Thyroid Hormone Stimulation of NADPH P450 Reductase Expression in Liver and Extrahepatic Tissues

REGULATION BY MULTIPLE MECHANISMS*

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The role of thyroid hormone in regulating the expression of the flavoprotein NADPH cytochrome P450 reductase was studied in adult rats. Depletion of circulating thyroid hormone by hypophysectomy, or more selectively, by treatment with the anti-thyroid drug methimazole led to a 75–85% depletion of hepatic microsomal P450 reductase activity and protein in both male and female rats. Thyroxine substantially restored P450 reductase activity at a dose that rendered the thyroid-depleted rats euthyroid. Microsomal P450 reductase activity in several extrahepatic tissues was also dependent on thyroid hormone, but to a lesser extent than in liver (30–50% decrease in kidney, adrenal, lung, and heart but not in testis from hypothyroid rats). Hepatic P450 reductase mRNA levels were also decreased in the hypothyroid state, indicating that the loss of P450 reductase activity is not a consequence of the associated decreased availability of the FMN and FAD cofactors of P450 reductase. Parallel analysis of S14 mRNA, which has been studied extensively as a model thyroid-regulated liver gene product, indicated that P450 reductase and S14 mRNA respond similarly to these changes in thyroid state. In contrast, while the expression of S14 and several other thyroid hormone-dependent hepatic mRNAs is stimulated by feeding a high carbohydrate, fat-free diet, hepatic P450 reductase expression was not increased by this lipogenic diet. Injection of hypothyroid rats with T3 at a supraphysiologic, receptor-saturating dose stimulated a major induction of hepatic P450 reductase mRNA that was detectable 4 h after the T3 injection, and peaked at ~650% of euthyroid levels by 12 h. However, this same treatment stimulated a biphasic increase in P450 reductase protein and activity that required 3 days to reach normal euthyroid levels. T3 treatment of euthyroid rats also stimulated a major induction of P450 reductase mRNA that was maximal (12-fold increase) by 12 h, but in this case no major increase in P450 reductase protein or activity was detectable over a 3-day period. Together, these studies establish that thyroid hormone regulates P450 reductase expression by pretranslational mechanisms. They also suggest that other regulatory mechanisms, which may involve changes in P450 reductase protein stability and/or changes in the translational efficiency of its mRNA, are likely to occur.

The thyroid hormones, T3 and T4, exert both catabolic and anabolic effects on the metabolism of proteins, carbohydrates, and lipids. T3 is generally considered the active ligand of the thyroid hormone receptors (Lazar and Chin, 1990), with T4 undergoing deiodination catalyzed by thyroxine 5'-deiodinase to yield T3 (Oppenheimer et al., 1987). In liver tissue, T3 and T4 regulate multiple metabolic processes, including lipogenesis (Tao and Towle, 1986), synthesis of vitamins and coenzymes (Lee and McCormick, 1985), and various P450-linked monoxygenations (Waxman et al., 1989). The molecular mechanisms of thyroid hormone action on hepatic gene expression have recently been elucidated for a number of genes which respond to T3 by transcriptional and/or post-transcriptional mechanisms (Wong and Oppenheimer, 1986; Back et al., 1986; Jump, 1989; Song et al., 1988). Well-studied examples of thyroid-responsive liver enzymes include malic enzyme, which furnishes NADPH for lipid synthesis, and S14, a thyroid-stimulated polypeptide that, like malic enzyme, is believed to play an important regulatory role in lipogenesis (Oppenheimer et al., 1987).

NADPH-cytochrome P450 reductase (P450 reductase) is a microsomal flavoprotein that transfers electrons from NADPH to microsomal cytochrome P450 (Peterson and Prough, 1986; Porter, 1991). P450 reductase is encoded by a single gene (Porter et al., 1990) and appears highly conserved across mammalian species. It is expressed in liver and many extrahepatic tissues and participates in monoxygenation reactions catalyzed by a large number of individual P450 enzymes. Although the regulation of individual P450s in response to exogenous and endogenous factors (e.g., drugs and other xenobiotics, developmental factors, and hormonal status) has been studied extensively (Gonzalez, 1988; Waxman, 1988), much less is known about the factors that regulate the expression of P450 reductase. Further studies in this area are clearly of importance, considering that P450 reductase is an obligatory, and often a rate-limiting, component of microsomal P450-dependent reactions (Miwa et al., 1978; Kaminsky and Guengerich, 1985), which include drug oxidations, steroid hydroxylations, and bioactivation of environmental carcinogens.

