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Hypophysectomy Differentially Alters P-450 Protein Levels and Enzyme Activities in Rat Liver: Pituitary Control of Hepatic NADPH Cytochrome P-450 Reductase

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SUMMARY

Pituitary-determined hormones regulate the expression of hepatic cytochromes P-450 through processes involving both negative and positive controls. Accordingly, protein levels of several P-450 forms are elevated in rat liver following hypophysectomy [P-450 forms designated 2a (gene IIIA2), RLM2 (gene IIA2), and PB-4 (gene IIB1)], whereas protein levels of others are suppressed [e.g., P-450 2c (gene IIC11)]. In the present study, microsomal steroid hydroxylase activities associated with these same P-450 forms were found to be decreased by hypophysectomy, despite elevations in protein levels for several of them. Studies were, therefore, undertaken to determine the biochemical basis for this decrease in microsomal P-450 enzyme specific activity. *In vivo* treatment of hypophysectomized rats with gonadotropin, under conditions that restore heme to testis P-450, and heme reconstitution experiments carried out with liver homogenates indicated that a deficiency in P-450-associated heme is unlikely to account for the observed decreases in liver P-450 enzyme specific activity. Analysis of the flavoprotein P-450 reductase, however, revealed that the reductase protein and its associated cytochrome *c* reductase activity are decreased by 50 to 75% in liver microsomes isolated from hypophysectomized rats. Moreover, supplementation of isolated liver microsomes with exogenous purified P-450 reductase stimulated microsomal steroid hydroxylase activity preferentially in the hypophysectomized rats, to levels consistent with the observed changes in P-450 protein levels. Thus,a deficiency in P-450 reductase, which is a rate-limiting component for many P-450-dependent hydroxylation reactions, appears to be responsible for the decrease in steroid hydroxylase specific activity in the hypophysectomized rats. Although growth hormone, adrenocorticotropic hormone, and chorionic gonadotropin were each ineffective at restoring hepatic P-450 reductase when administered to hypophysectomized rats, substantial restoration of P-450 reductase levels could be achieved by treatment of the hypophysectomized rats with thyroxine. Thyroxine treatment of these rats also elevated the microsomal steroid hydroxylase activities associated with the individual hepatic P-450 forms to levels commensurate with their respective P-450 protein levels. These results establish that hepatic P-450 reductase is subject to hormonal controls that are distinct from those governing cytochrome P-450 expression and further demonstrate the complexity of endocrine control of hepatic steroid hormone metabolism.

The mixed function oxidase system, located in the endoplasmic reticulum, consists of a family of enzymes that oxidatively metabolize many foreign substances, as well as a variety of endogenous compounds including steroid hormones and bile acids. The protein components of the mixed function oxidase system include the multiple forms of cytochrome P-450 and a flavoprotein, P-450 reductase, which catalyzes electron transfer from NADPH to cytochrome P-450 and is an obligatory component for all microsomal P-450-dependent hydroxylation reactions (1).

The expression of P-450 proteins in rodent liver is under complex hormonal control. In the rat, both gonadal and pituitary hormones contribute to the maintenance of constitutively expressed P-450 enzymes in adult liver through processes involving both positive and negative controls (2–5). Accordingly, levels of some P-450 forms[1] in adult rat liver are markedly decreased after hypophysectomy [P-450 2c (gene IIC11) and P-

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[1] The designations given in this report to individual rat hepatic P-450 forms (P-450 enzymes) can be compared with standard gene designations (6) and alternative protein nomenclature as summarized elsewhere (7). Briefly, P-450 2c (testosterone 2α/16α-hydroxylase; gene IIC11) (8) corresponds to the preparations designated P-450h (9), UT-A (10), RLM5 (11), male (3), M1 (12), and 16α (2). P-450 RLM2 (testosterone 15α-hydroxylase; gene IIA2) (5, 13) is designated according to Jansson *et al.* (14). P-450 PB-4 (testosterone 16β/16α-hydroxylase; gene IIB1) (15) corresponds to preparations designated P-450b (9) and PB-B (10). The term P-450 2a is used to designate the constitutive, adult male-specific liver microsomal P-450 enzyme catalyzing steroid hormone 6β-hydroxylation. A cDNA clone (PCN2) that appears to encode this enzyme has been identified and sequenced (gene IIIA2) (16). This form appears to be equivalent to a form recently designated 6β-1/PB1 (17, 18).

ABBREVIATIONS: P-450, cytochrome P-450; P-450 reductase, NADPH P-450 reductase; hCG, human chorionic gonadotropin; ACTH, adrenocorticotropic hormone.

