ANDROGEN METABOLISM IN THYMUS OF FETAL AND ADULT RATS
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ABSTRACT:
Cytochrome P450 (P450) monoxygenases play a role in target tissue metabolic activation of xenobiotics and/or endogenous compounds, such as vasoactive molecules or hormones. Indeed, tissue-specific metabolism of steroids is important in a variety of organs, including thymus, and may alter tissue-specific functions. Steroids have been shown to regulate thymus growth and function, but surprisingly little is known about expression of the responsible enzyme systems in thymus tissue, nor is the thymus-specific bio-

The thymus is an important immunologic lymphoepithelial organ, and hormones are thought to play a major role in the regulation of thymic function. Hormones may also trigger thymus atrophy in aged animals. Importantly, androgens regulate size and tissue composition of thymus. Depletion of androgens through castration of animals results in thymic enlargement; consequently, androgen replacement reverses these effects (Olsen et al., 1991). Tissue-specific metabolism of hormones might impact organ function (Pazirandeh et al., 1999), but little is known about expression and function of steroid-metabolizing enzymes in the thymus. It is, however, well established that cytochrome P450 (P450s) monoxygenases play a major role in the metabolism of steroids (Simpson, 1997; Capdevila et al., 2000; Thum and Borlak, 2000a, 2002). P450s are abundantly expressed in many different tissues including liver, lung, gut, placenta, heart, and endo-
thelium (Kivistö et al., 1996a,b; Thum et al., 2000; Thum and Borlak, 2000b; Borlak et al., 2003). Furthermore, expression of P450 monoxygenases and other steroid-metabolizing enzymes differs during fetal and postnatal development and during pregnancy and lactation, all of which will impact steroid metabolism (Borlakoglu et al., 1993b; Vianello et al., 1997; Van Der Eerden et al., 2002; Moran et al., 2002).

Because of the scarce information available and in view of its essential role in thymus biology, we investigated thymus-specific androgen metabolism and hormone receptor expression levels. We therefore studied gene and protein expression and enzyme activity of major P450 isoforms and key steroid-metabolizing enzymes, i.e., 17β- and 3β-hydroxysteroid dehydrogenases (HSDHs) and 5α-reductase, in thymus tissue of fetal and adult male rats. We also correlate gene and protein expression level and enzyme activity of steroid-metabolizing enzymes with expression of the androgen and estrogen receptor α.

Overall, our study aimed to investigate steroid metabolism in thymus for a better understanding of these messengers in organ function.

Materials and Methods

Animals. All animal procedures described in this article were approved by the ethical committee of the local government of Hannover, Germany. Male Sprague-Dawley or pregnant Sprague-Dawley rats weighing 200 to 250 g were obtained from Charles River (Sulzfeld, Germany). Food and water were given ad libitum. Rats were anesthetized with ketamine (anesthetic) and xylazine hydrochloride (muscle relaxant), with 0.1 ml of ketamine per 100 g body weight and 0.05 ml of xylazine hydrochloride per 100 g body weight. In addition, 2000 international units of heparin were given intraperitoneally before surgery. Thymus tissue was explanted under a magnifying glass to exclude contamination with nonthymic tissue. Then, all tissue samples were shock-frozen in liquid nitrogen and stored at −80°C to await further analysis.

Gene Expression Studies. RNA was isolated from thymus of fetal (gestation day 19; n = 5) and adult (9–12 months old; n = 3) male rats using the SV total RNA Isolation System (Macherey-Nagel, Düren, Germany) according to
the manufacturer’s recommendation. Quality of isolated RNA was checked using a 1% agarose gel. Total RNA (2 µg) from each sample was used for reverse transcription. RNA and random-primer (Roche, Mannheim, Germany) were preheated for 10 min at 70°C. Then, 5× reverse transcriptase-avian myeloblastosis virus buffer, deoxycytidine-5’-triphosphates (1 mM; Roche), 40 U RNase inhibitor (Strategene, Amsterdam, Netherlands), and 20 U avian myeloblastosis virus-reverse transcriptase (Promega, Mannheim, Germany) were added and volumes were adjusted to 20 µl with diethyl pyrocarbonate (Sigma Chemie, Deisenhofen, Germany)-treated water. Then, reverse transcription was carried out for 60 min at 42°C and was stopped by heating to 95°C for 5 min. The resulting cDNA was frozen at −20°C until further experimentation.

PCR reactions were carried out in a thermal cycler (T3; Biometra, Göttingen, Germany) using the melting, annealing, and extension cycling conditions, as shown in Table 1. DNA contamination was checked for by direct amplification of RNA extracts before conversion to cDNA, and any contamination of RNA extracts with genomic DNA could be excluded. For PCR amplification, a 20-µl reaction mixture was prepared containing 10 µl of HotStar TaqDNA Polymerase Mastermix (QIAGEN GmbH, Hilden, Germany), a 400 nM concentration of the 3’- and 5’-specific primer (see Table 1), and 1 µl of cDNA. PCRs were done within the linear range of amplification; amplification products were separated by using a 1.5% agarose gel and stained with ethidium bromide. Gels were photographed on a transilluminator (Kodak Image Station 440; Eastman Kodak, Rochester, NY; see Fig. 1).