In the course of our recent studies on the hormonal factors that regulate liver cytochrome P450 gene expression, we observed that hepatic levels of P450 reductase are decreased by up to 80% following hypophysectomy; moreover, expression of this enzyme in Hypox rat liver was substantially restored upon replacement of T4 but not other pituitary-dependent hormones (Waxman et al., 1989). Major questions remain,

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The abbreviations used are: T3, 3, 5-triiodo-L-thyronine; T4, L-thyroxine; P450 reductase, NADPH-cytochrome P450 reductase; Hypox rat, hypophysectomized rat; SDS, sodium dodecyl sulphate.
however, regarding the mechanism by which thyroid hormone regulates the expression of P450 reductase. For example, it is unclear whether the decreased expression of this flavoprotein in thyroid hormone-deficient animals reflects the reduced availability of flavins that is secondary to the decrease in hepatic flavokinase activity in the hypothyroid state (Rivlin and Langdon, 1969), or alternatively, whether it reflects a more direct requirement of thyroid hormone for P450 reductase gene expression. Also unknown is whether the responsiveness of P450 reductase to thyroid hormone is part of the adaptive hyperlipogenic response of the liver that is characteristic of several other thyroid-regulated hepatic proteins and mRNAs (Liu et al., 1983; Tao and Towe, 1986), and whether extrahepatic P450 reductase is subject to a similar hormone dependence. Finally, the implications of this thyroid hormone dependence for the toxiication and detoxification of foreign compounds subject to P450 metabolism, as well as for P450-independent reductive processes catalyzed by P450 reductase itself (e.g. Berlin and Hasecline, 1981) remain to be established. Several of these questions are examined in the present study which investigates the mechanisms by which thyroid hormone regulates the expression of P450 reductase in liver and extrahepatic tissues.

MATERIALS AND METHODS

Animals—Adult male and female Fischer 344 rats (Taconic, Inc., Germantown, NY) were maintained under standardized conditions of light and temperature. Hypothyroidism was induced in rats 8–9 weeks of age by treatment with 0.025% methimazole (w/v) in the drinking water for 16–24 days (Cooper et al., 1984; Ram and Waxman, 1990) as specified in individual experiments. Hypox rats (Ram and Waxman, 1990) and hypothyroid (methimazole-treated) rats were treated with T4 (Sigma, T-2501) administered at a replacement dose (Ram and Waxman, 1990, 1991) of 5 μg/100 g body weight (intraperitoneally) daily for 7 days. In some experiments, Hypox or hypothyroid rats were treated with growth hormone (hGH, 2.4 IU/mg; National Hormone and Pituitary Program) as a continuous infusion via an Alzet osmotic minipump (0.5 μg of growth hormone/rat/h) either alone or in combination with daily T4 injections (Ram and Waxman, 1990). T3 (Sigma, T-2752) was given to hypothyroid or euthyroid rats as a single intraperitoneal injection at a supraphysiological dose of 200 μg/100 g body weight (Oppenheimer et al., 1977), and groups of rats were sacrificed at times ranging from 1 to 78 h later, as specified in the text. Control animals received the same volume of vehicle (10 mM KP, pH 8.3, 0.9% NaCl). In other experiments, euthyroid and hypothyroid rats were fed a high carbohydrate, fat-free diet (60.2% carbohydrate, 19.2% protein, 8.0% fat, 11.6% fiber; Biochemicals, Diet 960235) for 1 or 2 weeks. Control rats were maintained on a regular laboratory diet (Arway PRO-LAB diet R-M-H-3000). Serum T4 levels were measured by laser immunoassay (Ram and Waxman, 1990).

Methods used for killing animals, washing and freezing of livers, and subsequent preparation of either microsomes or total RNA for Northern and slot blot analysis using portions of individual frozen livers are reported elsewhere (Ranz and Waxman, 1990). Extrahepatic tissues were washed in 1.15% KCl and stored at −80 °C. Microsomes were prepared from kidney, lung, testis, and heart using the same Ca++ precipitation method employed for preparation of liver microsomes, whereas adrenal microsomes were prepared by differential centrifugation.