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450 2d (gene IIC12) (2, 3)], whereas levels of other P-450 forms are significantly elevated [P-450 2a (gene IIIA2), P-450 RLM2 (gene IIA2), and P-450 PB-4 (gene IIB1) (5, 19)]. In several instances, however, the effects of hypophysectomy on P-450 protein levels are inconsistent with the observed response of the corresponding P-450-dependent microsomal hydroxylase activities to this surgical treatment. We have recently observed that rat hepatic P-450j (IIE1) is elevated 3- to 5-fold following hypophysectomy, without a significant increase in its associated microsomal aniline hydroxylase activity.[2] Similarly, hypophysectomy elevates P-450 2a protein about 2-fold in adult male rat liver (5) but microsomal androstenedione 6β-hydroxylation, which is P-450 2a-dependent in uninduced male rats (4), is decreased by 15–25% (20). Finally, P-450 forms 3 (gene IIA1)[2] and PB-4 (19) can be elevated severalfold by hypophysectomy, yet their respectively associated microsomal steroid 7α-hydroxylase and pentoxyresorufin *O*-dealkylase activities are largely unchanged. Hypophysectomy, thus, has effects on hepatic P-450 activities that cannot be explained on the basis of changes in P-450 apoprotein levels as determined by Western blot analysis. In the present study, the effect of hypophysectomy on the microsomal steroid hydroxylase specific activity of four individual P-450 forms is directly examined, and the biochemical basis for the observed discrepancy between P-450 protein and activity levels identified.

Materials and Methods

Animals. Untreated, sham-operated, and hypophysectomized Fischer 344 male and female rats were purchased from Taconic Inc. (Germantown, NY). Hypophysectomy was performed by the supplier at 8 weeks of age and the animals were provided with 5% glucose in water for the first week after surgery. The completeness of hypophysectomy was verified by the absence of weight gain over a 2-week period (≤5 g increase in weight). Hormonal treatments (see below) were initiated at 10–11 weeks of age and continued for 7 days. Animals were then sacrificed by cervical dislocation after asphyxiation under CO_2 and the livers were immediately removed, minced in ice cold 1.15% KCl, and then frozen in liquid nitrogen. Livers were subsequently stored at −80° until required for microsome preparation. Methods used for preparation of microsomes from individual livers are described elsewhere (21).

Hormone treatments. Human growth hormone (2.4 IU/mg) was obtained from the National Hormone and Pituitary Program and was administered (a) by twice daily injection as described previously (5) or (b) by continuous infusion at either 0.6 or 5 μg/rat/hr for 7 days via a subcutaneously implanted osmotic minipump (Model 2001; Alza Inc., Palo Alto, CA). Rats were anesthetized for the implantation using ketamine (44–50 mg/kg, intramuscularly). ACTH (10 IU/kg; Sigma Chemical Co., St. Louis, MO) and hCG (150 or 500 IU/kg; Sigma CG-5) were dissolved in 10 mM KP_i buffer (pH 8.3), 0.9% saline, and were administered to the hypophysectomized rats at doses based on earlier studies (21–23). Thyroxine (Sigma) was dissolved at 0.62 mg/ml in 10 mM KP_i (pH 9.5), 0.9% saline buffer, then diluted to 75 μg/ml by the addition of 10 mM KP_i buffer (pH 8.3), the final pH adjusted to 8.3 with HCl. The dose chosen (50 μg/kg) has been reported to render thyroidectomized rats euthyroid (24). Hormones or the appropriate vehicle controls were administered daily for 7 days as a subcutaneous injection in a total volume of 0.3 ml/150 g of rat.

Immunochemical and enzymatic analysis. Western blotting, immunochemical detection using antibodies to P-450 reductase and to P-450 forms 2a, RLM2, and 2c, and quantitation of the relative P-450 levels by laser densitometry of the nitrocellulose blots were carried out as described in detail elsewhere (5). Polyclonal antibody to rat liver P-450 reductase was a generous gift of Dr. F. P. Guengerich, Vanderbilt University. Liver microsomal testosterone 6β-, 15α-, and 16α-hydroxylase activities were measured as described (5), except that purified P-450 reductase was only included where indicated. Androstenedione 16β-hydroxylation was determined in a similar manner, except that the metabolites were resolved on silica gel thin layer plates developed in CH_2Cl_2/ethanol (97:3) followed by $CHCl_3$/ethyl acetate (1:1). The P-450 PB-4-dependence of androstenedione 16β-hydroxylase activity catalyzed by untreated and hypophysectomized rat liver microsomes was determined by antibody inhibition experiments (25). In some experiments, microsomal incubations containing 60 μg of protein in 0.4 ml were supplemented with up to 1.2 units of exogenous P-450 reductase, purified to apparent protein homogeneity from phenobarbital-induced rat or rabbit liver using methods described elsewhere (15) (1 unit = 1 μmol of cytochrome *c* reduced/min at pH 7.7 and 30°). P-450 reductase activity was assayed by monitoring the rate of cytochrome *c* reduction at 550 nm in 0.3 M KP_i, pH 7.7, at 30° in 1-ml incubations containing 20 μg of microsomal protein and 5 mg/ml cytochrome *c*. Serum testosterone levels were determined by solid phase radioimmunoassay using a commercially available kit (National Diagnostics Products Corp., Los Angeles, CA). All analytical results are presented as the mean ± standard error of individual microsome preparations isolated from *n* individual rat livers assayed in a given single experiment.

