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Insulin-like Growth Factor 1 (IGF-I) Effects on Sex-specific Cytochrome P450 Enzymes in Normal and Hypophysectomised Male Rats

Eva Rasmussen,§ Birgitta Ask,* Niklas Finnström,* Anna Skottner-Lundin†‡ and Anders Rane**

*Department of Clinical Pharmacology, University Hospital, S-751 85 Uppsala; and †Pharmacia and Upjohn Biological Sciences, Research and Development, S-112 87 Stockholm, Sweden

ABSTRACT. The role of growth hormone (GH) in the regulation of the sex-differentiated rat cytochrome P450 (CYP) enzymes has been extensively studied. However, little is known about the involvement of insulin-like growth factor I (IGF-I) as a mediator in this regulation. We wanted to study if IGF-I had effects on sex-differentiated CYP enzymes and to compare the effects of IGF-I to the effects of GH. IGF-I, GH or saline was administered continuously via osmotic minipumps to normal and hypophysectomised rats for seven days. After treatment, the expression of several sex-differentiated liver enzymes (CYP2C11, CYP2C12), the female-dominant steroid 5α-reductase, and the male-dominant CYP3A2 enzyme was studied at mRNA, protein and/or functional levels. Our results demonstrate that IGF-I has marked effects on the sex-specific expression of CYP2C11 and CYP2C12. The effects of IGF-I were similar to those of GH. In contrast, in hypophysectomised rats IGF-I gave effects opposite to those observed after GH treatment to normal rats on the CYP3A-associated cortisol 6β-hydroxylation. No effects of IGF-I on the steroid 5α-reductase activity were observed. BIOCHEM PHARMACOL **56**;4:459–466, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P450; GH; IGF-I; sex-differentiation; liver; rat

Several hepatic CYP¶ enzymes are sex-differentiated in the rat, e.g. CYP2C11 and CYP2C12, which are selectively expressed in male and female rats, respectively [1, 2]. Other enzymes are classified as male-dominant (CYP3A2) or female-dominant (CYP2C7) [3]. The sex differentiation is believed to be caused by the sexual dimorphism in the secretion pattern of GH [4]. The secretion of GH in adult male rats is characterised by high concentration peaks in the blood between which the baseline concentration is almost undetectable. The peaks appear approximately every third hour. In adult female rats, the peaks are lower but appear more frequently and the baseline concentration is higher than in males [5, 6].

Whether the effects of GH on the liver enzymes are mediated by the hormone itself, or by insulin-like growth factors I and II is not clear. The GH-induced effects on the CYP enzymes mentioned above have been studied extensively *in vivo* [1, 7] but the question as to the effects of IGF-I on liver enzymes has been addressed only in experiments using isolated or cultured hepatocytes [8].

To study this issue, the effects of s.c. GH and IGF-I infusion on specific enzymes of the CYP family were compared. In order to distinguish the IGF-I effects from those of GH, hypophysectomised animals were included to avoid the negative feedback of IGF-I on GH secretion [9]. The expression of several sex-differentiated, female-dominant or male-dominant enzymes (CYP2C11, CYP2C12, steroid 5α-reductase, CYP3A2) was investigated at mRNA, protein and/or functional levels. Codeine *N*-demethylation and androstenedione 16α-hydroxylation were used as marker reactions for CYP2C11 [10, 1], whereas androstenedione 5α-reduction was used to reflect the female-dominant steroid 5α-reductase [1]. Cortisol 6β-hydroxylation was also included, as it has been suggested to be catalysed by CYP3A [11].