Immunochromatographic and Enzymatic Analysis—Western blotting, immunochromatographic detection using polyclonal rabbit antibody raised to rat liver P450 reductase (Ng and Waxman, 1990), and quantification of relative P450 reductase protein levels by densitometry of nitrocellulose blots were carried out as reported previously (Waxman et al., 1989). P450 reductase activity in microsomal fractions isolated from liver and extrahepatic tissues was determined by monitoring the reduction of cytochrome c at 550 nm (ε = 21 mM−1 cm−1) in 0.3 M KF buffer, pH 7.7, and 30 °C. The cytochrome c reductase activity of microsomal fractions of extrahepatic tissue microsomes examined was shown to be catalyzed by P450 reductase, as judged by the specific inhibitory effects of anti-P450 reductase IgG (≥20–80% inhibition at 2 μg of IgG/pg microsomal protein). Moreover, Western blot analysis of kidney, lung, testis, and adrenal microsomes confirmed the presence of P450 reductase protein at an apparent molecular mass indistinguishable from that of liver P450 reductase (~80 kDa) and at a relative level consistent with the lower microsomal P450 reductase activities seen in each of these tissues (cf., Table II, below). All analytical results are presented as the mean ± S.D. for samples prepared from n = 3–4 individual rats/treatment group, unless indicated otherwise.

Northern and Slot-blot Analysis of P450 Reductase and S14 mRNA Levels—Oligonucleotide probes were synthesized, purified, and 32P-labeled as described elsewhere (Waxman, 1991). P450 reductase mRNA was detected using oligonucleotide ON-28 (5'-TAG-CCG-CCC-TTG-GTC-ATG-AG-3'), which corresponds to the exact complement of nucleotides 2000–2019 of rat liver P450 reductase cDNA (Porter and Kasper, 1985) and nucleotides 2002–2021 of human liver P450 reductase cDNA (Yamano et al., 1989). P450 C26 (PB1) oligonucleotide probe ON-4 (Sunset and Waxman, 1990) and rat α-tubulin oligonucleotide probe ON-30 (Ram and Waxman, 1990) were employed as control mRNA probes, to verify RNA load consistency and RNA integrity (Waxman, 1991); the P450 C26 and α-tubulin mRNAs were generally unresponsive to the treatments described in this study. S14 mRNA was detected by hybridization to the 5'–exonic probe pS14ExoPB1-9, which was provided by Dr. D. Jump, Michigan State University (Jump et al., 1988). The DNA insert (nucleotides +46 to +483 of the 5'-exon and cloned into the Smal-Sall site of pGEM1) was excised with HindIII + EcoRI, then gel-purified and labeled by random priming using a commercially available kit (Boehringer Mannheim). In brief, 20 μg of probe DNA was incubated with 3 μCi [γ-32P]dCTP, 0.2 μl of random primer buffer, 5 μl of α-[32P]dCTP (3000 Ci/mmol), and 1 μl of DNA polymerase I (Klenow fragment) in a total volume of 20 μl at 37 °C for 30 min. The radiolabeled probe was purified on a NENaorb column (Du Pont-New England Nuclear), then denatured by boiling for 5 min immediately before use.

Northern blotting was carried out as detailed elsewhere, with hybridizations to 32P-labeled oligonucleotide probes carried out in buffers containing 0–20% formamide at 40, 45, or 50 °C, depending on the oligonucleotide: ON-4: 0% formamide, 40 °C; ON-28: 10% formamide, 50 °C; ON-50: 20% formamide, 45 °C (Waxman, 1991). In the case of the S14 cDNA probe, nylon filters were hybridized under conditions modified from Greenberg and Ziff (1984). Filters were prehybridized at 42 °C for 12–16 h in a solution containing 50% formamide, 1% glycine, 6 mM SSC (1 mM NaCl, 15 mM sodium citrate, pH 7.0), 1× Denhardt's (1× Denhardt's = 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 0.1% SDS, 1× sodium phosphate, 50 mM NaPO4, pH 6.5, and 0.25 mg/ml denatured salmon sperm DNA. Hybridization was performed at 42 °C for 12–16 h in 50% formamide, 6× SSC, 0.8× Denhardt's, 0.1% SDS, 50 mM NaPO4, pH 6.5, 6.25 mg/ml denatured salmon sperm DNA, and 10% dextran sulfate in the presence of 1× Denhardt's. Blots after hybridization were washed sequentially with 2× SSC (5 min, 20–22 °C), 1× SSC, 0.1% SDS (30 min, 20–22 °C, twice), and 0.2× SSC, 0.1% SDS (30 min, 42 °C, twice). Slot-blots were prepared, hybridized, and relabeled mRNA levels quantitated by laser densitometry (Ram and Waxman, 1990; Waxman, 1991).

