Dose–response effects of a new growth hormone receptor antagonist (B2036-PEG) on circulating, hepatic and renal expression of the growth hormone/insulin-like growth factor system in adult mice

J W van Neck, N F J Dits, V Cingel, I A Hoppenbrouwers, S L S Drop and **A Flyvbjerg**[1]

Laboratory of Pediatrics, Subdivision of Molecular Endocrinology, Erasmus University/AZR-Sophia, 3015 GD Rotterdam, The Netherlands

[1]Medical Research Laboratories, Institute of Experimental Clinical Research, Aarhus University Hospital, DK-8000 Aarhus C, Denmark

(Requests for offprints should be addressed to J W van Neck, Laboratory of Pediatrics, Room Ee1500, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands)

Abstract

The effects of growth hormone (GH) in regulating the expression of the hepatic and renal GH and insulin-like growth factor (IGF) system were studied by administering a novel GH receptor antagonist (GHRA) (B2036-PEG) at different doses (0, 1·25, 2·5, 5 and 10 mg/kg/day) to mice for 7 days. No differences were observed in the groups with respect to body weight, food consumption or blood glucose. However, a dose-dependent decrease was observed in circulating IGF-I levels and in hepatic and renal IGF-I levels at the highest doses. In contrast, in the 5 and 10 mg/kg/day GHRA groups, circulating and hepatic transcriptional IGF binding protein-3 (IGFBP-3) levels were not modified, likely resulting in a significantly decreased IGF-I/IGFBP-3 ratio. Hepatic GH receptor (GHR) and GH binding protein (GHBP) mRNA levels increased significantly in all GHRA dosage groups. Endogenous circulatory GH levels increased significantly in the 2·5 and 5 mg/kg/day GHRA groups. Remarkably, increased circulating IGFBP-4 and hepatic IGFBP-4 mRNA levels were observed in all GHRA administration groups. Renal GHR and GHBP mRNA levels were not modified by GHRA administration at the highest doses. Also, renal IGFBP-3 mRNA levels remained unchanged in most GHRA administration groups, whereas IGFBP-1, -4 and -5 mRNA levels were significantly increased in the 5 and 10 mg/kg/day GHRA administration groups.

In conclusion, the effects of a specific GHR blockade on circulating, hepatic and renal GH/IGF axis reported here, may prove useful in the future clinical use of GHRAs.

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Introduction

The growth hormone (GH)/insulin-like growth factor (IGF) axis constitutes a complex system of peptides in the circulation, extracellular space and in most tissues. The classical endocrine effect of pituitary-secreted GH is the induction of IGF-I synthesis in various organs. The liver is believed to be the major source of circulating IGF-I which, in turn, is a negative feedback signal on GH secretion (Namba *et al.* 1989, Yamasaki *et al.* 1991).

IGF-I is a member of the IGF system that also consists of IGF-II, two types of IGF receptors and six different IGF binding proteins (IGFBPs) (Kelley *et al.* 1996). In addition, four IGFBP-related proteins have been described (Baxter *et al.* 1998).

The GH/IGF axis is important for normal cell growth and differentiation, but has also been shown to be involved in pathophysiological processes. Recently, a series of GH antagonists has been developed that specifically block the GH receptor (GHRAs) (Chen *et al.* 1991*b*, 1994). These GHRAs may be used as intervention in various diseases where GH action is known to play a pathophysiological role (e.g. acromegaly) (Trainer *et al.* 2000) or is suspected of being involved in organ specific damage (e.g. diabetic kidney disease) (Flyvbjerg *et al.* 1999*a*, Segev *et al.* 1999).

In order to get more detailed information about the effects of specific GH receptor (GHR) blockade on circulating and local components of the GH/IGF axis, a GHRA with an enhanced affinity for the human GHR (B2036-PEG) was studied at different doses in mice. The effects of GHRA administration on the expression of the GH/IGF components were investigated in the liver, thought to be the major regulator of circulating IGFs, and in the kidney, as various kidney diseases may be potential targets for GHRA treatment.

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Materials and Methods

Animals

Adult female Balb/C(a) mice with initial body weights of 16·4 ± 0·2 g (Bomholtgaard, Ry, Denmark) were used in the study. The animals were housed 7–8 per cage on white special spanwall bedding. They were fed a standard laboratory diet (Altromin No. 1310, Altromin, Lage, Germany), had free access to water and were kept at constant temperature (21 ± 1 °C), humidity (55 ± 5%) and under a 12-h light, 12-h darkness cycle (lights on from 0700 h to 1900 h). The study complied with Danish regulations for the care and use of laboratory animals.