Results

Hypophysectomy differentially affects P-450 protein levels and P-450-catalyzed steroid hydroxylase activities in isolated liver microsomes. Adult rats were hypophysectomized and the protein content and associated microsomal steroid hydroxylase activities of specific P-450 forms were then determined in isolated liver microsomes (Table 1). In each case examined, the observed changes in steroid hydroxylase activities after hypophysectomy did not reflect the accompanying changes in the corresponding P-450 protein levels. Thus, the male-specific P-450 forms 2a and RLM2 were elevated in male rats by hypophysectomy ~2-fold, whereas their respective associated testosterone 6β- and 15α-hydroxylase activities (4, 5) were decreased, by as much as 35%. Hypophysectomy of adult female rats dramatically induced both these P-450 forms (>10-fold increase), but only a 2-fold increase (6β-hydroxylation) or no change (15α-hydroxylation) in their microsomal steroid hydroxylase activities was observed (Table 1). Hypophysectomy of male rats decreased the microsomal content of the male-specific P-450 2c to about 30% of sham-operated controls, whereas the microsomal testosterone 16α-hydroxylase activity catalyzed by this P-450 form (8) was decreased more significantly, to ~18% of control for the experiment shown.[3] Overall, hypophysectomy reduced the microsomal specific activities of these three P-450 forms significantly (relative specific activity = 0.26–0.62; Table 1, *last column*). Finally, although the phenobarbital-inducible P-450 PB-4 was induced by hypophysectomy severalfold from very low constitutive levels, in both male and female rats (19), P-450 PB-4-dependent microsomal 16β-hydroxylase activity was largely unchanged (data not shown).

Effects of hypophysectomy on P-450 heme. In rat testes, hypophysectomy leads to a dramatic loss of microsomal P-450 heme and P-450-dependent steroid 17α-hydroxylase activity with no apparent effect on P-450 apoprotein levels (26). A similar loss of P-450 heme in liver could account for the

[2] D. J. Waxman, J. J. Morrissey, and G. A. LeBlanc. *Endocrinology* (in press).

[3] Although P-450 2c protein was decreased by a variable 60–85% following hypophysectomy, the decrease in P-450 2c-associated microsomal hydroxylase activities was always more extensive (cf., Table 4).

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TABLE 1

Influence of hypophysectomy on microsomal specific activities of rat liver P-450 forms

Liver microsomes were isolated from untreated, sham-operated, and hypophysectomized (hypox) adult male and female rats (n = 2 to 4 individual rats/group, as indicated). Microsomes were then analyzed for protein levels of P-450 forms 2a, RLM2, and 2c by quantitative Western blotting using specific antibodies (Protein content) and for the respective associated microsomal testosterone 6β, 15α, and 16α hydroxylase activities of these P-450s (Hydroxylase activity). Relative specific activities calculated from these data are shown in the last column.

Microsomes	P-450 form			Relative Specific activity[c]
	n	Protein content[a]	Hydroxylase activity[b]	
P-450 2a/testosterone 6β-hydroxylation				
Untreated male	2	100 ± 26	0.42 ± 0.08	=1.00
Sham male	2	108 ± 10	0.44 ± 0.01	0.97
Hypox male	4	191 ± 11	0.39 ± 0.09	0.49[d,e]
Untreated female	2	18 ± 3	0.13 ± 0.01	1.72[f]
Sham female	4	11 ± 3	0.10 ± 0.03	2.16[f]
Hypox female	3	200 ± 12	0.22 ± 0.01	0.26[d]
P-450 RLM2/testosterone 15α-hydroxylation				
Untreated male	2	100 ± 21	89 ± 2	=1.00
Sham male	2	107 ± 8	101 ± 3	1.06
Hypox male	4	198 ± 16	66 ± 14	0.37[d]
Untreated female	2	<3	23 ± 1	
Sham female	4	<3	25 ± 2	
Hypox female	3	31 ± 9	26 ± 3	0.94
P-450 2c/testosterone 16α-hydroxylation				
Untreated male	2	100 ± 2	2.26 ± 0.04	=1.00
Sham male	2	87 ± 5	2.06 ± 0.08	1.05
Hypox male	4	27 ± 5	0.38 ± 0.10	0.62[d]
Untreated female	2	<3	0.14 ± 0.02	
Sham female	4	<3	0.08 ± 0.02	
Hypox female	3	<3	0.08 ± 0.01	

[a] Relative P-450 protein levels (mean ± standard error for n individual rats), with the P-450 protein levels present in untreated male microsomes set at 100.

[b] Expressed as pmol (15α-hydroxylation) or nmol (6β- and 16α-hydroxylation) per min per mg of microsomal protein (mean ± standard error).

[c] Expressed relative to the untreated male group. Values calculated from the ratio $(A/B)/(C/D)$, where A = hydroxylase activity of the group, B = mean protein content of the group, C = hydroxylase activity of the untreated male group, and D = protein content of the untreated male group.

[d] A significant decrease in microsomal hydroxylase specific activity following hypophysectomy.

[e] Values are ~2-fold higher than the untreated and sham-operated male groups, suggesting that a portion of the low level 6β-hydroxylase activity of the female microsomes may be catalyzed by P-450 forms other than P-450 2a.

TABLE 2

Effect of hCG treatment on testosterone hydroxylase activities in hypophysectomized rats

Untreated and hypophysectomized rats were administered hCG (500 IU/kg, subcutaneously daily for 7 days before sacrifice). Liver microsomal testosterone (T) hydroxylation activities and serum testosterone levels were determined as described under Materials and Methods (mean ± standard error for n = 3 individual rats/group).