RESULTS

T4 Dependence of P450 Reductase Expression in Hypox and Hypothyroid Rats—Rats were depleted of circulating T4 by hypophysectomy, or by treatment for 16–24 days with the antithyroid drug methimazole, which induces hypothyroidism without the global loss of pituitary-dependent hormones that is associated with hypophysectomy (Cooper et al., 1984). Both models of hypothyroidism led to a substantial decrease (up to 85% loss) of liver P450 reductase activity (Fig. 1). These decreases were substantially restored by a replacement dose of T4, in agreement with our earlier finding that circulating thyroid hormone is required for full expression of liver P450 reductase (Waxman et al., 1989). Treatment with growth hormone, which is also depleted in both Hypox and hypothyroid rats, did not restore P450 reductase activity, but partially blocked the stimulatory effect of T4 replacement in the case of the Hypox rats (Fig. 1A). Western blot analysis revealed that these P450 reductase activity changes are associated with
**Figure 1. T4 dependence of P450 reductase activity in Hypox and hypothyroid rat liver.** Liver microsomes were isolated from individual rats \( (n = 4\) /group\) and cytochrome c reduction rates \( (\text{nmol cytochrome c reduced/min/mg microsomal protein}) \) determined as described under "Materials and Methods." Panel A, males and female rats were hypophysectomized \( (HX) \) or sham-operated then treated for 7 days with T4 and/or growth hormone using an osmotic mini-pump \( (GH) \) as described under "Materials and Methods." Panel B, male and female rats were treated with methimazole \( (MI) \) for 16 days \( (+) \) or 24 days \( (-) \) to induce hypothyroidism. Rats were given T4 by daily injection during the last 10 days of a 24-day methimazole treatment, and/or they were given growth hormone by osmotic mini-pump \( (GH) \) for the last 7 days. 

**Figure 2. Western blot analysis of P450 reductase protein in microsomes isolated from Hypox (panel A) and hypothyroid rat liver (panel B).** Liver microsomes were isolated from adult male rats that were either untreated \( (UT, \text{lanes } 1, 2, 7 \text{ and } 8) \), Hypox \( (HX, \text{lanes } 3-6) \), hypothyroid \( (\text{methimazole treatment for } 16 \text{ d (lanes } 9 \text{ and } 10) \) or 24 d (lanes 11-14)), and given a replacement dose of T4 \( (\text{lanes } 5, 6, 13 \text{ and } 14) \) as described under Fig. 1. Microsomes were then analyzed for P450 reductase protein by Western blotting as described under "Materials and Methods." 

**Figure 3. Influence of hypothyroidism and thyroid hormone replacement on liver P450 reductase and S14 mRNAs.** Shown are autoradiographs of a Northern blot analyzing total liver RNA samples \( (10 \mu\text{g}) \) isolated from individual untreated \( (UT) \) or methimazole-treated male and female rats and resolved on a formaldehyde/agarose gel. Rat treatments were as in Fig. 1B. Lanes 2 and 8, methimazole treatment for 24 d \( (+) \); lanes 3 and 9, methimazole treatment for 24 d \( (-) \); lanes 4–6 and 10–12, 24-day methimazole treatment combined with the indicated hormone replacements. Sequential probing of a nylon blot of the gel to detect mRNAs encoding P450 reductase, S14, and P450 2C6 (control for RNA integrity and loading) was performed as described under "Materials and Methods." Migration distances of the ribosomal RNAs are indicated at the left. 
mals, the stimulatory effect of T4 on S14 mRNA appeared to be further enhanced by the presence of GH, an effect reported for several other thyroid-responsive liver mRNAs (Liaw et al., 1983; Ram and Waxman, 1990). These findings were confirmed by parallel analysis of P450 reductase and S14 mRNA levels in Hypox rats: hypophysectomy led to a decrease in both P450 reductase mRNA and S14 mRNA expression and these decreases were reversed by T4 replacement (data not shown).

Response of P450 Reductase to Dietary Carbohydrate—Dietary carbohydrate can interact synergistically with thyroid hormone to stimulate expression of several liver mRNAs, including S14 mRNA (Mariash et al., 1986; Tao and Towle, 1986). In order to establish whether P450 reductase expression is also stimulated by a lipogenic diet, we examined the effect of feeding a fat-free, high carbohydrate diet on liver expression of S14 and P450 reductase. Fig. 4 shows that while the high carbohydrate diet triggered the expected increase in S14 mRNA levels, it did not increase P450 reductase mRNA (middle panel versus top panel). Although increases in circulating T4 were observed in both female (+20%) and male rats fed the lipogenic diet (+50-60%), liver microsomal P450 reductase activity was only slightly increased (females) or was largely unaffected (males) (Table I). In hypothyroid rats, S14 mRNA was also induced by feeding the lipogenic diet (Fig. 4, lane 4), in accord with earlier reports (e.g. Tao and Towle, 1986), even though circulating T4 was still undetectable (Table I).