Study design

The mice were randomly allocated into five groups of eight animals: (1) animals injected on days 0, 2, 4 and 6 with 0·154 mol/l NaCl, the vehicle for the other treatments (control group); (2) animals injected on days 0, 2, 4 and 6 with 2·5 mg/kg human GHR antagonist (GHRA): B2036-PEG (1·25 mg/kg/day GHRA group); (3) animals injected on days 0, 2, 4 and 6 with 5 mg/kg GHRA (2·5 mg/kg/day GHRA group); (4) animals injected on days 0, 2, 4 and 6 with 10 mg/kg GHRA (5 mg/kg/day GHRA group); (5) animals injected on days 0, 2, 4 and 6 with 20 mg/kg GHRA (10 mg/kg/day GHRA group).

Human GHRA (B2036-PEG) was kindly provided by Sensus Drug Development Corporation (Austin, TX, USA) (Fuh *et al.* 1992). The molecule has a modification in the first GHR binding site resulting in a 30–50 times increased affinity for the human GHR. In addition, in the second GHR binding site, amino acid 120 is modified preventing GHR dimerization (Chen *et al.* 1991*c*). GHRA was provided in a pegylated formula to warrant prolonged biological action of the molecule (Clark *et al.* 1996). The GHRA was dissolved in 0·154 mol/l NaCl and injected s.c. on days 0, 2, 4, and 6 in an injection volume of 0·5 ml. The animals were weighed and their food consumption and blood glucose was determined on days 0, 2, 4, and 7. On day 7, the animals were anesthetized with pentobarbital (50 mg/kg i.p.) and non fasting blood samples were collected exactly 5 min later from the retrobulbar plexus through heparinized capillary tubes under light ether anesthesia. The serum samples were kept at − 80 °C for later analysis. Whole liver and the left kidney were removed and snap frozen in liquid nitrogen.

Blood glucose

Blood glucose was measured in unanesthetized animals in tail vein blood by Haemoglucotest 1–44 and Reflolux II reflectance meter (Boehringer-Mannheim, Mannheim, Germany).

Serum GH and IGF-I, and hepatic and kidney IGF-I determinations

Serum GH was measured by a radioimmunoassay (RIA) as described previously (Flyvbjerg *et al.* 1999*a*). Potential cross-reactivity of the GHRA (B2036-PEG) with the rodent specific GH assay was precluded, as addition in the assay of the GHRA at multiple concentrations (over a range from 0·5–5000 μg/l) did not reveal any significant binding. Serum IGF-I was measured after extraction using acid-ethanol (Flyvbjerg *et al.* 1999*a*). The intra-assay and interassay coefficients of variation (CV) were 5% and 10% respectively. Tissue extraction of renal and hepatic IGF-I was performed according to D'Ercole *et al.* (1984) and corrected for the contribution of entrapped serum IGF-I (Flyvbjerg *et al.* 1992*b*).

Serum IGFBPs

SDS-PAGE and Western ligand blotting (WLB) analysis were executed according to the method of Hossenlopp *et al.* (1986) as described previously (Flyvbjerg *et al.* 1992*a*).

Gene expression of GH and IGF system (mRNA) in tissues

Gene expression of IGFBP-1 to -6 (mRNA) was measured by Northern blot analysis. Total RNA was extracted from kidney and liver samples by the guanidinium thiocyanate method (Chomczynski & Sacchi 1987). Glyoxylated-RNA samples were electrophoresed in 1% agarose gels submerged in 10 mM sodium phosphate pH 7·2 and transferred to nylon membranes (Hybond N^+, Amersham, 's Hertogenbosch, The Netherlands). Filters were hybridized with 1–2 × 10^6 c.p.m. per ml ^{32}P-labeled cDNA fragments encoding for each of the six mouse IGFBPs (Schuller *et al.* 1994), mouse IGF-I and -II (kindly provided by Dr G I Bell, Howard Hughes Institute, Chicago, IL, USA), rat IGF-I receptor (kindly provided by Dr H Werner and Dr D LeRoith, National Institutes of Health, Bethesda, MD, USA), GHR, GHBP (Mathews *et al.* 1989) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 65 °C according to the method of Church & Gilbert (1984).

Quantification

Autoradiographs of WLBs were scanned using a laser densitometer (Shimadzu model CS 90001 PC, Shimadzu Europe GmbH, Duisburg, Germany) and the relative densities of the bands expressed in pixels. Northern blots were scanned on a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software. All measured mRNA results were expressed relative to GAPDH mRNA levels.

Statistical analysis

Data were examined for distribution, variance homogeneity (F-test) and analyzed by one-way analysis of variance followed by pair-wise comparisons with the least-significant difference method. All data are expressed as means ± S.E.M., with *n* indicating the number of mice studied. *P* values less than 0·05 are considered significant.