	6β-OH-T	15α-OH-T	16α-OH-T	Serum testosterone
	nmol/min/mg			*ng/ml*
Untreated male	0.53 ± 0.08	0.11 ± 0.02	1.71 ± 0.06	1.6 ± 0.9
Untreated male + hCG	0.51 ± 0.03	0.12 ± 0.01	1.80 ± 0.05	22 ± 6
Hypox male	0.36 ± 0.02	0.06 ± 0.01	0.14 ± 0.02	<0.15
Hypox male + hCG	0.39 ± 0.04	0.07 ± 0.01	0.20 ± 0.05	25 ± 11
Hypox/untreated (−hCG)[a]	0.68	0.54	0.08	
Hypox/untreated (+hCG)	0.76	0.58	0.11	

[a] Ratio of hydroxylase activities in the hypophysectomized group as compared with the untreated group. These ratios indicate that the low hydroxylase activities of the hypophysectomized rat liver microsomes (Hypox/Untreated <1) are not appreciably increased following hCG treatment.

decreases in hepatic microsomal steroid hydroxylase specific activity described above. Administration of the gonadotropin hCG reverses these effects of hypophysectomy on the testis P-450 by restoration of P-450 heme as well as P-450-dependent steroid 17α-hydroxylase activity (26). hCG treatment of adult male hypophysectomized rats did not, however, reverse the decrease in liver microsomal steroid hydroxylase specific activities, as evidenced by its lack of effect on microsomal steroid 6β-, 15α-, and 16α-hydroxylation (Table 2) and on the microsomal specific content of P-450 forms 2a, RLM2, and 2c (data not shown). Serum testosterone levels, however, were elevated significantly by the hCG treatment, indicating that testis P-450 heme and steroid 17α-hydroxylase activity were effectively restored following gonadotropin administration.

The inability of hCG to restore hepatic steroid hydroxylase activity in hypophysectomized rats might indicate, however, that the association of heme with P-450 is controlled by different hormonal factors in liver, compared with testes. Attempts were, therefore, made to directly supplement liver homogenates prepared from both untreated and hypophysectomized rats with exogenous hemin (50 μM) in buffer containing reduced glutathione (10 mM) using a procedure employed previously to restore heme to microsomal P-450 isolated from allylisopropylacetamide-treated rats (27). Microsomes were isolated from hemin-incubated liver homogenates and then assayed for steroid hydroxylase activity. No hemin stimulation of microsomal hydroxylase activities was observed and, indeed, no increase in microsomal P-450 heme was achieved (data not shown). These findings indicate that a deficiency in P-450-associated heme is unlikely to be the basis for the observed low steroid hydroxylase specific activities in hypophysectomized rat liver microsomes.

Pituitary dependence of P-450 reductase expression. Steroid hydroxylation in liver microsomes requires both the P-450 hemeprotein and electron input from NADPH mediated by a flavoprotein, P-450 reductase. This reductase is under developmental control and appears to be hormonally regulated in rat liver (e.g., Refs. 28 and 29), suggesting that it may be responsive to some of the pituitary factors that are known to regulate the cytochromes P-450 and other hepatic proteins. The effect of hypophysectomy on liver microsomal P-450 reductase activity and protein levels was, therefore, assayed. Three weeks after hypophysectomy, P-450 reductase activity in isolated liver microsomes was decreased by ~75% in males and by ~50% in females (Fig. 1A). Western blot analysis revealed that this activity decrease is associated with a parallel decrease in P-450 reductase protein (Fig. 1B). Thus, the decrease in microsomal P-450 reductase activity in the hypophysectomized rats is not due to loss of a flavin cofactor or deactivation of existing reductase protein but probably results from a decrease in P-450 reductase expression.

Restoration of microsomal steroid hydroxylase activities in hypophysectomized rat liver microsomes by P-450 reductase supplementation. The above findings suggest that the low steroid hydroxylase specific activity in hypophysectomized rat liver microsomes directly results from a loss of P-450 reductase protein. This hypothesis was tested by supplementation of isolated liver microsomes with purified P-450 reductase. As seen in Fig. 2A, exogenous purified P-450 reduc-