Induction of P450 Reductase mRNA by Hyperthyroidism: Kinetic Analysis—To ascertain whether the induction of P450 reductase mRNA by thyroid hormone involves a direct, rapid effect, hypothyroid rats were injected with T3 at a dose (200 µg/100 g body weight, intraperitoneal) that is sufficient to maintain 80% saturation of liver thyroid hormone receptors for an estimated 40-48 h (Oppenheimer et al., 1977). T3 injection under these conditions stimulated a major induction of P450 reductase mRNA that was first detected at 4 h (Fig. 5A) and increased to ~650% of normal euthyroid levels by 48 h (Fig. 6A). By 24-48 h after T3 injection, however, P450 reductase mRNA dropped to a level that was at most ~2-fold higher than the untreated euthyroid controls. Analysis of S14 mRNA on the same blots revealed that it responded to T3 more rapidly than P450 reductase mRNA, with near maximal induction achieved by 4 h (Figs. 5A and 6A). The kinetics of induction of P450 reductase mRNA seen in this experiment, although slower than that of S14 mRNA, are consistent with the involvement of thyroid hormone receptor in the induction response.

In contrast to this striking induction of high levels of P450 reductase mRNA, T3 stimulated increases in P450 reductase protein and activity that were much more modest and required a full 3 days to reach normal, euthyroid levels (Fig. 6B). These increases were biphasic, with an initial, up to 2-fold increase above the basal, hypothyroid levels occurring within 1 h of T3 stimulation, followed by a slower increase that was not evident until the 24 h time point. The magnitude and time dependence of this latter increase in P450 reductase protein and activity correlated with the persistence of normal thyroid to slightly hyperthyroid P450 reductase mRNA levels from 24-78 h after T3 injection, rather than with the major induction of this mRNA at the 12 h time point (Fig. 6, B versus A). Interestingly, the rapid, initial induction of P450 reductase protein at 1.0 h preceded any detectable increase in P450 reductase mRNA (Fig. 5, B versus A). This discordant response of P450 reductase protein versus mRNA at the 1 h time point was evident in each of three individual rats (Fig. 5) and was confirmed in a series of separate experiments in four additional rats (data not shown). No further increase in P450 reductase protein occurred at the 4, 8, and 12 h points (Figs. 5B and 6B) despite the major elevation of P450 reduc-

**TABLE I**

| Influence of lipogenic diet on hepatic P450 reductase activity and circulating T4 levels |
|---------------------------------------------------------------|---------------|
| Euthyroid and hypothyroid rats (24-day methimazole treatment at the conclusion of the experiment) were fed normal rat chow or a high carbohydrate, fat-free (lipogenic) diet for 1 or 2 weeks as described under “Materials and Methods.” Liver microsomal P450 reductase activity and serum T4 levels were assayed as described under “Materials and Methods.” |
| Liver P450 reductase activity | Serum T4 |
|-------------------------------|----------|
|                               | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid |
|                               | nmol/min/mg | ng/ml |
| Female rats                   |           |        |
| Normal diet                   | 173 ± 7   | 57 ± 6  | 39 ± 6   | <3 |
| Lipogenic diet (1 week)       | 190 ± 5*  | 39 ± 8  | 47 ± 3   | <3 |
| Male rats                     |           |        |
| Normal diet                   | 277 ± 7   | ND      | 43 ± 4   | ND |
| Lipogenic diet (1 week)       | 263 ± 6   | ND      | 69 ± 9*  | ND |
| Lipogenic diet (2 weeks)      | 243 ± 14  | ND      | 65 ± 6*  | ND |

*Significant increase compared to the corresponding normal diet groups at p < 0.01. All other normal diet versus lipogenic diet comparisons were not significant (p > 0.1).

*ND, not determined.
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Fig. 5. Induction of P450 reductase mRNA (panel A) and protein (panel B) in T3-stimulated hypothyroid rats. Hypothyroid male rats (16-day methimazole treatment) were injected with a single, receptor-saturating dose of T3 (200 μg/100 g body weight, intraperitoneal) and killed at the indicated times. For controls (0 h T3 treatment), sham-injected hypothyroid rats received vehicle alone (10 mM KPi, pH 8.3, 0.9% NaCI) and were sacrificed 4 h later. Panel A, total liver RNA isolated from individual animals and analyzed for P450 reductase and S14 mRNA by Northern blotting, as in Fig. 3. Panel B, liver microsomes isolated from individual rats and analyzed for P450 reductase protein by Western blotting in comparison to untreated euthyroid controls (Eu). Comparison of panels A and B highlights the discrepancy between the response of P450 reductase mRNA and protein to T3 at the t = 1 h time point. 

