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THE ROLE AND MECHANISM OF GROWTH HORMONE IN THE REGULATION OF SEXUALLY DIMORPHIC *P*450 ENZYMES IN RAT LIVER

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Summary—The determination of sexually dimorphic hepatic steroid metabolism in rat liver has been shown to involve growth hormone. However, the mechanisms by which growth hormone controls the cytochrome *P*450 enzymes responsible for this dimorphic steroid metabolism is largely unknown. In this review we discuss different levels of growth hormone signal transduction, including receptor binding, signal transduction and activation of target genes by growth hormone.

OUTLINE

1. INTRODUCTION

The sexually dimorphic pattern of growth hormone (GH) secretion in rodents exerts profound effects on the hepatic steroid metabolism, resulting in a sexually differentiated expression of individual *P*450 cytochromes. The induction of the *P*450 enzymes is temporally correlated with the onset of the sexually differentiated secretion of GH at puberty and becomes manifest in adult animals [1]. This sexual differentiation, however, is predetermined neonatally when testicular androgens imprint the ability to express a male pattern of metabolism later in life [2]. Hence, neonatal castration of a male rat leads to a fully feminized pattern of hepatic steroid metabolism in the adult animal, whereas castration in adult animals only results in a partial effect [3]. Ovarian estrogens seem to have less influence on hepatic steroid metabolism, but the ovaries have an inhibitory effect on neonatal androgen imprinting in female rats [4].

The influence of gonadal steroids on sex-specific liver metabolism in the adult animal appears to be an indirect effect on the liver involving hypothalamic factors. GH secretion is regulated by the interplay of two hypothalamic neuropeptides, somatostatin and growth hormone releasing hormone, which controls the release of GH from the anterior pituitary [5]. Acting reciprocally, somatostatin inhibits and growth hormone releasing hormone stimulates GH secretion to coordinate its episodic release into the peripheral circulation. However, the mechanisms underlying the sex difference in GH secretion patterns are not well understood. The adult male rat has a regular pulsatile pattern of GH secretion with high amplitude pulses occurring at intervals of approx. 3.5 h separated by low or undetectable baseline levels. In contrast, the adult female rat exhibits irregular, low amplitude GH pulses, superimposed on a significantly elevated baseline level [6].

The pronounced sex differences of the male-specific *P*4502C11 (catalysing 16α-hydroxylation of testosterone) and the female-specific *P*4502C12 (catalysing 15β-hydroxylation of steroid sulphates) have made these isozymes valuable markers in regulatory studies of hepatic sexual differentiation. Removal of the pituitary gland leads to low levels of *P*4502C12 and *P*4502C11, but continuous or pulsatile treatment with GH reestablishes levels to those found in intact female or male animals, respectively [7–9]. Likewise, *P*4502C7, a female

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predominant enzyme active in retinol and retinoic acid hydroxylation, is induced by the more continuous female-specific GH secretion pattern [10, 11]. On the other hand *P*4502C13, a male-specific enzyme possessing steroid 6β-hydroxylase activity, is suppressed by the continuous presence of GH [12, 13] and induced by the pulsatile male-specific pattern of GH [14]. See also the paper by D. J. Waxman in this issue.

2. GH REGULATION

The GH regulation of these sexually differentiated hepatic cytochrome *P*4502C enzymes is summarized in Table 1. Apparently, GH has opposing effects on a target tissue depending on the way the hormone is presented to the animal. Furthermore, dwarf rats maintain sex differences in GH secretion and liver steroid metabolism typical of normal rats in spite of a 95% reduction in pituitary GH levels [15, 14]. This, together with the fact that single daily injections of GH are sufficient to masculinize the liver of a hypophysectomized rat, indicate that neither the frequency nor the amplitude of the GH pulse is recognized as male or female by the hepatocyte, but rather the complete and prolonged suppression (in males) or the persistence (in females) of circulating GH during the trough period following a GH surge. Thus, the GH secretory rhythm is pivotal in determination of sexually differentiated *P*450 expression.