MATERIALS AND METHODS

Animals

Twenty-four hypophysectomised and 24 normal postpubertal (7-week-old) Sprague–Dawley male rats were included in the study. Hypophysectomy was performed at 6 weeks of age under halothane anesthesia. The absence of the pituitary was verified by growth measurements. In order to acclimatize, the rats were maintained in the laboratory one

‡ Present address: Astra Hässle AB, S-431 83 Mölndal, Sweden.
§ Corresponding author: Eva Rasmussen, Department of Clinical Pharmacology, University Hospital, S-751 85 Uppsala, Sweden. Tel. +46-18-66 49 35; FAX +46-18-51 92 37.
¶ *Abbreviations:* CYP, cytochrome P450; IGF-I, insulin-like growth factor; GH, growth hormone; rhGH, recombinant human GH; and rhIGF-I, recombinant human IGF-I.
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week before the beginning of the study. The rats were housed in a room with a 12 hr light-dark cycle. They were fed standard laboratory rat diet and water *ad lib*. The temperature and humidity in the storage room were registered daily and were 22–25° and 35–40%, respectively.

Drug Treatment and Study Protocol

Normal and hypophysectomised rats were treated with rhGH 1.0 IU/kg/day (N = 8 + 8), rhIGF-I 1.0 mg/kg/day (N = 8 + 8) or saline (N = 8 + 8) as continuous s.c. infusion via Alzet® osmotic minipumps for 7 days. In the Results and Discussion sections rhGH and rhIGF-I will be referred to as GH and IGF-I, respectively. The minipumps were implanted s.c. in the neck under halothane anesthesia. The rats were sacrificed by decapitation on the morning of the eighth day. The livers were collected, immediately frozen in liquid nitrogen and stored at −70°. The project was approved by the local ethics committee for animal experimentation.

mRNA Solution Hybridisation

*CYP*2C11 and *CYP*2C12 specific mRNAs were assayed by solution hybridisation according to Durham *et al.* [12] and Melton *et al.* [13], as described previously [14]. cRNA probes were synthesized *in vitro* using the Riboprobe Gemini system (Promega) and radiolabeled with ^{35}S-UTP(Amersham International). A 50-base oligonucleotide synthesized from the sequence of a *CYP*2C12 gene fragment and a 190-base fragment corresponding to the 3′-part of the *CYP*2C11 gene were used as templates for cRNA synthesis as described previously [15].

Enzyme Assays

Microsomes were prepared from rat liver according to standard procedures [10]. Protein determinations were performed according to Lowry *et al.* [16] using BSA as standard. The microsomes were stored at −70° until analysis.

Codeine incubations were performed at a final codeine concentration of 1 mM. The incubates were extracted and analysed for norcodeine on a reversed phase HPLC system with UV detection according to Ladona *et al.* [17].

Microsome incubations with [4-^{14}C]-androstene-3,17-dione and analysis of 16α-OH-androstenedione were performed as described previously [7]. The radioactive metabolites were quantified using a PhosphorImager (Molecular Dynamics Inc.).

Incubations with cortisol, 0.5 mM, were performed according to Rane and Ask [10], based on the method of Taylor *et al.* [18]. Briefly, the cortisol 6β-hydroxylation assay was performed as follows: 0.1 mL of the incubate or the standards (containing various concentrations of 6β-hydroxycortisol in aqueous solution) were diluted to 1.0 mL with distilled water and extracted with 5 mL of dichloromethane by slow shaking for 15 min. The samples were centrifuged at 200 rpm for 5 min at 4° (Hettich). The aqueous phase was aspirated and 4 mL of the dichloromethane phase was transferred to new tubes for evaporation under nitrogen at 35–40°. The residue was kept dry at −20°. For analysis, the residue was dissolved in 125 μL of the eluent (10% acetonitrile in water) and 50 μL was injected onto the column. Chromatography was performed on a 15-cm Spherisorb Phenyl 5-μm column (Jones Chromatography) at an eluent flow rate of 0.7 mL/min. UV detection was carried out at a wavelength of 243 nm (limit of quantitation 5 ng/mL) and the retention time of 6β-hydroxycortisol was 6 min.