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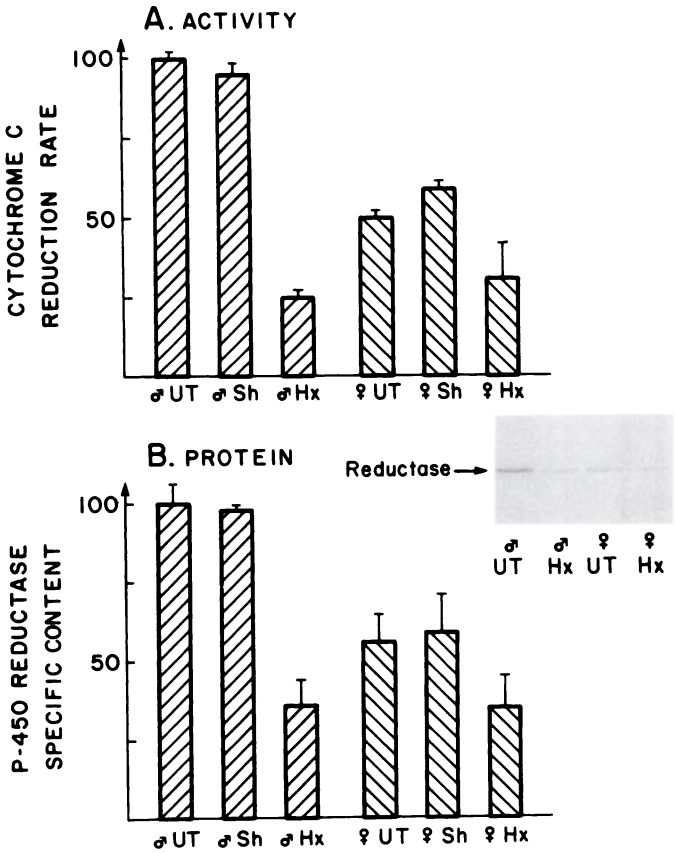

Fig. 1. Loss of liver microsomal P-450 reductase activity (A) and P-450 reductase protein (B) following hypophysectomy of adult rats. Male and female rats were untreated (*UT*), (n = 2/group), sham-operated (*Sh*) (n = 3), or hypophysectomized (*Hx*) (n = 4) and liver microsomes were prepared as described under Materials and Methods. Shown are the relative cytochrome *c* reduction rates (UT male group, set at 100 = 319 nmol/min/mg) and P-450 reductase specific contents (UT male group, set at 100 = 0.15 nmol/mg) determined in the isolated liver microsomes by Western blotting, in comparison with a purified rat liver P-450 reductase standard. Values are presented as mean ± standard error for n individual samples assayed in a given single experiment. Shown in the *inset* of B is a portion of a representative Western blot of P-450 reductase protein in liver microsomes from untreated and hypophysectomized male and female rats.

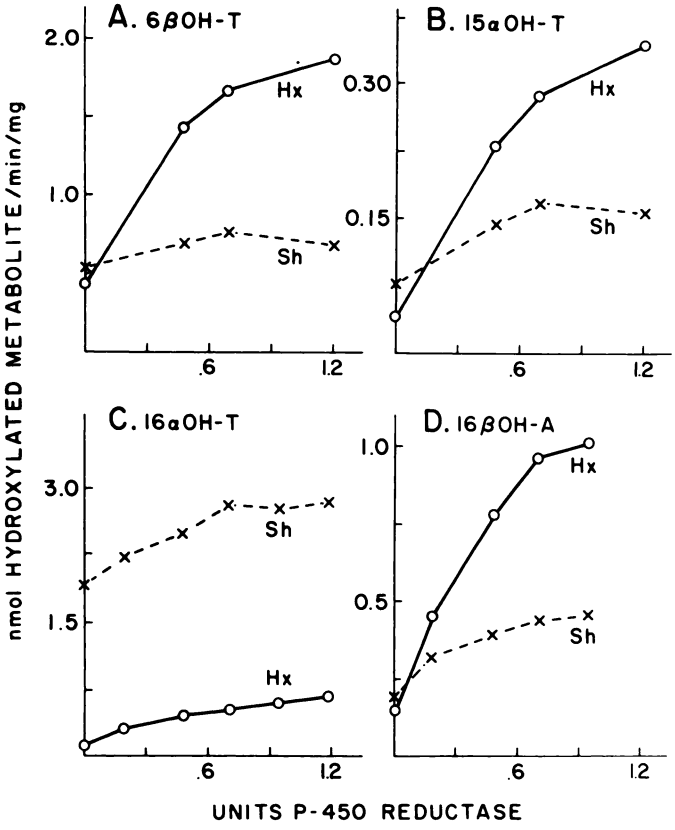

Fig. 2. Effect of P-450 reductase supplementation on microsomal steroid hydroxylation rates: preferential stimulation of activities catalyzed by hypophysectomized rat liver microsomes. Liver microsomes (60 μg of protein pooled from three or four sham-operated (*Sh*) or hypophysectomized (*Hx*) adult male rats were assayed for hydroxylation of testosterone (*T*) at the 6β- (A), 15α- (B), and 16α-positions (C) and for androstenedione 16β-hydroxylation (D). Assays were performed in the presence of 0 to 1.2 units of P-450 reductase purified from rat liver (A and B) and rabbit liver (C and D). Both preparations of P-450 reductase preferentially stimulated, to a similar degree, steroid hydroxylation catalyzed by the hypophysectomized rat liver microsomes. Antibody inhibition experiments (not shown) revealed that the portion of microsomal androstenedione 16β-hydroxylase activity that is P-450 PB-4-dependent (see Materials and Methods) measured in the presence of 0.75 units of purified P-450 reductase was ~3-fold higher in the hypophysectomized liver microsomes (0.48 nmol/min/mg) as compared with the sham-operated controls (0.15 nmol/min/mg). In contrast, in the absence of added reductase, the P-450 PB-4-dependent 16β-hydroxylase activity was similar in the two groups (0.07 to 0.08 nmol/min/mg).