The differential secretory profile of GH as a regulator of sex-specific liver functions also applies to sex differences in hepatic fatty acid metabolism, expression of the α_2-microglobulin and the level of prolactin receptors [16–18]. Regulatory studies of sex-specific hepatic *P*450 enzymes have also been carried out in species other than the rat. In mice the major sex-specific cytochrome *P*450 isozymes seem to be partially dependent on the mode of GH administration [19]. A male-specific 16α-hydroxylase has been isolated from mouse liver and characterized. This 16α-hydroxylase is, however, not a homolog of the rat *P*4502C11, but a member of the *P*4502D gene subfamily [20]. In some inbred mouse strains such as BALB/cJ and C57BL/6J there is also considerable 16α-hydroxylase activity in females. This activity is due to another *P*450 enzyme termed *P*4502b-9, expressed specifically in females [19]. Furthermore, female mice have a high testosterone 15α-hydroxylase activity in the liver relative to the male mice. Two highly homologous (98.3%) *P*450 15α cDNAs have been characterized in mouse (Cyp2a-4 and Cyp2a-5) [21]. With regard to humans there are indications that estrogens have a stimulatory effect on GH secretion (for references see [22]), but no dramatic sex difference in GH secretion has been shown in man. However, GH administration to GH-deficient children has been shown to alter hepatic drug metabolism [23].

Table 1. GH regulation of sexually differentiated rat hepatic cytochrome *P*4502C enzymes

Enzyme	Sex specificity	GH-infused hypox. rats	GH-injected hypox. rats
*P*4502C12	F $\gg$ M	++	No change
*P*4502C7	F > M	+	–
*P*4502C11	F $\ll$ M	– –	++
*P*4502C13	F $\ll$ M	–	+

Symbols: F = female and M = male, + and – represent an increase and decrease, respectively, of the enzyme in question when compared to the situation in hypophysectomized (hypox.) rats of either sex. This is a summary of the results from several studies performed in our lab, which are discussed in the text. See text for references.

The four rat *P*450 isozymes 2C11, 2C12, 2C13, and 2C7 have been purified to electrophoretic homogeneity [24–26] and full-length cDNA clones have been isolated [13, 27–31]. The cDNAs have been used to study the GH regulation of these genes [10, 12, 13, 27, 32, 33]. The physiological significance of the pronounced sex difference in expression of these individual enzymes remains unknown, but one may speculate that these differences are important in maintaining appropriate handling of metabolic demands during certain conditions or functions such as growth, reproduction, pregnancy or lactation. Although it is thought that hepatic *P*450 hydroxylations are part of a degradative pathway, one cannot exclude the possibility that some of the metabolites formed may have physiological functions, especially in view of the precise age- and sex-dependent regulation of these enzymes.

The GH regulation of the *P*4502C subfamily appears to occur at the transcriptional level [10, 32]. This fact prompted us to isolate the 5′ flanking region of the genes in order to further investigate the mechanisms of GH action at the molecular level [10, 32, 34]. In addition, a culture system with primary liver cells has been established. By culturing primary liver cells on a laminin rich extracellular matrix in serum-free medium, the cells maintain their adult liver-characteristic phenotype and respond to various inducers of *P*450 enzymes [35, 36]. This *in vitro* system has been characterized in terms of GH responsiveness,

i.e. specific binding of [^{125}I]hGH to the cells, and mRNA expression of both the GH receptor and the GH serum binding protein [37]. The female-specific *P*4502C12 and the female predominant *P*4502C7 are transcriptionally induced by GH in this system (20- and 2-fold, respectively). The male-specific *P*4502C11 and *P*4502C13 are both down-regulated by the continuous presence of GH in the culture medium (90 and 50%, respectively) [38]. This demonstrates that the regulation of these *P*450 enzymes by GH represents a direct action on the hepatocytes. So far it has not been possible to induce the male-specific enzymes by intermittent exposure of cultured hepatocytes to GH. The primary hepatocyte system and the 5′ flanking regions of the CYP2C genes offer versatile tools to elucidate second messenger pathway(s) and transcription factor(s) involved in GH action.

A central problem in eukaryotic molecular biology is the elucidation of molecules and mechanisms that mediate specific gene regulation in response to exogenous inducers such as hormones and growth factors. The pathway(s) by which GH controls the activation or repression of different genes in the hepatocyte is largely unknown. However, transduction of the GH signal should at least include: (1) binding of GH to its cognate receptor; (2) transduction of the binding signal via intracellular messengers; and (3) activation of target genes.