Immunoblotting Procedure

Samples of pooled microsomes containing 10 μg of protein were subjected to SDS-PAGG (12%) according to Laemmli *et al.* [19]. The transfer to nitrocellulose filter was performed in a miniblot apparatus (Bio-Rad) essentially as described by Towbin *et al.* [20] and modified according to Ladona *et al.* [21]. Primary antibodies (raised in rabbits) against *CYP*2C11 were incubated with the filter for 2 hr followed by incubation with secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG) for 1 hr. The *CYP*2C11 specific bands were visualized with the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad). Scanning of immunoblots was performed as described elsewhere [22]. The immunoblotting was repeated twice.

Determination of rhGH, Rat IGF-I and rhIGF-I in Serum

After decapitation, blood was collected and serum prepared for analysis of rhGH and rhIGF-1. The serum was stored at −20° until analysis. Human GH was determined using a DELFIA kit (Wallac Oy). Briefly, the DELFIA kit assay is a solid phase, two-site fluoroimmunometric assay based on the sandwich technique in which two monoclonal antibodies directed against two separate antigenic determinants on the rhGH molecule are used. Measuring range was 0.03–100 mIU/L. Intra-assay coefficient of variation was between 1 and 1.6%, and inter-assay coefficient of variation between 4.2 and 6.3% at high and low concentrations of rhGH, respectively.

Recombinant hIGF-I and rat IGF-I were both analysed by radioimmunoassay according to Bang et al. [23]. The influence of IGF-I binding proteins was reduced by acid ethanol extraction and cryoprecipitation prior to assay, and by use of the truncated des [1–3] IGF-I analogue as tracer. The limit of quantitation of rhIGF-I was 8 μg/L. Intra- and interassay coefficients of variation of the rhIGF-I analyses were 4 and 11%, respectively. Recombinant human IGF-I was used as standard in the rat IGF-I assay because no rat IGF-I standard was available. Thus, only relative group comparisons were performed regarding rat IGF-I.

Chemicals and Reagents

Recombinant human GH and rhIGF-I were provided by Pharmacia and Upjohn. Norcodeine HCl was purchased from the National Institute of Drug Abuse and [4-^{14}C]-androstene-3,17-dione from Amersham. The antibodies against *CYP*2C11 were kindly provided by Prof. C. R. Wolf (Biomedical Research Centre, University of Dundee, Scotland, U.K.). Plasmids containing the *CYP*2C11 and *CYP*2C12 specific fragments were kindly provided by Dr. Inger Porsch-Hällström (Karolinska Institute, Stockholm, Sweden). All other chemicals were of analytical grade and available from commercial sources.

Statistical Analysis

Statistical analysis was performed using the unpaired Student's *t*-test in the software Statview 4.02 (Abacus Concepts Inc.). The results were expressed as mean ± SD. The levels of significance were set at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

RESULTS

Effects of Hypophysectomy on Adult Male Rats

CYP2C11. The amount of *CYP*2C11-specific mRNA decreased by 58% after hypophysectomy. The corresponding decrease in androstenedione 16α-hydroxylation and codeine *N*-demethylation activities were 87 and 76%, respectively. The intensity of the *CYP*2C11 protein band was 77% lower in hypophysectomised than in normal rats.

CYP2C12. The amount of *CYP*2C12-specific mRNA was 256% higher in hypophysectomised than in normal rats.

5α-Reductase and Cortisol 6β-Hydroxylation. The 5α-reductase activity was 95% lower in hypophysectomised compared to normal rats. In contrast, there were no differences between hypophysectomised and normal rats in the *CYP*3A2-associated cortisol 6β-hydroxylation activity.

IGF-I Serum Concentrations. The rat IGF-I serum concentrations decreased from 522 ± 53 μg/L in normal control rats to 23 ± 2 μg/L in hypophysectomised control rats.

Effects of IGF-I Infusion Compared to GH Infusion in Normal Adult Male Rats

CYP2C11. Treatment with IGF-I decreased the *CYP*2C11-specific mRNA by 53% ($P < 0.01$). This mRNA was reduced by 97% ($P < 0.001$) after GH treatment (Fig. 1A). The down-regulation of *CYP*2C11 mRNA observed after IGF-I and GH treatment was associated with a decrease in codeine *N*-demethylation by 32 ($P < 0.01$) and 82% ($P < 0.001$), respectively (Fig. 1B). The *CYP*2C11-associated 16α-hydroxylation of androstenedione decreased by 93% after GH treatment ($P < 0.001$), whereas no change was observed after IGF-I treatment (Fig. 1C).