Results

Body weight and organ weights

The body weights of the different study groups at the beginning (day 0) and end (day 7) of the experimental period were determined. The mean body weights on day 0 were 16·4 ± 0·2 g with no differences between the groups. At day 7, mean body weight increased to 18·9 ± 0·3 g in control animals with no differences in the different groups (data not shown). At the end of the experimental period, liver and kidney weights were determined. Liver weight and the weight of the kidneys in the control group were 926 ± 35 mg and 237 ± 3 mg respectively. The GHRA administration regimen demonstrated no significant effects on the weight of liver or kidneys (data not shown).

Food consumption and blood glucose

Food consumption over 24 h was measured during the study period on a group basis. Mean food intake per mouse of the control group was 4·2 ± 0·1 g per 24 h. Food consumption did not differ significantly during the experimental period in any of the groups (data not shown). Blood glucose values were measured in all animals at the end of the experimental period and did not change significantly in any of the experimental groups (data not shown).

Serum IGF-I and GH

At the end of the study period serum IGF-I levels were determined in all groups (Fig. 1A). Compared with placebo control levels, in the 1·25 mg GHRA group serum IGF-I levels were unchanged, whereas in the 2·5, 5 and 10 mg GHRA groups serum IGF-I levels were reduced to 75% ($P=0·006$), 70% ($P=0·001$) and 51% ($P<0·001$) of control values respectively (Fig. 1A). Compared with the other groups significant and dose-dependent decreases in circulatory IGF-I levels were observed (1·25 mg versus 2·5 mg GHRA group, $P<0·008$; 5 mg versus 10 mg GHRA group, $P=0·011$). Figure 1B shows serum GH levels in the five experimental groups by the end of the study (day 7). It has been demonstrated previously that barbital anesthesia induces a marked rise in GH levels that lasts for up to 90 min (Takahashi *et al.* 1971). Therefore, the endogenous GH levels given in Fig. 1B are stimulated values. Increased serum GH levels amounting to 183% ($P=0·003$) and 145% ($P=0·01$) of control values were seen in the 2·5 mg GHRA and 5 mg GHRA groups respectively.

Serum IGFBPs

Using WLB, four distinct bands were obtained. A double band at 38–42 kDa representing IGFBP-3, a single 30 kDa band (IGFBP-1 and -2) and a 24 kDa band identified as IGFBP-4.

GHRA administration did not affect IGFBP-3 levels with the exception of the group receiving 2·5 mg GHRA. In this group IGFBP-3 levels were significantly decreased to 78% of control levels ($P=0·04$) (Fig. 1C). Circulatory 30 kDa (IGFBP-1 and -2) levels were unchanged in all groups (Fig. 1C). IGFBP-4 levels were significantly increased in the 1·25, 2·5 and 5 mg GHRA groups to 143% ($P=0·008$), 142% ($P=0·04$) and 159% ($P=0·001$) of control levels. In the 10 mg GHRA group, IGFBP-4 levels were near significantly increased to 125% ($P=0·06$) of control levels (Fig. 1C). In the 5 and 10 mg GHRA groups the calculated ratio of IGF-I to IGFBP-3 was significantly decreased to 71% ($P=0·001$) and 53% ($P<0·001$) of control values.

Hepatic GH/IGF system expression

GHRA administration for 7 days significantly increased hepatic GHR mRNA expression at all doses tested (Fig. 2B). GHBP mRNA expression was dose-dependently increased in the 1·25 mg, 2·5 mg and 5 mg GHRA groups (control versus 1·25 mg GHRA group, $P<0·001$; 1·25 mg versus 2·5 mg GHRA group, $P<0·001$; 2·5 mg versus 5 mg GHRA group, $P=0·044$). A representative Northern blot is given in Fig. 2A.

Hepatic IGF-I protein levels, however, demonstrated a tendency to decline in all GHRA administration groups, only reaching significance in the 10 mg GHRA group ($P=0·004$) (see Fig. 4A). Hepatic IGF-I mRNA levels (predominantly 1·2 kb and 7 kb) also had a tendency to decline in all GHRA administration groups reaching significance in the 2·5 mg GHRA ($P=0·01$) and 10 mg GHRA ($P=0·05$) groups (Fig. 2C).

Compared with control animals, hepatic IGFBP-1 mRNA levels had a tendency to increase, only reaching significance in the 2·5 mg GHRA group ($P<0·03$) (Fig. 2D). Hepatic IGFBP-2 mRNA levels were significantly decreased in the 1·25, 2·5 and 5 mg GHRA groups ($P<0·001$, $P=0·002$ and $P=0·001$ respectively) (Fig. 2D). IGFBP-3 mRNA levels were unchanged during GHRA administration, whereas IGFBP-4 mRNA levels were significantly increased in all GHRA administration groups (Fig. 2D). Hepatic IGFBP-5 and -6, IGF-II and IGF-I receptor mRNA levels were not detectable in any of the groups.