3. THE GH RECEPTOR

Specific binding sites for GH have been demonstrated in a variety of tissues from different species [39], and expression of the GH receptor mRNA has been observed in every tissue examined [40]. Though many details of the mechanism of action of GH are as yet unknown, it is widely assumed that the biological action of GH is initiated by the binding of the hormone to specific receptors on the plasma membrane of target cells, and that this binding is followed by a series of postreceptor events leading to the generation of one or more of the multiple effects of GH. Cloning of the GH receptor from several species [41, 40], including the rat, has placed the GH receptor in the cytokine receptor superfamily. This family of single transmembrane hormone receptors includes the GH, prolactin and at least eight other cytokine and hematopoietic receptors. These receptors contain homologous extracellular hormone binding domains and highly variable intracellular domains that are not homologous to any known tyrosine kinase or other protein [42]. In addition to the membrane localized receptors on target cells, specific soluble binding proteins have been demonstrated in serum for several of these cytokines [43, 44]. The GH serum binding protein has been identified as the extracellular hormone binding domain of the membrane bound GH receptor, and indirect evidence suggests that the rat serum GH binding protein is generated by differential splicing of a primary transcript encoding both the GH receptor and binding protein [45, 46].

Although the signal transduction mechanism for these cytokine receptors is unknown, there is evidence that after initial binding of human GH (hGH) [47] and other related cytokines [48] to their receptors, an accessory receptor may bind to mediate the biological signal. It has recently been shown that hGH-induced dimerization of the hGH receptor occurs through a sequential binding mechanism [47]. In the first step, an 1:1 complex is formed between hGH and the extracellular domain of its receptor-binding protein (hGHbp) (expressed in *Escherichia coli*), and in the second step an additional hGHbp is bound to give a 1:2 complex with hGH and hGHbp. Furthermore, it was demonstrated that a large excess of hGH dissociates the 1:2 complex into a monomeric complex.

Following binding, the GH receptor has been shown to be internalized [49]. The exact cellular mechanism is unknown but it is assumed to occur via cell surface mobilization and micro-aggregation into coated pits. Studies on the intracellular processing of GH by cultured rat adipocytes have shown that approx. 75% of internalized GH and GH receptors are targeted for lysosomal degradation [50]. The fate of the remaining 25% of the internalized GH receptors is currently unknown, but recent data from immunohistochemical studies indicate a nuclear localization of GH receptors in both rat and rabbit tissues [51], indicating that GH action is mediated at the nuclear level. Whether an activated GH receptor or GH binding protein can bind to specific regulatory DNA sequences, or nuclear transcription factors, has to await further investigation.

As mentioned in the introduction, some biological effects of GH seem to be dependent upon levels of circulating GH, whereas others depend on the pattern of GH secretion. It has therefore been suggested that different subtypes of GH receptors might exist, mediating different

functions of GH. Several studies have indicated the presence of structurally heterogeneous receptors with differing affinity for GH [39, 52], which might in part be explained by the existence of both monomeric and dimeric receptor complexes. The possibility that different receptor variants might give rise to different intracellular signals, led us to investigate whether a sex difference at the GH receptor level could explain the sex-specific steroid metabolism in rat liver. However, no major sex differences were found regarding number or affinity of GH binding sites in rat liver [53], neither is the ratio GH receptor/GH binding protein mRNA sexually differentiated (personal communication; B. Enberg and G. Norstedt). It remains to be elucidated whether there exist any differences in the fate of the internalized receptor that could explain the sexually differentiated expression of *P*4502C enzymes.

4. INTRACELLULAR MESSENGER MOLECULES

Although the GH receptor has been cloned from both human and rodent liver the signal transduction mechanisms for GH are poorly understood, particularly since the cloned GH receptor lacks sequence homology with receptors of known signal transduction mechanisms. Despite the lack of consensus sequence for tyrosine kinase, a primary event after GH receptor occupancy seems to be increased phosphorylation of the receptor on tyrosyl residues [54]. The tyrosine kinase activity is evidently tightly associated with the receptor, and the association of GH receptors with tyrosine kinase activity has been demonstrated in various cell types from different species [55]. These observations raise the possibility that there are different kinds of receptors for GH; one represented by the cloned liver GH receptor, and another by a tyrosine kinase-containing GH receptor. Whether this could explain some of the different effects on GH in rat liver has to await further investigation. No GH-mediated effects have as yet been shown to be dependent on tyrosine kinase activity. To study a potential role of such kinase in the GH-mediated induction of *P*4502C12, experiments have been initiated in which cultured primary rat hepatocytes have been treated with different tyrosine kinase inhibitors prior to addition of GH. No inhibitory compound tested so far has affected the GH response of *P*4502C12.