CYP2C12. Infusion treatment with IGF-I gave an eight-fold increase in the expression of *CYP*2C12-specific mRNA ($P < 0.001$). This increase was of the same order of magnitude as that induced by GH treatment (Fig. 2).

5α-Reductase and Cortisol 6β-Hydroxylation. GH increased the androstenedione 5α-reduction *ca.* four-fold ($P < 0.001$) (Fig. 3) and decreased 6β-hydroxylation of cortisol by 35% ($P < 0.001$) (Fig. 4), whereas IGF-I had no effects on these reactions.

CYP2C11 Protein. The anti-*CYP*2C11 antibody-identified protein band in Western blots was markedly down-regulated by IGF-I and GH treatment (by 70 and 96%, respectively).

IGF-I and GH Serum Concentrations. Serum concentrations of rat IGF-I were similar in rhGH- and saline-treated normal rats (516 ± 34 vs 522 ± 53 μg/L). The serum concentration of rhIGF-I in the rhIGF-I-treated normal animals was not determined specifically, because the assay also co-measures endogenous rat IGF-I. The mean serum concentration of rhGH after rhGH treatment was 16 ± 9 mU/L in the normal rats.

Effects of IGF-I Infusion Compared to GH Infusion in Hypophysectomised Adult Male Rats

CYP2C11. In hypophysectomised rats, a down-regulation of *CYP*2C11-specific mRNA by 63% ($P < 0.01$) was observed after IGF-I treatment (Fig. 1A). A marked decrease in these mRNA levels (by 92%; $P < 0.001$) was also observed during GH treatment. Both codeine *N*-demethylation and androstenedione 16α-hydroxylation were decreased (by 30 and 64%, respectively, $P < 0.05$, $P < 0.01$) in hypophysectomised rats treated with GH (Fig. 1B, C). No such effects by IGF-I were observed in these rats.

CYP2C12. Consistent with our findings in normal rats, *CYP*2C12-specific mRNA was up-regulated by 80% ($P < 0.01$) in hypophysectomised rats after IGF-I treatment (Fig. 2). The *CYP*2C12-specific mRNA was also increased after GH administration.

5α-Reductase and Cortisol 6β-Hydroxylation. GH increased androstenedione 5α-reduction three-fold, whereas IGF-I had no effect on this reaction (Fig. 3). Cortisol 6β-hydroxylase activity was increased by 48% in hypophysectomised rats after IGF-I treatment ($P < 0.001$) (Fig. 4). However, no effects of GH on this activity were observed in hypophysectomised rats (Fig. 4).