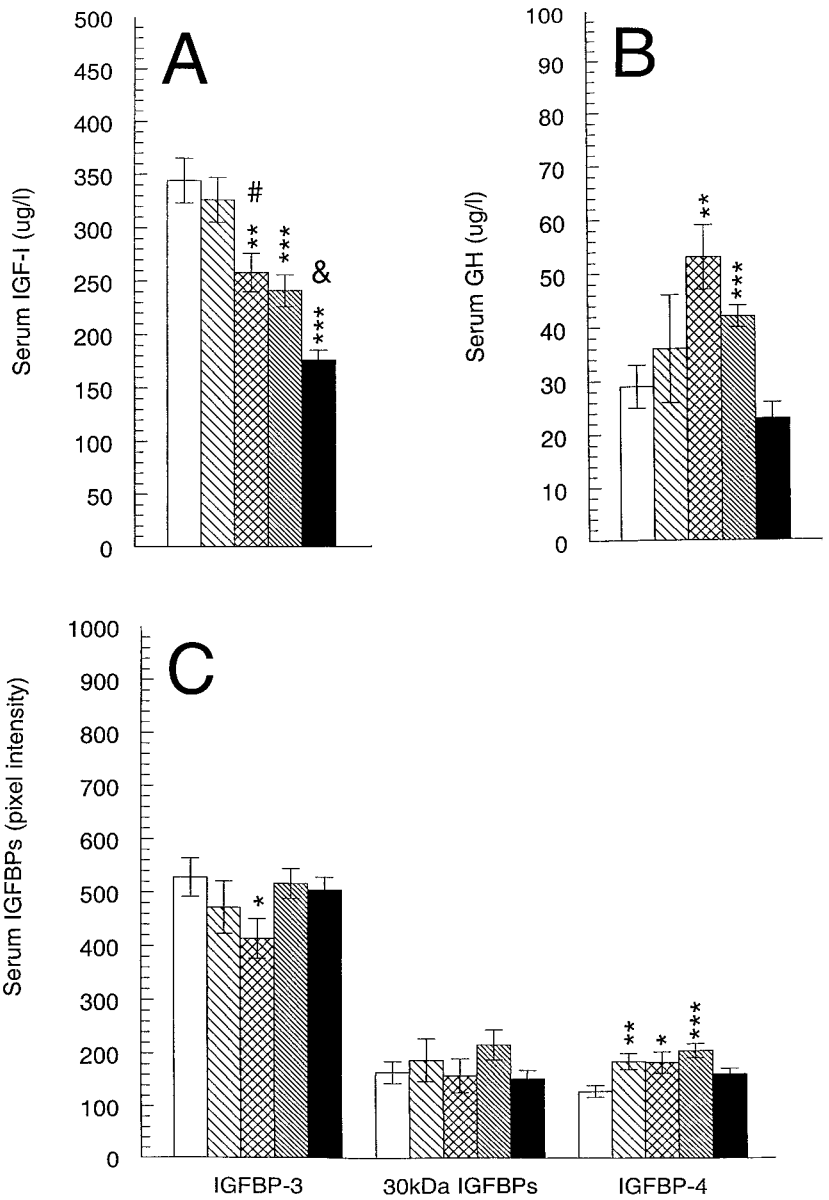


Figure 1 Circulatory levels of (A) IGF-I, (B) GH, and (C) IGFBP in adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are means ± S.E.M. ($n=8$). *$P=0·04$, **$P<0·01$, ***$P\leq 0·001$, statistical significance level between the indicated GHRA group and the placebo control. $^{\#}P=0·008$ significance level between the 1·25 mg and the 2·5 mg GHRA group; $^{\&}P=0·011$ significance level between the 5 mg and 10 mg GHRA group.

Renal GH/IGF system expression

GHRA administration did not modify renal GHR mRNA levels except for the 1·25 mg GHRA group where a significantly decreased GHR mRNA level was observed ($P=0·03$) (Fig. 3B). A representative Northern blot is given in Fig. 3A.

GHRA administration also did not modify renal GHBP mRNA levels in most of the groups. Only the 10 mg GHRA group demonstrated a significantly increased GHBP level ($P=0·005$) (Fig. 3B).