Fig. 6. Time course of T3-induced expression of P450 reductase and S14 in hypothyroid rat liver. Shown are quantitative data from the same experiment described in Fig. 5 and including data for T3-treated hypothyroid rats sacrificed at times up to 78 h after the single T3 injection. Relative levels of liver P450 reductase mRNA (closed circles) and S14 mRNA (open triangles) (assayed by slot-blotting, panel A), and relative levels of liver microsomal P450 reductase activity (cytochrome c reduction, open squares), and P450 reductase protein (by Western blotting, closed triangles) (panel B), were determined as described under “Materials and Methods.” Data are for n = 3 rats sacrificed at each time point and are expressed relative to untreated euthyroid controls (=100%) (mean ± S.E.). 100% P450 reductase activity = 311 ± 15 nmol cytochrome c reduced/min/mg microsomal protein.

Fig. 7. Major induction of P450 reductase mRNA (panel A) but not protein or activity (panel B) following T3 treatment of euthyroid male rats. Rats were injected with a receptor-saturating dose of T3 at t = 0 and groups of rats (n = 3) sacrificed at the indicated time points, exactly as described for the hypothyroid rat experiment shown in Figs. 5 and 6. Shown are P450 reductase mRNA (panel A), protein, and activity levels (panel B) relative to sham-injected euthyroid controls. 100% P450 reductase activity = 328 ± 20 nmol cytochrome c reduced/min/mg microsomal protein.
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**TABLE II**

*P450 reductase activity in extrahepatic tissues of methimazole (MI)-treated rats: thyroid hormone dependence*

P450 reductase activity was determined for liver microsomes isolated from the indicated tissues, obtained from male rats, except as noted. Values (mean ± S.D. for n = 3–4 individual rats/treatment group) are expressed as a percentage of the euthyroid controls. Rats were treated with methimazole for 16 or 24 days, as indicated. T3 was administered as a single injection, as in Figs. 5 and 6, and tissues were isolated 24–78 h later, as indicated (Experiment 2).

| Tissue            | Euthyroid | MI (16 days) | MI (24 days) | MI (24 days) + T3 |
|-------------------|-----------|--------------|--------------|------------------|
| Kidney            | 100 ± 7 (60) | 80 ± 20 | 59 ± 9* | 92 ± 11*         |
| Kidney (female)   | 100 ± 16 (68) | 57 ± 7* | 51 ± 7* | 106 ± 12*        |
| Lung              | 100 ± 8 (77) | 88 ± 32 | 63 ± 4* | 83 ± 12**        |
| Lung (female)     | 100 ± 15 (95) | 94 ± 5 | 73 ± 8** | 109 ± 6*         |
| Testis            | 100 ± 2 (40) | ND | 88 ± 7 | 90 ± 7          |
| Adrenal           | 100 ± 15 (125) | ND | 55 ± 6* | 101 ± 12*        |

**Experiment 1: T4 replacement**

| Tissue            | Euthyroid | MI (16 days) | +T3 (24 h) | +T3 (48 h) | +T3 (78 h) |
|-------------------|-----------|--------------|------------|------------|------------|
| Kidney            | 100 ± 4 (49) | 65 ± 2* | 111 ± 8* | 138 ± 6** | 141 ± 10*  |
| Lung              | 100 ± 17 (107) | 96 ± 7 | 91 ± 3 | 111 ± 24 | 124 ± 4.4H |
| Testis            | 100 ± 11 (53) | 89 ± 0 | 96 ± 6 | 93 ± 6 | 105 ± 7**  |
| Heart             | 100 ± 20 (15) | 69 ± 7** | 105 ± 7* | 128 ± 27* | 126 ± 0.4H |

*Statistical comparisons by student's t test: *p < 0.01; **p < 0.05, significant decrease compared to euthyroid controls; * p < 0.01; ** p < 0.05, significant increase following T4 or T3 treatment compared to corresponding methimazole control group. §§ p < 0.01; §§§ p < 0.005, significant increase above euthyroid control.

*Absolute P450 reductase activities for the euthyroid controls (nmol cytochrome c reduced/min/mg microsomal protein) are shown in parentheses.

*ND, not determined.

same animals (Fig. 6B). Expression of P450 reductase activity in lung microsomes was also thyroid hormone dependent, although this dependence was only evident in rats maintained on methimazole for 24 days but not in 16 day-treated rats (Experiment 1). As in kidney and heart, hyperthyroidism increased lung P450 reductase to levels above the corresponding euthyroid controls (Experiment 2). In contrast, testis P450 reductase activity was unresponsive, both to thyroid hormone depletion and to T3-induced hyperthyroidism (Table II). Together, these findings indicate that while the thyroid responsiveness of P450 reductase is not restricted to liver, there are tissue-specific differences in its regulation by thyroid hormones.