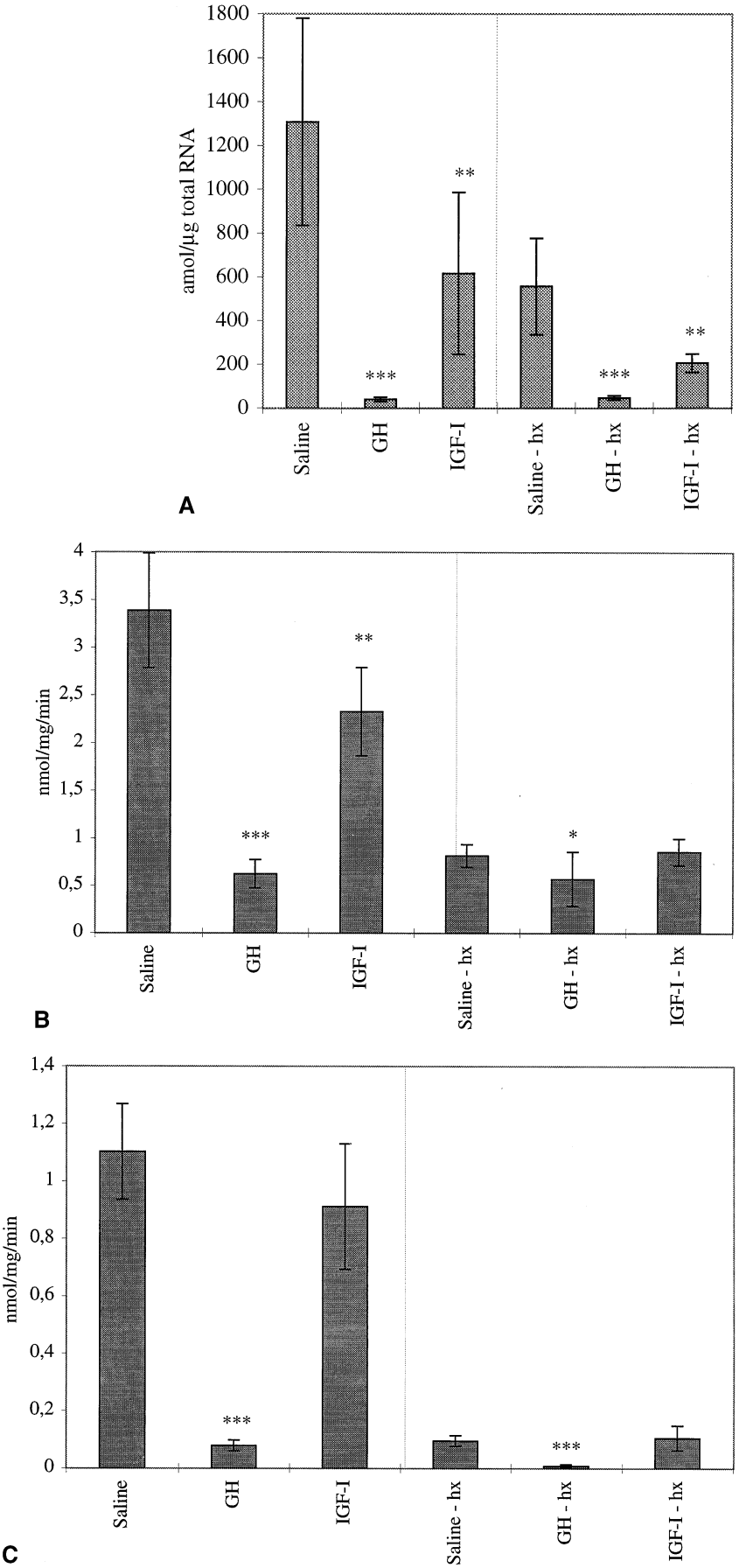

FIG. 1. The effects of IGF-I or GH infusion treatment on hepatic CYP2C11 showing (A) CYP2C11-specific mRNA; (B) codeine N-demethylation; and (C) androstenedione 16α-hydroxylation in normal and hypophysectomised (hx) adult male rats.

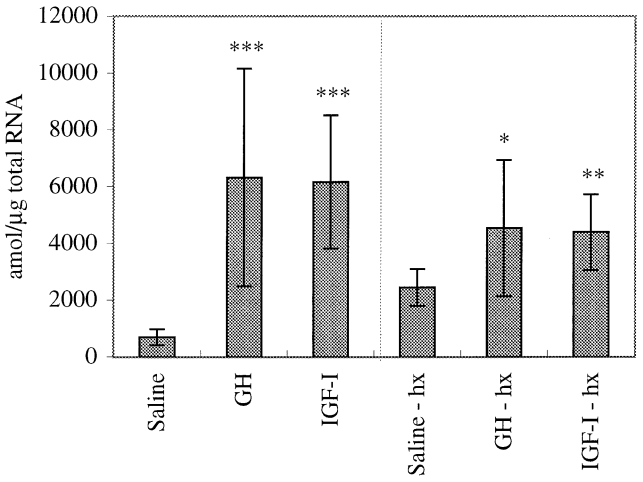

FIG. 2. The effects of IGF-I or GH infusion treatment on hepatic CYP2C12-specific mRNA in normal and hypophysectomised (hx) adult male rats.