Compared with the placebo control, in the 5 and 10 mg GHRA groups renal IGF-I protein levels significantly

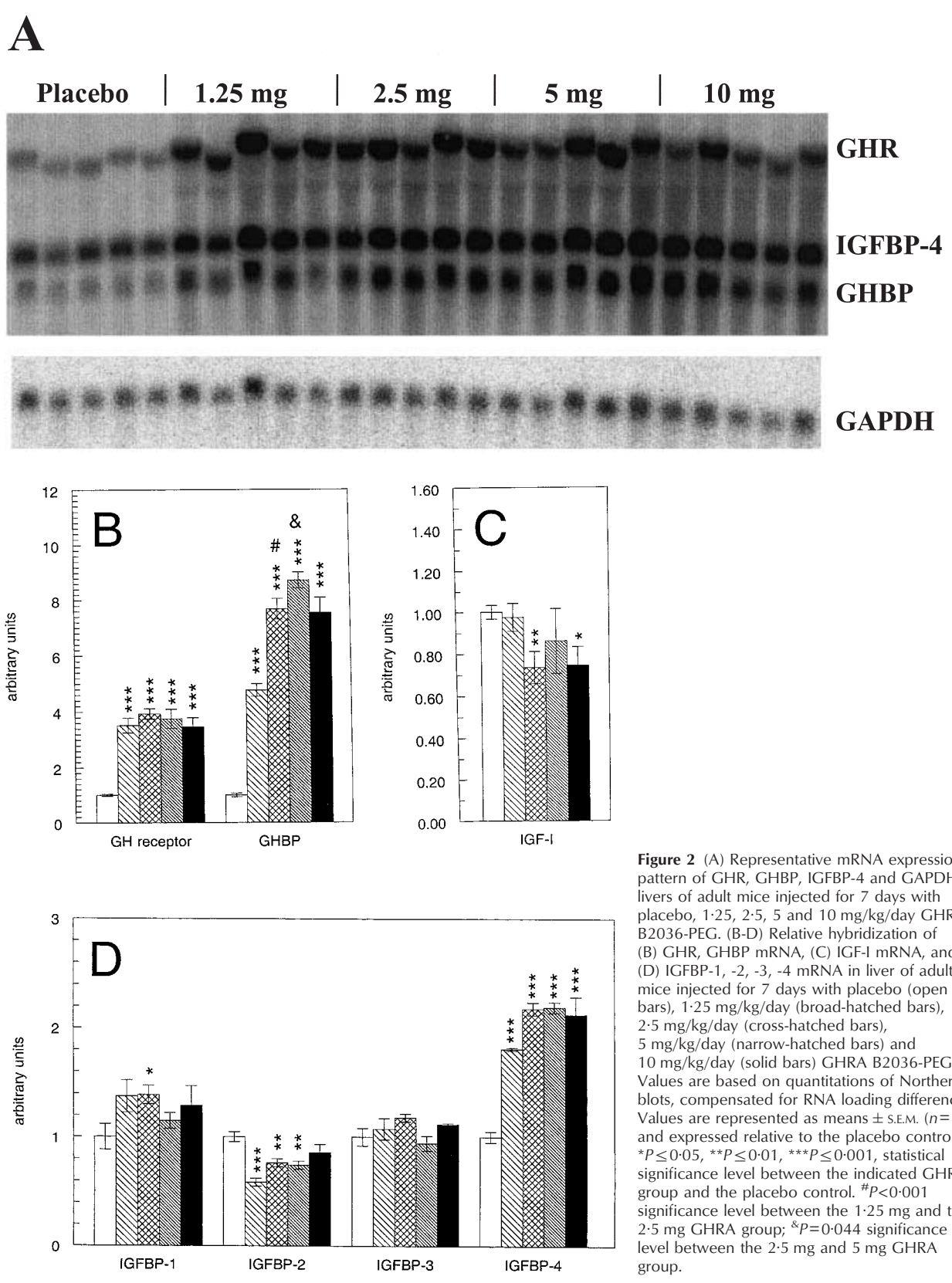
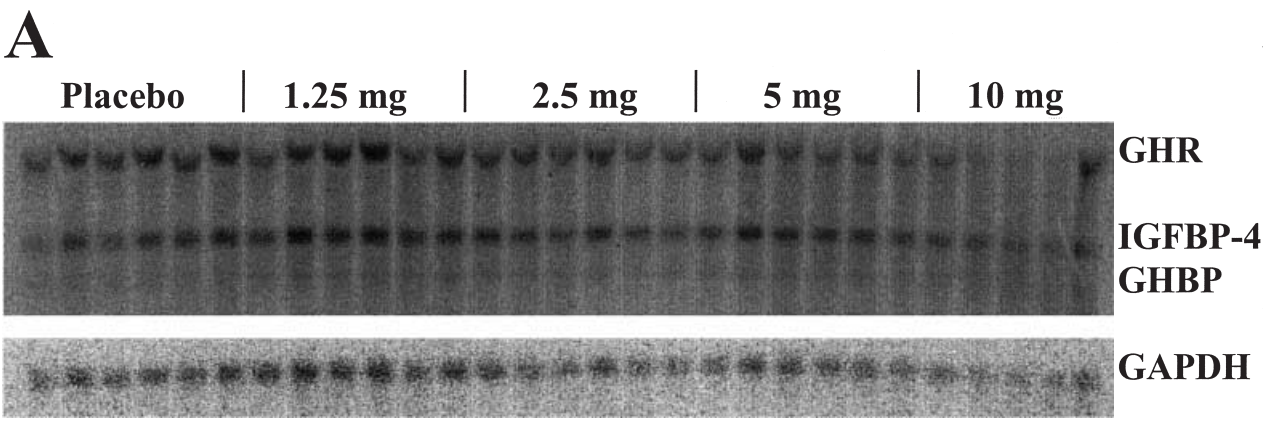
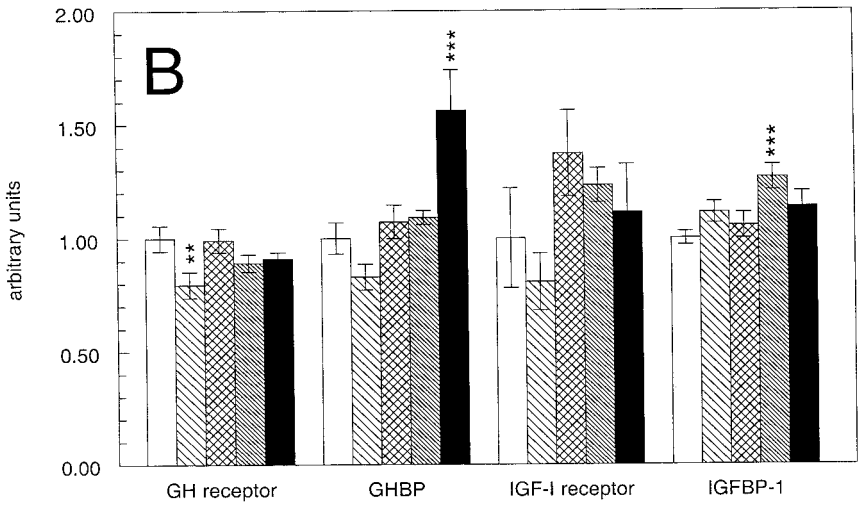


