum from rhGH-treated hypox rats in the presence of 5 mM EDTA. % Proteolysis (bottom line): the integrated peaks of the relative optical densities in the cleavage product bands are given as percentage of the total optical density in each lane.

3. RESULTS

3.1. *Western ligand analysis of rat sera*

In hypox rats, serum levels of IGFBP-3 are low (Fig. 1). Both rhGH and rhIGF I increase IGFBP-3 levels. Densitometric quantitation of the bands shows a significantly greater increase in IGFBP-3 levels in GH- as compared to IGF I-infused animals.

3.2. *Analysis of IGFBP-3 proteolytic activity in rat sera*

Fig. 2 shows the patterns of intact and degraded [^{125}I]rhIGFBP-3 obtained by incubation with various serum pools. Incubation with serum from normal adult rats yields three distinct bands of degraded IGFBP-3 with approx. M_r of 30,000, 22,000, and 17,000 besides the M_r 42,000/45,000 doublet of intact IGFBP-3. The degradation products are barely detectable after incubation with serum from vehicle- or IGF I-treated hypox

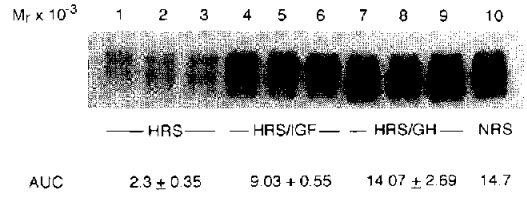

Fig. 1. Western ligand blot from individual hypox and treated hypox rat sera. Each lane shows the IGFBP-3 signal ('triplet'), representing 3 different glycosylation variants. Lanes 1–3, vehicle-treated hypox rats (HRS); lanes 4–6, rhIGF I-treated hypox rats (HRS/IGF); lanes 7–9, rhGH-treated hypox rats (HRS/GH); lane 10, serum pooled from 3 normal adult rats (NRS). AUC denominates the area under the relative OD peaks obtained by densitometric scanning of each triplet (mean ± S.D.).

rats, but they reappear after incubation with serum from GH-treated hypox rats. Degradation is prevented by boiling the serum for 5 min prior to the incubation or by the addition of 5 mM EDTA to the incubation mixture. The same M_r cleavage products are found after incubation of [^{125}I]rhIGFBP-3 with serum from term pregnant women. However, degradation is more pronounced than after incubation with rat sera. Human non-pregnancy serum contains no IGFBP-3 proteolytic activity. Cleavage of radiolabeled rhIGFBP-3 by serum from GH-treated hypox rats is completely abolished by 10 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM benzamidine, two serine protease inhibitors, but not by 10 μM leupeptine, a serine/cysteine protease inhibitor (Fig. 3). The above concentrations of PMSF and benzamidine effectively inhibit proteases in sera [24] or fibroblast conditioned media [21]. Proteolytic cleavage is detectable after 1 h incubation. It reaches its maximum after 8 h. No further degradation appears after 18 h. When radiolabeled rhIGFBP-3 is incubated at pH 3, the three typical degradation bands are no longer detectable (data not shown).

To test the specificity of the IGFBP-3-degrading activity, reactions were carried out in the presence and absence of various concentrations of unlabeled rhIGFBPs (Fig. 4). Proteolysis of [^{125}I]rhIGFBP-3 by normal rat serum is inhibited completely by 1 μM rhIGFBP-3 and nearly completely by 1 μM rhIGFBP-4 and 5. In contrast, rhIGFBP-6 does not inhibit degradation (upper panel). Identical results are found with serum from rhGH-treated hypox rats (not shown). Proteolysis by human term pregnancy serum is competed