CYP2C11 Protein. Both IGF-I and GH treatment gave a down-regulation of the Western blot-identified CYP2C11 protein band by 60 and 80%, respectively.

IGF-I and GH Serum Concentrations. In hypophysectomised rats, the serum levels of rat IGF-I (as measured with our rhIGF-I standards) were 11-fold higher after rhGH treatment as compared to saline-treated animals (257 ± 27 vs 23 ± 2 µg/L). The rhIGF-I concentration in the rhIGF-I treated hypophysectomised rats was estimated as 145 ± 32 µg/L without correction for interference by endogenous rat IGF-I. The mean serum concentration of rhGH after rhGH treatment was 19 ± 10 mU/L in the hypophysectomised rats.

DISCUSSION

This investigation was designed to study the effects of IGF-I on different CYP enzymes. For this purpose, the gene expression, the activity and/or the immuno-identified protein of the sex-differentiated CYP2C11 and CYP2C12 enzymes, as well as of the male-dominant CYP3A2 enzyme, were chosen. In addition, the female-dominant steroid 5α-reductase was studied.

Our results demonstrate for the first time that treatment with IGF-I has similar effects as treatment with GH (both given as infusion) on the mRNA levels of the sex-specific CYP2C11 and CYP2C12 enzymes. Whereas the IGF-I effect on CYP2C11 mRNA was less pronounced than that of GH, the expression of female-specific CYP2C12 mRNA was equally enhanced by GH and IGF-I. These data were supported by our findings in the Western blots showing a decrease in the CYP2C11 enzyme protein band. The serum concentration of rat IGF-I did not increase after GH treatment, indicating that the GH effects were not mediated by IGF-I. Nevertheless, it is possible that IGF-I may mediate the effects of GH through a local release in the liver.

In contrast to the marked effects of IGF-I on CYP2C11-specific mRNA only a tendency toward a lower activity of the CYP2C11-associated 16α-hydroxylation of androstenedione after treatment with IGF-I was observed. An effect of IGF-I on the codeine *N*-demethylation was, however, noted consistent with the fact that 75% of this reaction is accounted for by CYP2C11 [10]. The difference between the observed effects of IGF-I on the two CYP2C11 marker reactions is difficult to explain. The variability in the 16α-hydroxylation reaction may be too high for a modest effect to be observed. A dose-dependent effect on the metabolic enzyme function may be assumed, since in another experiment with a higher dose of IGF-I (5 µg/kg/day), a 34% down-regulation ($P < 0.01$) of the androstenedione 16α-hydroxylation was observed (Rasmussen E, Ask B, Finnström N, Scottner A and Rane A, unpublished results).