tase preferentially stimulates testosterone 6β-hydroxylation catalyzed by the hypophysectomized rat liver microsomes. In the presence of saturating levels of added P-450 reductase, hypophysectomy is seen to elevate microsomal 6β-hydroxylation about 2.5-fold, a finding consistent with the 2-fold elevation of P-450 2a protein shown in Table 1. Likewise, when assayed in the presence of added P-450 reductase, P-450 RLM2-catalyzed microsomal testosterone 15α-hydroxylation is elevated to levels that better reflect the increase in P-450 RLM2 protein (Fig. 2B). Testosterone 16α-hydroxylase activity, which is almost undetectable in hypophysectomized animals in the absence of added P-450 reductase, is restored to about 25% of control values upon reductase supplementation (Fig. 2C), better reflecting the partial loss of P-450 2c following hypophysectomy (cf., Table 1). Finally, an increase in P-450 PB-4-dependent androstenedione 16β-hydroxylase activity is also achieved in the hypophysectomized rat liver microsomes, but only when the assays are carried out in the presence of exogenous P-450 reductase (Fig. 2D).

Hormonal control of P-450 reductase. The significant loss of P-450 reductase following hypophysectomy indicates that this enzyme is dependent on some pituitary-derived factor, either directly or indirectly, for maintenance of full expression. Experiments were, therefore, carried out to identify the pituitary factors required for hepatic expression of P-450 reductase. Initially, the effect of growth hormone was examined, because this pituitary product is known to be involved in both positive and negative regulation of a number of liver proteins, including several forms of cytochrome P-450. However, growth hormone replacement in hypophysectomized rats did little to restore microsomal P-450 reductase, independent of whether the hormone was given (a) by intermittent (twice daily) injection, a protocol (2) designed to mimic the pulsatile growth hormone secretion pattern of male rats (Table 3, Experiment A), or (b) by continuous infusion, to achieve the more continuous pattern of growth hormone secretion that is characteristic of adult female rats (Fig. 3). In fact, continuous infusion of growth

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TABLE 3

Effect of pituitary-determined hormones on P-450 reductase activity in hypophysectomized adult male rats

Hormones were administered to untreated, sham-operated and hypophysectomized (hypox) rats daily for 7 days as described under Materials and Methods, (n = 2 to 5 individual rats/group, as indicated). Growth hormone was given by twice daily injection at 50 μg/rat (GHi) or by continuous infusion via osmotic minipumps at 0.6 μg of hormone/hr (GHp), hCG at 150 IU/kg, and thyroxine (T4) at 50 μg/kg. Liver microsomes were then isolated and cytochrome c reduction rates were determined as described under Materials and Methods.

Microsomes	n	Reductase activity	
		nmol/min/mg	%
Experiment A			
Sham male	2	305 ± 13	=100
Hypox male	4	80 ± 5	26
+ GHi	3	92 ± 4	30
Experiment B			
Untreated male	3	329 ± 4	=100
+ hCG	3	324 ± 27	98
+ T4	3	498 ± 22[a]	151
Hypox male	3	57 ± 5	17
+ hCG	3	69 ± 4	21
+ T4	3	215 ± 6[b]	65
Experiment C			
Sham male	5	310 ± 25	=100
Hypox male	2	83 ± 6	27
+ GHp	3	78 ± 12	25
+ hCG	2	93 ± 3	30
+ ACTH	2	130 ± 15	42
+ T4	2	186 ± 3[b]	60
+ T4 + GHp	3	180 ± 10[b]	58
+ all four[c]	3	230 ± 25[b,d]	74

[a] Significantly greater than untreated male group at $p \leq 0.01$ (Student's t test).
[b] Significantly greater than hypox treatment alone at $p \leq 0.01$ (Student's t test).
[c] GHp + hCG + ACTH + T4.
[d] Not significantly different than T4 treatment alone at $p \leq 0.05$ (Student's t test).

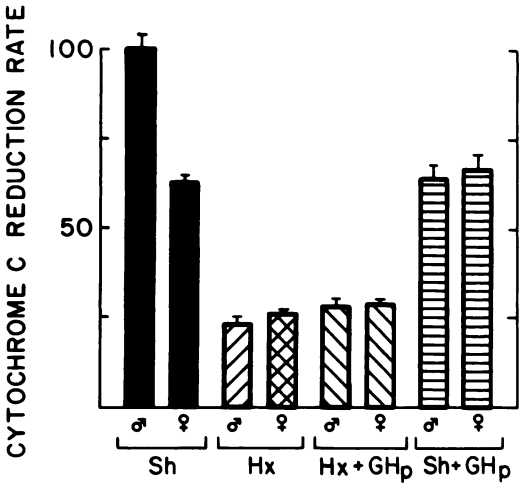

Fig. 3. Influence of growth hormone on P-450 reductase levels. Shown are relative rates of cytochrome c reduction determined for liver microsomes isolated from rats that were sham-operated (*Sh*) or hypophysectomized (*Hx*) and (as indicated) were subsequently infused with growth hormone (5 μg/hr for 7 days) via osmotic minipumps (Hx + GHp and Sh + GHp, respectively) (n = 2 to 4 rats/group). Similar effects were seen when growth hormone was administered at an 8-fold lower dose (0.6 μg/hr for 7 days), which was also found to be sufficient for the feminization of P-450 enzyme expression in the same liver samples (not shown).[2] Reductase activity of the sham-operated male group (value set at 100) was equal to 311 nmol of cytochrome c reduced/min/mg of microsomal protein.

hormone in intact male rats decreased P-450 reductase by ~35%, i.e., to the lower level characteristic of adult female rats (Fig. 3). This indicates that continuous serum growth hormone may exert a suppressive influence that contributes to the lower level of P-450 reductase expression in female rat liver but is not involved in maintaining full expression of the reductase in intact rats.