Figure 2 (A) Representative mRNA expression pattern of GHR, GHBP, IGFBP-4 and GAPDH in livers of adult mice injected for 7 days with placebo, 1·25, 2·5, 5 and 10 mg/kg/day GHRA B2036-PEG. (B-D) Relative hybridization of (B) GHR, GHBP mRNA, (C) IGF-I mRNA, and (D) IGFBP-1, -2, -3, -4 mRNA in liver of adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are represented as means ± S.E.M. ($n=8$) and expressed relative to the placebo control. $^{*}P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$, statistical significance level between the indicated GHRA group and the placebo control. $^{\#}P<0{\cdot}001$ significance level between the 1·25 mg and the 2·5 mg GHRA group; $^{\&}P=0{\cdot}044$ significance level between the 2·5 mg and 5 mg GHRA group.





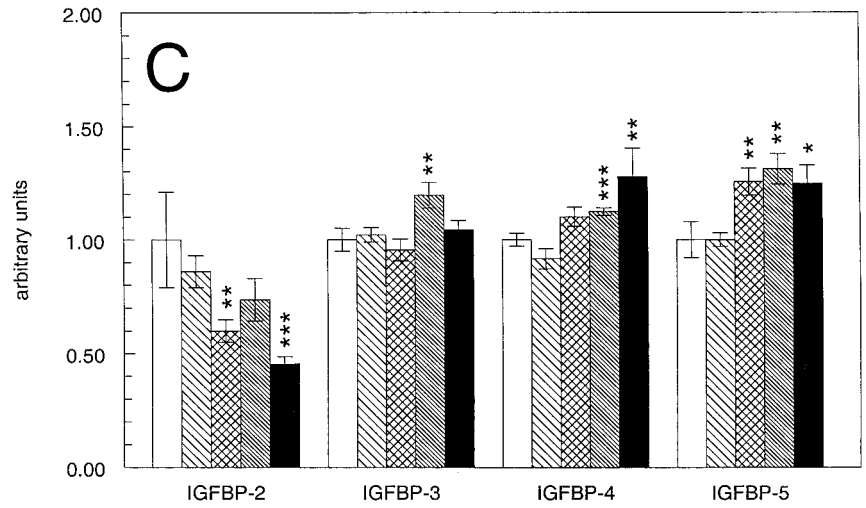


Figure 3 (A) Representative mRNA expression pattern of GHR, GHBP, IGFBP-4 and GAPDH in kidneys of adult mice injected for 7 days with placebo, 1·25, 2·5, 5 and 10 mg/kg/day GHRA B2036-PEG. (B and C) Relative hybridization of (B) GH receptor, GHBP, IGF-I receptor and IGFBP-1 mRNA and (C) IGFBP-2, -3, -4 and -5 mRNA in kidneys of adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are based on quantitations of Northern blots, compensated for RNA loading differences. Values are represented as means ± S.E.M. ($n=8$) and expressed relative to the placebo control. *$P=0{\cdot}05$, **$P\leq 0{\cdot}03$, ***$P\leq 0{\cdot}005$, statistical significance level between the indicated GHRA group and the placebo control.