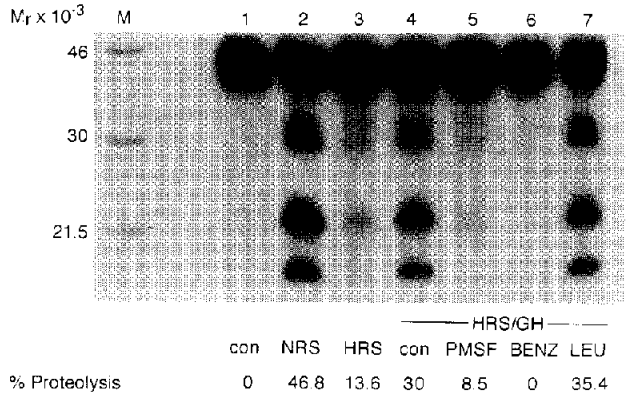

Fig. 3. SDS-PAGE of [^{125}I]rhIGFBP-3 after incubation for 8 h with various serum pools. M: molecular weight marker. Lane 1, buffer control (con); lane 2, serum from normal rats (NRS); lane 3, serum from hypox rats treated with vehicle (HRS); lanes 4–7, serum from rhGH-treated hypox rats (HRS/GH) in the absence (con) or presence of 10 mM PMSF; 50 mM benzamidine (BENZ) and 10 μM leupeptine (LEU). % Proteolysis (bottom line): the percentage of proteolysis for each lane, determined as described in Fig. 2.

by 1 μM rhIGFBP-3, but not by 1 μM rhIGFBP-4, -5, and -6 (lower panel). rhIGFBPs at 1 nM and bovine serum albumin in concentrations of up to 10 μM (not shown) do not inhibit degradation.

4. DISCUSSION

Normal adult rat serum contains an enzymatic activity that cleaves [^{125}I]rhIGFBP-3 into three proteolytic fragments. This serum activity is lacking in hypox rats and restored by rhGH-, but not rhIGF I infusion. Partial characterization of the activity suggests that it is a cation-dependent serine protease. However, we cannot conclude from our experiments whether GH exerts its effects on this activity by inducing the protease or by suppressing a putative enzyme inhibitor.

A cation-dependent serine protease has been suggested to degrade IGFBP-3 in human term pregnancy serum [13,24]. However, IGFBP-3 proteolysis by human and rat serum differ in several aspects: (i) whereas the degradation of radiolabeled IGFBP-3 by human term pregnancy serum is almost complete, it is incomplete with serum from normal or GH-treated hypox rats. A possible explanation for this is inhibition of the enzyme by cleavage products, although we have no data to support this hypothesis; (ii) in contrast to rat serum, human non-pregnancy serum contains no proteolytic activity; (iii) the activity found in human serum appears to be more specific than that in rat serum, since it can be competed only by an excess of unlabeled IGFBP-3, whereas other IGFBPs also inhibit degradation by the rat serum protease. We conclude from these findings that the enzymes responsible for the degradation of IGFBP-3 by human term pregnancy and adult rat serum are different.

The absence of IGFBP-3-degrading activity in serum from IGF I-treated hypox rats could result from higher serum levels of IGFBP-3 in IGF I- as compared to GH-treated animals. High concentrations of IGFBP-3 would compete for the protease in our assay. However, as evidenced by the ligand analysis of the rat sera (Fig. 1), serum levels of IGFBP-3 are even higher in the GH- than in the IGF I-treated hypox rats. Thus, the proteolytic serum activity is induced by GH, and its induction does not appear to be mediated by IGF I.

Disappearance of the M_r 150,000 ternary complex with a concomitant shift of IGF to an M_r 40,000 IGF/IGFBP complex and truncation of IGFBPs are two independent mechanisms which can modulate IGF bioavailability. For example, hypox rats lack the M_r 150,000 ternary serum complex [6] and carry all serum IGF in the M_r 40,000 complex which, in contrast to the former, passes the capillaries. In these animals, intravenously injected IGF has a much shorter half-life than in normal rats [8] and the animals are more susceptible to acute insulin-like effects of IGF I and II than normal rats [8]. Other studies suggest that truncated IGFBPs bind IGFs with lower affinity than the intact forms [14,26,27], although this has been challenged [28]. Thus,