Previous information on IGF-I effects on hepatic CYP

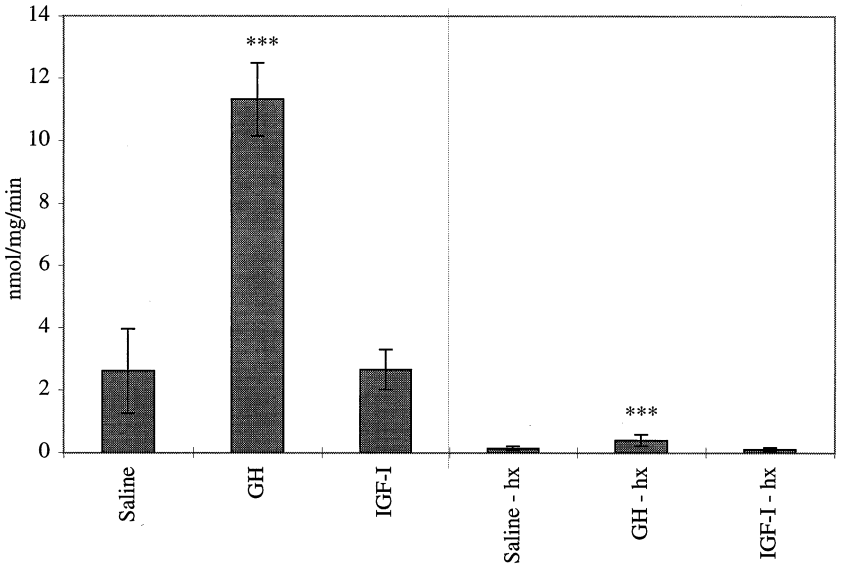

FIG. 3. The effects of IGF-I or GH infusion treatment on hepatic androstenedione 5α-reduction in normal and hypophysectomised (hx) adult male rats.

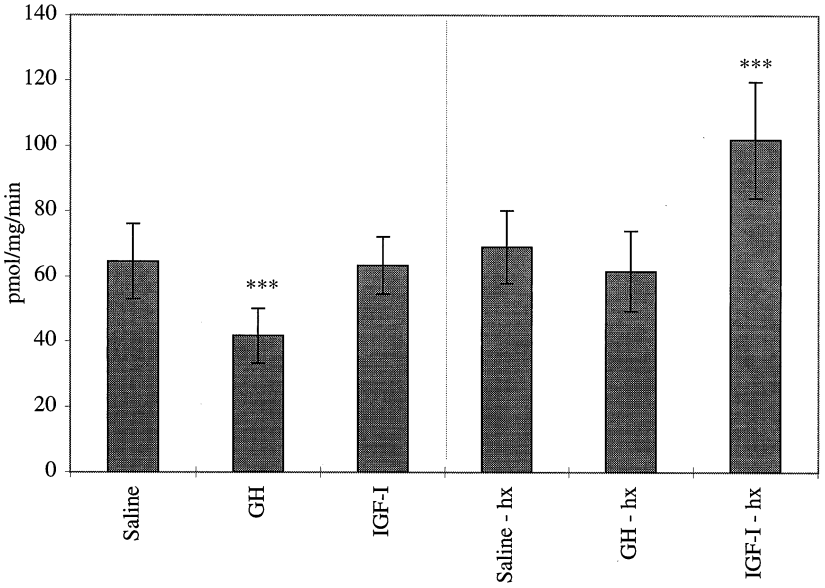

FIG. 4. The effects of IGF-I or GH infusion treatment on the CYP3A2-associated cortisol 6β-hydroxylation in normal and hypophysectomised (hx) adult male rats.

enzymes has been obtained from *in vitro* studies in primary rat hepatocyte cultures [8]. These authors did not find any effect of IGF-I on *CYP*2C12 mRNA, but IGF-I potentiated the effect of GH on this mRNA species. It was concluded that GH alone is responsible for the induction of *CYP*2C12 mRNA synthesis. The reason for the apparent discrepancy between our results and those of Tollet *et al.* [8] is unclear. However, it should be pointed out that these authors tested IGF-I *in vitro*, and that IGF-I was allowed to act on the hepatocytes for only 24 hr. Our IGF-I treatment continued for 7 days, and we propose that the difference may be due to an alteration of the hepatic IGF-I receptors during the course of the treatment. It is possible that continuous administration of IGF-I enhances the IGF-I receptor binding, similar to what has been described for GH receptors after continuous GH treatment [24, 25]. Another explanation for the discrepancy between our results and those of Tollet *et al.* could be that the IGF-I effects observed by us are indirect and mediated by other regulatory factors.

While IGF-I increased the female-specific *CYP*2C12 mRNA, no effect was observed on the female-dominant 5α-reduction of androstenedione. Although the regulation of these two enzymes by GH shows several similarities, their expression appears to be differently affected by hypophysectomy. The 5α-reductase activity was almost extinguished in hypophysectomised animals, while the *CYP*2C12-specific mRNA was markedly higher in hypophysectomised rats than in normal rats.