Several investigations suggest that phospholipid hydrolysis is of importance in the GH signaling process. We have shown that treatment of the cultured primary hepatocytes with GH for 30 s results in a 5-fold increase in diacylglycerol production [56]. In agreement with this, others have shown that GH administration to freshly isolated rat hepatocytes results in increased diacylglycerol formation [57]. These researchers did not find any evidence for simultaneous production of inositol phosphates, indicating a source of diacylglycerol other than phosphatidylinositol. In adipocytes, GH has been shown to stimulate phospholipase C-catalysed hydrolysis of phosphatidylcholine [58], but further studies are needed to find the source of diacylglycerol produced as an early response to GH in the liver.

The accumulating evidence for GH-induced production of diacylglycerol indicates that protein kinase C (PKC) could be a mediator of GH effects. However, when the primary hepatocytes were treated with the PKC activators 4β-phorbol 12-myristate 13-acetate (PMA) or *sn*-1,2-dioctanoylglycerol, in dose and time-course experiments in the presence or absence of ionomycin, no induction of *P*4502C12 was obtained [56]. On the other hand, down-regulation of PKC by PMA treatment, i.e. 24 h pretreatment, attenuated the ability of GH to induce *P*4502C12 mRNA expression. As shown in Fig. 1 the effect of PMA was dose-dependent, and approx. 50% inhibition of the GH effect

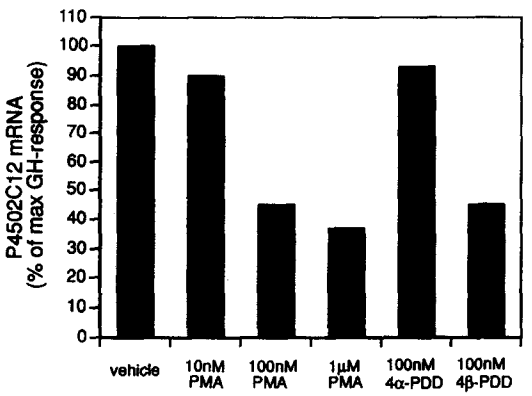

Fig. 1. Effects of PKC down-regulation by phorbol-ester pretreatment on GH-induction of steady state mRNA levels of *P*4502C12 in rat hepatocytes. At 66 h of culture age primary rat hepatocytes were incubated in the presence of PMA, 4α-phorbol 12,13-didecanoate (4α-PDD) or 4β-phorbol 12,13-didecanoate (4β-PDD). 24 h later the cells were incubated with or without GH for 8 h, in the presence or absence of phorbol-ester. Cells were harvested, total nucleic acid samples prepared and analysed for *P*4502C12 mRNA levels by solution hybridization. Results are expressed as % of the maximal GH response.

was observed at 100 nM PMA. The 4α-isomer of phorbol 12,13-didecanoate, inactive in PKC modulation, did not interfere with the GH-induction of *P*4502C12, whereas the active 4β-congener was as efficient as PMA in this context (Fig. 1). The potent kinase inhibitor staurosporine also interfered with the ability of GH to induce *P*4502C12 mRNA. These data indicate a permissive role for PKC in the GH-mediated induction of *P*4502C12 mRNA, and that another factor(s) is required together with PKC. Down-regulation of PKC was also shown to impair GH-induced mRNA expression of the insulin-like growth factor I (IGF-I) in the hepatocytes. Similar results have been reported from others studying GH effects in adipose tissue. GH effects on lipogenesis [59], lipolysis [60] and expression of the protooncogene c-*fos* [61] in rat adipose tissue and preadipocytes have been shown to be blocked by inhibitors of PKC. Taken together, PKC might act as a common mediator of different GH effects.