**DISCUSSION**

Previous studies carried out in Hypox rats have indicated that thyroid hormone is required for full expression of P450 reductase in liver tissue (Waxman et al., 1989). Those observations are confirmed and extended by the present demonstration that an 80–85% decrease in hepatic levels of P450 reductase can also be achieved in adult rats rendered hypothyroid by treatment with the anti-thyroid drug methimazole, and that this decrease is readily reversed by restoration of thyroxine (T4) to physiological levels. The methimazole-treated rat provides a simplified model system for studying the mechanisms by which thyroid hormones (i.e. T3 and T4) stimulate hepatic P450 reductase expression, insofar as the global loss of pituitary and pituitary-dependent hormones that occurs in Hypox rats is avoided, and the stress associated with surgery is eliminated. Two possible mechanisms for this thyroid dependence of P450 reductase were considered. First, the observed decrease in P450 reductase in the hypothyroid state could involve a decrease in P450 reductase mRNA, leading to decreased synthesis of P450 reductase protein. Alternatively, newly synthesized P450 reductase apoprotein might be more rapidly degraded in hypothyroid rats, perhaps owing to a decrease in the availability of the FMN and FAD cofactors of this flavoprotein in the hypothyroid state (Lee and McCormick, 1985). Synthesis of these flavin cofactors from riboflavin is known to be stimulated by thyroid hormone through the hormone's effects on the enzyme flavokinase (Rivlin and Langdon, 1969). Northern analysis of hepatic P450 reductase mRNA levels indicated that the first of these two mechanisms is operative insofar as P450 reductase mRNA levels varied in parallel with P450 reductase protein and activity in going from the hypothyroid to the euthyroid state. Although this thyroid regulation of P450 reductase mRNA probably involves transcriptional mechanisms, post-transcriptional regulation could also be occurring, as suggested by studies of other thyroid-dependent hepatic mRNA's, including those that encode the S14 protein (Jump, 1989) and malic enzyme (Song et al., 1988).

Thyroid hormone-inducible hepatic genes vary greatly in the kinetics of their response to thyroid hormone stimulation. For example, rat hepatic S14 gene expression is rapidly induced following treatment of hypothryroid rats with T3 (elevation of gene transcription rate seen within 15 min) (Jump, 1989), while induction of S11 mRNA exhibits a lag time of 6 h (Wong and Oppenheimer, 1986). Hepatic HMG-CoA reductase mRNA responds much more slowly (30 h required for full response) and occurs secondary to thyroid hormone-stimulated biliary cholesterol secretion (Day et al., 1989). The present demonstration that T3 rapidly induces P450 reductase mRNA (detectable within 4 h and maximal by 12 h in both hypothyroid and euthyroid rats) is consistent with a direct, primary response to thyroid hormone. The apparent rapid decrease in P450 reductase mRNA from 12 to 24 h following T3 injection (Figs. 6A and 7A) suggests a short half-life for this mRNA in the hypothyroid state (t₁/₂ ~ 5 h). This decrease occurred at a point in time (24 h after T3 injection) when circulating T3 levels are reportedly still high (Oppenheimer et al., 1977), suggesting that chronically high
levels of T3 may render the P450 reductase gene refractory to induction. Although the induction of S14 mRNA by T3, which preceded that of P450 reductase mRNA, was somewhat slower than reported previously (Jump et al., 1984), this could relate to the route of T3 administration: intraperitoneal injection (this study) versus intravenous injection (Jump et al., 1984).

While thyroid hormone status thus has a major effect on P450 reductase mRNA levels, it is also apparent from the present study that thyroid hormone can rapidly increase (within 1 h) P450 reductase protein in a manner that is independent of the ensuing changes in P450 reductase mRNA. This observation implies that thyroid hormone can confer a translational or post-translational regulation on P450 reductase. Possible mechanisms for this rapid effect of thyroid hormone could conceivably include stabilization of P450 reductase apoprotein, perhaps via a T3-induced increase in the availability of the reductase's FMN and FAD cofactors, or alternatively, an increase in the translatability of the pre-existing P450 reductase mRNA pool following T3 treatment of hypothyroid rats.