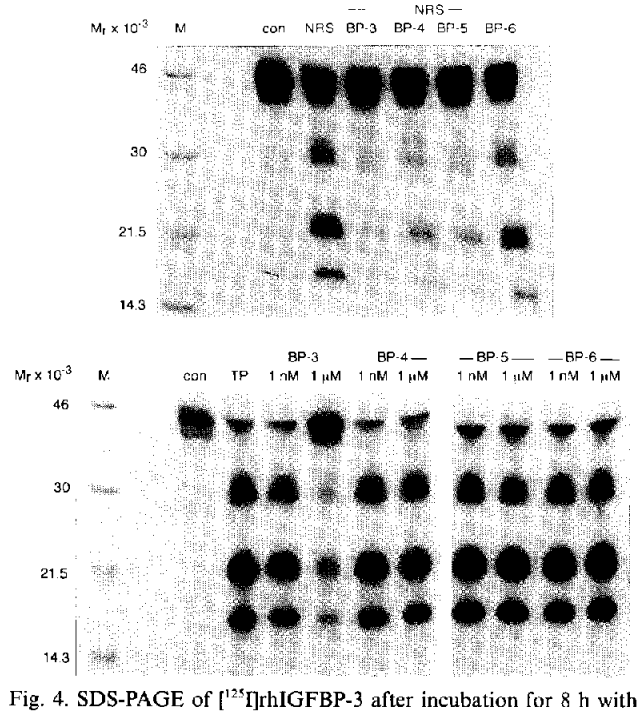

Fig. 4. SDS-PAGE of [^{125}I]rhIGFBP-3 after incubation for 8 h with rat and human serum pools in the absence and presence of excess unlabeled rhIGFBPs. Upper panel: effects 1 μM rhBP-3, -4, -5 and -6 on proteolytic degradation of [^{125}I]rhIGFBP-3 by normal rat serum (NRS). M, molecular weight marker; Con, buffer control. If the reactions were done with serum from rhGH-treated hypox rats, an identical pattern of proteolytic bands was observed (not shown). Lower panel: effects of various concentrations of rhIGFBPs -3, -4, -5 and -6 on proteolytic degradation of radiolabeled rhIGFBP-3 by human term pregnancy serum. M and con, molecular weight marker and buffer control, respectively.

intravenously injected IGF I has a longer serum elimination half-life in non-pregnant as compared to pregnant rats, in which intact IGFBP-3 is dramatically reduced [15]. Our data provide evidence that proteolysis of IGFBP-3 is under hormonal control. Thus, GH may augment concentrations of IGFs both systemically and locally: (i) directly by inducing all components of the ternary serum complex (IGF, IGFBP-3, and the acid-labile subunit, ALS) and increasing IGF synthesis in certain tissues; (ii) indirectly by inducing an IGFBP-3 proteolytic activity, thereby facilitating the dissociation of IGF from IGFBP-3, which is a constituent of the ternary complex both in its intact and truncated form [26,29,30].

Our study does not address the question to which extent ALS may protect IGFBP-3 from proteolysis. Radiolabeled IGFBP-3 is incorporated into the ternary serum complex [31]; however, it is unknown if degradation occurs before or after complex formation. We suspect that ALS has little, if any, role in preventing enzymatic degradation of IGFBP-3: although ALS is present in

GH-, but not in IGF I-infused hypox rats [6], degradation occurs only in GH-treated animals. Furthermore, in humans, concentrations of immunoreactive ALS increase linearly during pregnancy [29], whereas concomitantly most of the circulating IGFBP-3 is degraded to lower molecular weight forms.

In conclusion, an IGFBP-3 proteolytic serum activity, presumably a cation-dependent serine protease distinct from that in human term pregnancy serum, correlates with the GH-status of adult rats. Its induction by GH does not appear to be mediated by IGF I. Thus, GH may modulate IGF actions by a mechanism distinct from effects on IGF or IGF-receptor concentrations.

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