Since different signal transduction pathways can interact to regulate cell function, and since staurosporine also inhibits other kinases than PKC, the possible involvement of cyclic AMP-dependent protein kinase (PKA), in the PKC-dependent GH induction of *P*4502C12 has been investigated [56]. The combination of the PKA activator forskolin and PKC activators did not cause any accumulation of *P*4502C12 mRNA. Instead, a reduced effect on the basal expression of *P*4502C12 was observed when the cells were treated with forskolin, independently of simultaneous PKC stimulation. Furthermore, when forskolin was added together with GH, the induction of *P*4502C12 mRNA was reduced by 50%. When the cyclic AMP analogue 8-Br-cAMP was used instead of forskolin, similar data were obtained. Interestingly, GH-induced IGF-I mRNA expression was potentiated 2-fold by elevated cyclic AMP levels in the cells.

The GH-induced accumulation of *P*4502C12 mRNA has been shown to be dependent on on-going protein synthesis [37]. In the same experiments, the GH-stimulated induction of IGF-I mRNA did not show cycloheximide sensitivity. Thus, it would appear that GH can activate more than one signaling pathway in the hepatocytes. However, whether different GH-activated signaling molecules are mediating the different modes of GH regulation on the CYP2C genes is as yet not known.

5. TRANSCRIPTIONAL ACTIVATION OF THE CYP2C GENES

Whether the direct and divergent effects of GH on the hepatic expression of *P*4502C7, 2C11, 2C12 and 2C13 are exerted at the transcriptional level has been studied both *in vivo* and in primary cultures of hepatocytes [14]. By using a specific and sensitive run-on assay it was clearly demonstrated that GH exerts its actions via transcriptional control of CYP2C7, 2C11, 2C12 and 2C13. The GH-mediated effects of transcriptional activation on these genes *in vivo* are summarized in Table 2.

Hypophysectomy dramatically suppresses the transcription of CYP2C11 in males, CYP2C12 in females, CYP2C7 in both males and females, and induces the transcription of CYP2C11 and CYP2C13 in females. Hypophysectomized rats were treated with GH continuously or discontinuously to mimic the sex-specific secretory pattern of GH. A male-like pattern was created by single daily injections of GH subcutaneously into hypophysectomized animals of both sexes. This pattern induced the transcriptional rate of CYP2C11 in both male

Table 2. Transcriptional regulation of CYP2C7, CYP2C11, CYP2C12 and CYP2C13 gene expression *in vivo* by treatment of hypophysectomized rats with hGH

	CYP2C12	CYP2C7	CYP2C11	CYP2C13
Normal male	5	30	100	100
Normal female	100	100	10	7
Hypox. male	5	6	15	70
GH-injected hypox. male	8	4	120	140
GH-infused hypox. male	80	330	12	5
Hypox. female	5	4	11	50
GH-injected hypox. female	7	4	157	90
GH-infused hypox. female	190	350	13	10

Hypophysectomized (hypox.) rats were treated with hGH (1.0 IU/kg) either by continuous infusion from osmotic minipumps, or in a pulsatile fashion by daily subcutanuous injections for 6 days. Liver nuclei were purified and the rate of transcription from each gene was determined in run-on analysis (Legraverend *et al.*, 1991). Values in normal males (CYP2C11 and CYP2C13) or normal females (CYP2C12 and CYP2C7) are given as 100.

and female hypophysectomized rats. The transcription of CYP2C13 was increased by hypophysectomy in both sexes, and a small increase in transcription after discontinuous treatment with GH could be seen. The male-like pattern of GH administration did not increase the transcription of CYP2C12 in hypophysectomized animals, neither did it activate CYP2C7. The fact that transcription of CYP2C7 is not restored by this treatment is remarkable since this gene is actively transcribed in the normal male, and it is known that the same treatment activates CYP2C11. This is, however, in agreement with previously published data on *P*4502C7 [10], which indicates that the discontinuous mode of GH administration used is not sufficient to induce *P*4502C7. In relation to this, Sasamura *et al.* [11] managed to increase *P*4502C7 mRNA in hypophysectomized males by two daily injections, although not to the same extent as continuous GH administration. The female-like pattern of GH in serum was created by implantation of an osmotic minipump which gives a constant level of GH in serum. This pattern increased transcription of CYP2C12 and CYP2C7 above the level of normal animals after 6 days of treatment. The transcription from the CYP2C11 gene was not affected by this mode of GH administration, but CYP2C13 was dramatically repressed by this treatment.