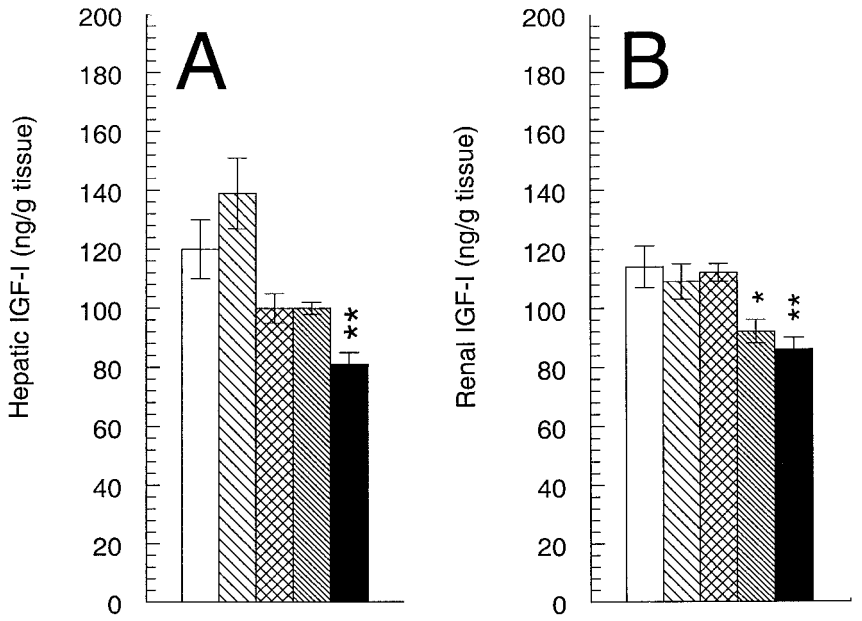


Figure 4 (A) Hepatic and (B) renal IGF-I protein levels in adult mice injected for 7 days with placebo (open bars), 1·25 mg/kg/day (broad-hatched bars), 2·5 mg/kg/day (cross-hatched bars), 5 mg/kg/day (narrow-hatched bars) and 10 mg/kg/day (solid bars) GHRA B2036-PEG. Values are means ± S.E.M. ($n=8$). *$P=0·02$, **$P \leq 0·005$, statistical significance level between the indicated GHRA group and the placebo control.

decreased ($P=0·02$ and $P=0·005$ respectively) (Fig. 4B). IGFBP-1 and -3 mRNA levels were not modified by GHRA administration except for the 5 mg GHRA administration group where a significant increase was observed ($P=0·03$ and $P=0·005$ respectively) (Fig. 3C). IGFBP-2 mRNA levels decreased dose-dependently with increasing GHRA concentrations which, however, only reached significance in the 2·5 and 10 mg GHRA groups ($P=0·03$ and $P=0·005$ respectively) (Fig. 3C). IGFBP-4 mRNA levels significantly increased in the 5 and 10 mg GHRA groups ($P=0·004$ and $P=0·03$ respectively) and IGFBP-5 mRNA levels significantly increased in the 2·5, 5 and 10 mg GHRA groups ($P=0·03$, $P=0·01$ and $P=0·05$ respectively) (Fig. 3C). Renal IGF-I receptor expression did not significantly change with any of the treatments. Renal IGF-I, IGF-II and IGFBP-6 mRNA levels were not detectable in any of the groups.

Discussion

In the present study, for the first time, the specific blockage of GH at the organ level was studied using a novel GHRA (B2036-PEG). B2036-PEG is a member of a GHRA family recently developed for the human GHR that retained its activity in mice. Studies using GHRAs *in vitro* demonstrated high affinity binding to the GH receptor (Chen *et al.* 1991*b*,*c*, Fuh *et al.* 1992). Transgenic animals expressing GHRAs phenotypically resembled dwarf animals with reduced circulating IGF-I levels (Chen *et al.* 1990, 1991*a*). When GHRA transgenic animals were made diabetic, protection to GH- and IGF-I-induced renal damage was observed (Chen *et al.* 1995, 1996). Furthermore, GHRA treatment protected diabetic mice from renal damage (Flyvbjerg *et al.* 1999*a*, Segev *et al.* 1999) and abolished compensatory renal growth in uninephrectomized mice (Flyvbjerg *et al.* 1999*b*).