In contrast to the effects of IGF-I on the sex-specific *CYP* enzymes, no effect of IGF-I was observed on the *CYP*3A2-associated 6β-hydroxylation of cortisol in normal rats, even though GH treatment gave a moderate decrease (52%) in the enzyme activity. These observations are interesting and may be related to the different regulation of *CYP*3A2, which is not dependent on the male-specific GH secretory pattern for its expression even though it is a male-dominant enzyme [26, 27]. We also found that the cortisol 6β-hydroxylation responded differently to hypophysectomy compared to the male-specific *CYP*2C11 enzyme. In addition, and in contrast to GH, IGF-I gave a significant increase in the 6β-hydroxylation activity in hypophysectomised rats.

Any treatment with IGF-I in normal rats will tend to decrease the levels of GH by a feedback mechanism [9]. Our results in hypophysectomised rats will minimize an effect of such a mechanism. The down-regulating effects of IGF-I on *CYP*2C11 mRNA levels were in the same range in normal and hypophysectomised rats. In the absence of a possible decrease in the GH secretion after IGF-I treatment, the effects of IGF-I on *CYP*2C11 would be more marked than in normal rats. The mRNA levels of this enzyme were decreased by 63% in hypophysectomised rats and by 53% in normal rats.

The effect of both IGF-I and GH on *CYP*2C12 mRNA was less pronounced in hypophysectomised rats, indicating that contribution of another pituitary-dependent factor to the regulation of this enzyme may exist.

The effects of IGF-I and GH on the male-dominant *CYP*3A2 enzyme were more complex. As expected, treatment with GH decreased the cortisol 6β-hydroxylation activity in normal rats. In contrast, IGF-I had no effect on this activity in normal rats but increased the activity in hypophysectomised rats. The lack of increase in this activity by IGF-I in normal rats may have been masked by GH suppression of the enzyme, even in the presence of a negative feedback of IGF-I on GH secretion.

The success of the surgical hypophysectomy was verified by the low rat IGF-I serum concentrations observed in hypophysectomised control rats. The rat IGF-I was measured with a human standard (rhIGF-I) under the assumption that endogenous rat IGF-I in the hypophysectomised rats would contribute negligibly to the concentrations. As expected, there was a large difference between the hypophysectomised and normal control rats. Hypophysec-

tomy itself decreased the *CYP*2C11 mRNA and the *CYP*2C11 marker activities, probably due to lack of stimulation from intermittent GH secretion. The more profound decrease in *CYP*2C11 enzyme activity may be caused by an effect of hypophysectomy on other enzymes such as P450 NADPH reductase, which is essential for the catalytic function of the P450 enzymes and which has been found to be markedly down-regulated after hypophysectomy [28, 29]. The expression of *CYP*2C11 is promoted by the intermittent GH pulses characteristic for the male rat and is suppressed by the higher sustained GH secretion found in female rats. GH infusion treatment, which mimics the female secretion pattern, suppressed the *CYP*2C11 activity. The *CYP*2C12-specific mRNA was markedly higher in hypophysectomised rats compared to normal rats, indicating GH-independent expression of this enzyme. In contrast, the female-dominant 5α-reductase activity was very low in hypophysectomised animals. The male-dominant cortisol 6β-hydroxylation activity was similar in hypophysectomised and normal animals. This was expected, because the expression of *CYP*3A2 is independent of the male GH secretory pattern [26, 27].

In conclusion, our results demonstrate that treatment with IGF-I has marked effects on the sex-specific expression of *CYP*2C11 and *CYP*2C12 both in normal and in hypophysectomised rats. The effects of IGF-I were similar to those of GH. In contrast, in hypophysectomised rats IGF-I increased the *CYP*3A2-associated 6β-hydroxylation of cortisol, while GH did not affect this reaction in hypophysectomised rats and decreased this activity in normal rats. Further studies are needed to clarify the interactive effects of GH and IGF-I on sex-dependent and non-sex-dependent enzymes in the *CYP* enzyme family.

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