In the system for primary culture of adult rat hepatocytes described above, it has been possible to demonstrate a direct action of GH on these CYP2C genes. The continuous presence of GH added to a hormone-free medium was sufficient to induce the transcription of CYP2C12 and to repress the transcription of CYP2C11 and CYP2C13 in male hepatocytes, which agrees very well with our *in vivo* findings. As mentioned above, the GH-induced accumulation of *P*4502C12 mRNA is blocked in cycloheximide treated cells. This need for on-going protein synthesis has also been shown at the level of transcription, indicating that newly synthesized proteins are required in the transcriptional activation of CYP2C12 by GH. Interesting, Yoon *et al.* [62] have presented evidence for a GH-activated hepatic nuclear factor interacting with a regulatory sequence in the serine protase inhibitor 2.1 gene. The activity of this as yet unidentified factor is not blocked by protein synthesis inhibition, suggesting that a preexisting factor mediates this effect of GH. Taken together, the different inductive and repressive effects of GH on these genes are all exerted at the transcriptional level, and both the inductive and repressive effects of the continuous presence of GH are direct effects on the hepatocyte.

6. IDENTIFICATION OF GH RESPONSIVE ELEMENT(S)

One of our immediate goals is to identify *cis*-acting elements and their corresponding *trans*-acting factors responsible for the GH-dependent regulation of members of the *P*4502C subfamily. One question arising from the *in vivo* and *in vitro* studies of the GH regulation of *P*4502C enzymes is: how does one and the same hormone (i.e. GH) by means of differential secretory pattern control the expression of individual *P*450 enzymes? At the moment this is an open question, but it seems reasonable to assume that more than one GH signal transduction pathway operates in the liver, to account for the divergent effects of GH on individual *P*4502C expression. No GH response element has thus far been identified, but much effort is now being concentrated on the 5′-flanking regions of the CYP2C11 and CYP2C12 genes.

In an attempt to determine whether regulatory DNA sequences responsible for the GH-dependent as well as liver- and sex-specific regulation of the CYP2C12 gene could reside close to the promoter, we used 700 base pairs of the 5′-flanking region in an *in vitro* transcription assay. This was done by using liver and kidney nuclear extracts prepared from intact male and female rats according to the procedure described by Gorski *et al.* [63], and the G-less cassette of Sawadogo and Roeder [64]. The AdML-50 plasmid containing a 180 bp G-less cassette driven by the AdML minimal promoter served as an internal control for polymerase II efficiency. After normalization against the activity of the AdML-50 promoter, no difference in transcription could be seen either between liver nuclear extracts from intact or hypophysectomized rats of both sexes or between liver and kidney extracts from intact females. Another example where the sex-specific transcription of a gene could not be reconstituted *in vitro* is the mouse testosterone 16α-hydroxylase (Cyp2d-9) [65]. The lack of regulation of the CYP2C12 gene *in vitro* could be explained by the fact that important

sequence(s) or factors for GH regulation might be missing, or that a certain chromatin structure is needed to obtain regulation.

The 5′-flanking regions of CYP2C11 and CYP2C12 are now being transiently transfected into cells driving the expression of the reporter gene for chloramphenicol acetyltransferase (CAT). Both primary hepatocytes and Chinese hamster ovary (CHO) cells have been used for transient transfection experiments. CAT-constructs with 6kb of the 5′-flanking region of CYP2C12 transfected into primary hepatocytes show low basal activity, not affected by GH. Although the CYP2C12 gene is liver-specific, CAT-constructs with up to 6kb of the 5′-flanking region of this gene are expressed at high levels in both transiently and stably transfected CHO cells, suggesting that the liver-specificity determining DNA motif might reside further upstream than 6kb, or within the gene. One example of a gene that has its liver-specificity controlling element far upstream from the promoter is the tyrosine amino transferase gene [66]. The lack of liver-specificity of CYP2C12 expression in transient transfections is in agreement with the results from the *in vitro* transcription study referred to above.

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