To elucidate further the effects of GHRA, the present study was performed with exogenous administration of a long-acting formula of GHRA at increasing doses in adult animals. The effects on body weight and on the circulating, hepatic and renal GH and IGF systems were analyzed.

In our study, increasing GHRA doses proportionally decreased circulating and hepatic IGF-I levels. In contrast, circulatory IGFBP-3 levels remained constant. The IGF-I and IGFBP-3 serum values matched hepatic mRNA levels as a dose-dependent decrease in hepatic IGF-I mRNA values was observed, whereas the IGFBP-3 mRNA levels were unchanged at any of the GHRA doses used. In the circulation, this might indicate a decrease in IGF-I bioavailability. In rodents, there is evidence that serum IGFBP-3 is regulated directly by IGF-I and is independent of GH (Clemmons *et al.* 1989, Camacho-Hubner *et al.* 1991). However, in our study circulatory IGFBP-3 protein and hepatic IGFBP-3 mRNA levels remained unchanged despite increased GH and decreased

IGF-I levels. The latter finding is in contrast to the human, where serum IGFBP-3 levels directly reflected GH levels (Blum *et al.* 1993). In addition, patients lacking a functional GHR demonstrated high serum GH and low serum IGF-I and IGFBP-3 levels (Cotterill *et al.* 1992, Gargosky *et al.* 1993). The reasons for these differences in serum IGFBP-3 regulation are unclear.

GHRA administration significantly increased circulatory GH levels, although at the highest GHRA concentration, GH levels were decreased to control values. We cannot readily explain this latter observation. As expected, the increased blocking of the GHRs was reflected by a dose-dependent decrease in circulatory IGF-I levels. Therefore, a dose-dependent increase in circulating GH levels was expected. It could be argued that, due to cross-reactivity of the GHRA and GH, the GHRA interfered in our GH assay. However, this possibility had to be excluded as we could not demonstrate any cross-reactivity between the human GHRA and the mouse GH in the rodent specific RIA used for the measurements of circulatory GH levels. Furthermore, one could argue that a single GH determination is not the best estimate of GH secretion. However, as shown before, a single barbital-stimulated GH measurement may be used as an estimate of GH levels (Takahashi *et al.* 1971).

GHRA administration significantly increased hepatic GHR and GHBP mRNA levels, whereas renal GHR and GHBP mRNA levels did not change significantly. These results are in agreement with other studies examining renal GHR/GHBP mRNA levels (Chen *et al.* 1997, Flyvbjerg *et al.* 1999*b*, Segev *et al.* 1999). Our study may represent a compensatory upregulation of the GHR gene expression in response to the functional blockade of the GHR by the GHRA. However, in contrast to our findings, Chen *et al.* (1997) using GHRA expressing transgenic mice did not observe increased hepatic GHR/GHBP mRNA expression although strongly enhanced liver GHR and serum GHBP protein levels were found in these same GHRA transgenic animals (Chen *et al.* 1997, Sotelo *et al.* 1998).

In GH-deficient dwarf rats, decreased renal IGF-I mRNA and increased renal IGFBP-1 mRNA levels were demonstrated that were normalized with exogenous GH administration (Kobayashi *et al.* 1995). In our study, increased GHRA doses did not have an effect on circulatory 30 kDa IGFBPs or hepatic and renal IGFBP-1 mRNA levels. Remarkably, GHRA administration did increase circulatory IGFBP-4 protein, hepatic IGFBP-4 mRNA and, at higher doses, renal IGFBP-4 mRNA levels also. This stimulation of IGFBP-4 expression, in addition to the decrease in the IGF-I/IGFBP-3 ratio, may lead to a further reduction in IGF-I bioavailability in GHRA-treated animals. Kobayashi *et al.* (1995) also demonstrated increased IGFBP-4 mRNA levels in dwarf rats that, however, were not influenced by GH administration. Therefore, multiple mechanisms may be operative in regulating the expression of this gene.

In summary, in this study we demonstrated that administration of a specific GHRA dose-dependently decreased hepatic and serum IGF-I with no effect on the expression of hepatic or renal IGFBP-1 and -3 levels, while stimulating hepatic and circulatory IGFBP-4 levels, likely creating a significant decrease in IGF-I bioavailability. Our findings provide a framework for understanding the usefulness of GHRA as a therapeutic drug. GHRA treatment may be beneficial in inhibiting the GH/IGF-I axis in diseases in which its involvement is implicated, such as acromegaly and GH/IGF-I sensitive tumors. Furthermore, GHRA administration may influence organ specific, direct, GH action in conditions characterized by renal damage such as diabetic nephropathy.

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