Because the above experiments indicate that the loss of growth hormone is not responsible for the decrease in P-450 reductase following hypophysectomy, other pituitary-dependent hormones were considered for their effect on the expression of hepatic P-450 reductase (Table 3, Experiments B and C). Administration of hCG and the resultant stimulation of testosterone production by the testes had no significant effect on P-450 reductase levels in hypophysectomized rats. This is consistent with the minor effect that castration has on P-450 reductase levels (e.g., Ref. 4). In contrast, ACTH stimulation of adrenal steroid hormone production resulted in a small elevation of P-450 reductase levels in the hypophysectomized rats. A more significant (albeit still incomplete) reversal of the decrease in P-450 reductase expression following hypophysectomy was obtained by treatment with thyroxine, which elevated P-450 reductase activity levels from 17–27% of the sham-operated control to 60–65% of control (Table 3). P-450 reductase protein was similarly elevated by thyroxine treatment of the hypophysectomized rats (increase from 21% to 78% of the sham-operated control level; data not shown). Thyroxine was also effective in stimulating P-450 reductase expression in intact rats to 50% above control values (Table 3, Experiment B). The combined administration of thyroxine with growth hormone, hCG, and ACTH resulted in a somewhat more complete restoration (~75%) of P-450 reductase activity in hypophysectomized rats but was not statistically different than the response to thyroxine alone (Table 3, Experiment C).

Microsomal steroid hydroxylation in thyroxine-treated rats. Untreated and hypophysectomized rats were treated with thyroxine under conditions that resulted in an elevation of microsomal P-450 reductase. The effect of this treatment on the microsomal levels of P-450 forms 2a, RLM2, and 2c and their associated testosterone 6β-, 15α-, and 16α-hydroxylase activities was then determined (Table 4). Although thyroxine treatment had a variable effect on P-450 protein levels in the hypophysectomized rats (25% decrease in P-450 2a, 45% decrease in P-450 RLM2, and no effect on P-450 2c), in every case microsomal testosterone hydroxylase specific activities were elevated from the low levels seen in the hypophysectomized rats (relative specific activity = 29–43% of untreated adult males; Table 4, column 3) to values comparable to those of the untreated controls (relative specific activity = 91–121% of adult male control; Table 4, column 4). Moreover, thyroxine treatment stimulated microsomal steroid hydroxylase specific activities in intact adult male rats as much as 2.9-fold (Table 4, column 2). These findings demonstrate that thyroid hormone, via its effects on microsomal P-450 reductase levels, can markedly alter microsomal specific activities of hepatic P-450 enzymes.

Discussion

In the current study, the biochemical basis for the discrepancy in hypophysectomized rats between the levels of several hepatic P-450 proteins and their associated microsomal steroid

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TABLE 4

Modulation of microsomal steroid hydroxylase specific activity by thyroxine administration

Liver microsomes were isolated from untreated and hypophysectomized (hypox) adult male rats treated with thyroxine (7 daily injections at 50 μg/kg; see Materials and Methods) as indicated (n = 3 individual rats/group). Microsomes were then analyzed for testosterone hydroxylase activities and relative P-450 protein content, and specific activities relative to the untreated male rat group were then calculated, as described under Table 1. Testosterone hydroxylase activities are expressed in units of pmol (15α-hydroxy-T) or nmol (6β- and 16α-hydroxy-T) of hydroxy metabolite formed/min/mg of microsomal protein (mean ± standard error). P-450 protein levels present in the untreated male microsome group are set at 100.

	Untreated male	Untreated male + thyroxine	Hypox male	Hypox male + thyroxine
6β-Hydroxy-T*	0.49 ± 0.05	0.38 ± 0.03	0.35 ± 0.01	0.65 ± 0.09
P-450 2a protein	100 ± 32	27 ± 3	167 ± 5	125 ± 18
Relative specific activity	=1.00	2.87	0.43	1.06
15α-Hydroxy-T	87 ± 8	140 ± 2	42 ± 1	97 ± 18
P-450 RLM2 protein	100 ± 21	91 ± 19	167 ± 10	92 ± 15
Relative specific activity	=1.00	1.77	0.29	1.21
16α-Hydroxy-T	2.05 ± 0.27	2.05 ± 0.06	0.12 ± 0.02	0.30 ± 0.12
P-450 2c protein	100 ± 12	62 ± 6	17 ± 3	16 ± 8
Relative specific activity	=1.00	1.61	0.35	0.91

* T, testosterone.