Although a major increase in P450 reductase mRNA was obtained within 8–12 h after T3 was given to hypothyroid rats, no significant changes in P450 reductase protein or activity were detected from 1 to 12 h following the T3 injection. Subsequent increases in P450 reductase protein were modest and were first observed at 24–78 h, i.e., after the major T3-induced rise in P450 reductase mRNA had largely been reversed (Fig. 6). The apparent lag between T3-induction of P450 reductase mRNA and T3 induction of immunodetectable P450 reductase protein is reminiscent of a lag with respect to the translation of S14 mRNA into S14 protein in the same hyperthyroid rat model (Kinlaw et al., 1989). In the case of S14, the apparent translation block has been proposed to be a consequence of prolonged hyperthyroidism since no lag in S14 protein synthesis occurs when euthyroid or short term hypothyroid rats are treated with T3 (Kinlaw et al., 1989; Strait et al., 1989). In the case of P450 reductase, however, major mRNA increases induced by T3 treatment of euthyroid rats did not lead to significant changes in protein levels (Fig. 7). These findings imply that hyperthyroidism is associated with a block in the translation of P450 reductase mRNA and/or destabilization of newly synthesized P450 reductase protein. More detailed kinetic analyses, including measurements of P450 reductase protein synthesis and degradation rates, will be required for a full mechanistic understanding of these effects of thyroid hormone.

Many thyroid hormone-responsive hepatic mRNAs are regulated by thyroid hormone and dietary carbohydrate in a coordinate, and often synergistic, manner (Tao and Towle, 1986). Included are several enzymes that are key to lipogenesis (e.g. malic enzyme, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase), and proteins that are otherwise involved in fat metabolism or storage. Since P450 reductase plays an obligatory role in hepatic P450-catalyzed oxidation of a broad variety of fatty acids and their derivatives, including lauric acid, arachidonate, and various prostaglandins (e.g. Capdevila et al., 1990; Kupfer et al., 1988), we considered whether P450 reductase might be induced as part of the adaptive hyperlipogenic response of the liver. However, no significant changes in hepatic P450 reductase activity or mRNA were observed in adult rats maintained on a high carbohydrate, fat-free diet. Thus, P450 reductase expression and P450 reductase-dependent liver P450 monooxygenase activity are not likely to be greatly influenced by lipogenic dietary conditions. It is still possible, however, that the expression of individual cytochromes P450 is influenced by these dietary factors.

The tissue specificity of P450-catalyzed foreign compound metabolism is an important determinant of the organ-specific toxicities associated with many deleterious environmental agents. In addition to liver, P450 reductase is expressed in many extrahepatic tissues, including lung, kidney, and the gonads, where it participates in P450-linked xenobiotic metabolism. It is therefore significant that a thyroid hormone dependent expression of P450 reductase was also found in thyroid-responsive extrahepatic tissues, namely, adrenal, kidney, and heart, albeit to a lesser extent and with different kinetics of T3 induction as compared to liver. Thyroid hormone-dependent expression of P450 reductase was also apparent in lung, but not testis, despite the fact that both these tissues are classified as thyroid nonresponsive (cf., Dozin et al., 1985; Oppenheimer et al., 1974). This raises the interesting possibility that thyroid hormone regulates P450 reductase in lung and perhaps other extrahepatic tissues by mechanisms that are distinct from those operative in the liver.

Although P450 reductase is known to be rate limiting for many liver P450-catalyzed biotransformations (e.g. Miwa et al., 1978; Waxman et al., 1989), the situation in extrahepatic tissues has yet to be examined in detail. In liver, individual P450 forms can be differentially affected by a decrease in total P450 reductase levels, possibly owing to differences in their association constants for P450 reductase complexation (e.g. Kaminsky and Guengerich, 1985). Since different P450 forms contribute to the bioactivation of different carcinogens (e.g. benzo(a)pyrene activation preferentially catalyzed by P450 1A1 versus aflatoxin B1 activation catalyzed by P450 3A in both humans and rats; e.g. Shimada et al., 1989), it is conceivable that alteration of microsomal P450 reductase levels in response to changes in thyroid hormone state can have effects that vary from one carcinogen to the next. Circulating thyroid hormone levels can be altered not only by thyroid diseases but also by many common physiological and environmental factors, including pregnancy, malnutrition, environmental temperature, and physiological stress caused by non-thyroid illnesses (e.g. Mandel et al., 1990). A more detailed understanding of the molecular mechanisms by which thyroid hormones regulate P450 reductase in liver and extrahepatic tissues may therefore help to identify individuals who are more susceptible to deleterious effects of drugs or other xenobiotics metabolized by the P450 reductase-cytochrome P450 system as a consequence of alterations in thyroid hormone status.

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