hydroxylase activities was identified. P-450 reductase, an essential component for all P-450-catalyzed microsomal hydroxylation reactions, was found to be markedly reduced upon hypophysectomy. Although a similar loss of P-450-dependent testicular steroid 17α-hydroxylase activity occurs in hypophysectomized rats without a corresponding loss in testicular P-450 protein and can be attributed to a selective loss of P-450 heme (26), neither hemin reconstitution of liver homogenates nor *in vivo* treatment of hypophysectomized rats with the gonadotropin hCG, which restores heme to testicular P-450, was effective in restoring liver microsomal P-450 activities to levels commensurate with their corresponding P-450 protein levels. That the loss of microsomal P-450 reductase upon hypophysectomy is directly responsible for the failure of P-450-catalyzed steroid hydroxylase activities to parallel the corresponding changes in P-450 protein levels is supported by two observations. First, *in vitro* supplementation of liver microsomes from hypophysectomized rats with exogenous P-450 reductase increased steroid hydroxylase specific activities to levels commensurate with their respective protein levels. Second, significant increases in specific activities were achieved by *in vivo* administration of thyroxine to hypophysectomized rats under conditions that substantially restored hepatic P-450 reductase levels.

The loss of hepatic P-450 reductase following hypophysectomy described in the current study may help to explain apparently conflicting findings on the pituitary dependence of P-450 2a and its associated microsomal steroid 6β-hydroxylase activity. Recent studies from this laboratory have shown that the adult male-specific P-450 2a is distinguished from another adult male-specific P-450, form 2c, by not requiring pulsatile plasma growth hormone for full expression (5). In fact, P-450 2a appears to be suppressed by growth hormone, as suggested by the 50–100% elevation of this protein in liver microsomes isolated from hypophysectomized rats, and by the partial reversal of these effects of hypophysectomy by growth hormone replacement (5). This elevation of P-450 2a in hypophysectomized rats was unexpected, however, because Mode *et al.* (20) had previously reported that microsomal androstenedione 6β-hydroxylase activity, which is P-450 2a-dependent in uninduced rat liver (4), is decreased following hypophysectomy. The present study indicates, however, that these two sets of observations are not in conflict, because P-450 reductase levels are greatly reduced following hypophysectomy and, consequently, P-450 2a-dependent steroid 6β-hydroxylase activity is reduced in isolated liver microsomes unless the microsomes are supplemented with exogenous purified P-450 reductase. Thus, in experiments carried out with unsupplemented microsomes, a decrease in steroid 6β-hydroxylase activity is associated with hypophysectomy (20) whereas an increase in this hydroxylase activity is observed when assays are performed using P-450 reductase-supplemented microsomal preparations (5).

Expression of P-450 reductase in liver microsomes appears to be controlled by one or more pituitary-dependent factors, as indicated by the significant decrease in hepatic levels of this enzyme following hypophysectomy and the significant restoration achieved following administration of thyroxine. Although pituitary growth hormone and its characteristic sex-dependent secretory profiles (pulsatile in adult males versus more continuous in adult females) play a key role in regulating the expression of a number of P-450 enzymes in liver tissues (2, 3, 5, 7), the present studies suggest that growth hormone does not make positive contributions to the regulation of P-450 reductase. Thus, growth hormone treatment of hypophysectomized rats was ineffective in reversing the loss of P-450 reductase. In intact male rats, however, continuous growth hormone treatment suppressed hepatic P-450 reductase expression by ~35%, to levels found in untreated adult female rat liver. This suggests that the more continuous pattern of serum growth hormone found in the adult females may exert a suppressive effect that contributes to the lower expressed level of this enzyme in female rats, as compared with males. Growth hormone treatment of hypophysectomized rats did not further suppress hepatic P-450 reductase, however, suggesting that the suppressive activity of this hormone in the intact rats may require the presence of other pituitary-dependent hormones.

The present demonstration that thyroxine, and to a lesser extent ACTH, can significantly restore P-450 reductase protein levels in hypophysectomized rats is consistent with earlier reports that hepatic P-450 reductase activity is partially decreased by thyroidectomy and adrenalectomy and can be elevated in the surgically altered animals by treatment with thyroxine and cortisone, respectively (29, 30). In the present study, changes in P-450 reductase protein levels in response to hormonal manipulations paralleled the changes in P-450 reductase activity levels, indicating that the hormonal control of this enzyme occurs at the level of protein expression rather than by activation and deactivation of existing reductase protein, for instance, by modulation of its flavin content. Administration of thyroxine to intact male rats produced a further elevation over control levels that is analogous to the elevation seen in hypophysectomized rats. This suggests that thyroxine may exert positive regulatory effects on P-450 reductase that are independent of other pituitary factors and that, perhaps, occur by direct action of this hormone on the hepatocyte. In contrast, thyroxine can exhibit negative regulatory effects on the expres-

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sion of cytochrome P-450, as indicated by its suppression of total hepatic P-450 levels at high doses (24, 31) and by its selective suppression of individual P-450 forms when given at physiological doses (Table 4). The precise mechanism(s) by which thyroxine regulates the expression of P-450 reductase and individual P-450 proteins in liver tissue are presently unknown.

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