Western Immunoblotting. Microsomal extracts from thymus (100 µg) of adult rats were immediately denatured at 95°C for 5 min, followed by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels, and blotted onto a polyvinylidene difluoride membrane (PerkinElmer Life and Analytical Sciences, Rodgau-Jugenheim, Germany) at 350 mA for 2 h from each sample. Microsomal protein concentrations were determined according to the method of Smith et al. (1985) using the BCA test, a modification of the Lowry test (Lowry et al., 1951).

Protein content was adjusted to approximately 1 mg of protein/ml. Microsomal samples were diluted in 0.1 M Tris buffer (pH 7.4) to approximately 1 mg of protein/ml and the diluted sample was added to both sample and reference cuvettes to record the baseline between 400 and 500 nm. Sodium dithionite was added to both cuvettes and the sample cuvette was gently bubbled with carbon monoxide for approximately 1 min. The spectrum was then scanned from 400 to 500 nm, and the absorbance difference between 450 and 490 nm was calculated. Using Beer’s Law, the cytochrome P450 concentration was measured by the following equation: Abs.Diff = 450 nm - 490 nm (extinction coefficient of 91 for the absorbance difference between 450 and 490 nm; see Omura and Sato, 1964).

Metabolism Experiments with Testosterone. One milligram of microsomal protein was incubated with 100 µM testosterone and 1 mg/ml NADPH in 1 ml of Tris buffer (20 mM; Sigma Chemie) for 4 h at 37°C in a shaking water bath. Samples were shock-frozen in liquid nitrogen and were stored at −80°C until further analysis.

Testosterone and its metabolites were analyzed by high-performance liquid chromatography, according to the method of Arlott et al. (1991) with slight

<table>
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<th>Accession Number</th>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<td></td>
<td>base pairs</td>
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**TABLE 1**

Oligonucleotide primer used in the gene expression study

PCR conditions: predenaturation 95°C, 120 s; denaturation 94°C, 60 s; annealing (see table) for 60 s, and extension 12°C for 60 s.
modifications as described in Thum and Borlak (2002). Hydroxyprogesterone was used as an internal standard for the quantitative determination of testosterone.

Statistical Analysis. Data represent mean ± standard deviation. The Student’s t-test was used and differences were considered significant at $p < 0.05$.

Results

Gene Expression. Cytochrome P450 monooxygenases. We detected transcript copies of CYP1A2, CYP2A1/2, CYP3A1, CYP3A2, or CYP4A1 was below the limit of detection. In contrast, all of the P450 isoforms investigated are expressed in rat liver or cultured rat hepatocytes, as reported recently (Borlak and Thum, 2001). Transcripts of CYP2A1/2 were significantly ($p < 0.05$) higher in thymus tissue of adult rats, whereas CYP2J3 mRNA levels were significantly ($p < 0.05$) more abundantly expressed in thymus tissue of fetal rats (see Figs. 1 and 2a). Furthermore, CYP2E1 was 2-fold more highly expressed in fetal thymic tissue, but no statistical significance was reached.

Hydroxysteroid dehydrogenases, 5α-steroid reductase estrogen, and androgen receptor. When compared with thymus tissue of fetal rats, the 17β-hydroxysteroid dehydrogenase isofrom 1 (17β-HSDH1) and the androgen receptor were expressed >3-fold higher in adult rats, whereas expression of 17β-HSDH2, 17β-HSD3, and 5α-reductase were 12%, 0%, and 32% higher, respectively. No significant change in expression levels of 17β-HSDH4, 3β-HSDH1, or the estrogen receptor α was observed when fetal and adult rat thymus were compared (see Figs. 1 and 2a and b).

Protein Expression. We detected CYP2A and CYP2B expression in thymus tissue of adult rats. CYP2E1 protein expression was low, and in the case of CYP1A1, levels were below the limit of detection (see Fig. 3). Protein expression of P450 monooxygenases could not be determined in fetal thymus because of the small amounts of tissue available.

Androgen Metabolism in the Thymus. Total P450 content and enzyme activity could only be measured in thymus and liver of adult animals, since the tissue weight of fetal thymic tissue was too small to enable microsomal enzyme assays.

We used a considerably long incubation time to gain maximal metabolic production; therefore, the enzymatic activities were determined under nonlinear conditions. In thymus, total P450 content was about 8% of liver (see Fig. 3a), and testosterone was metabolized to 6α-HT, 7α-HT, 16α-HT, 2α-HT, and androstenedione as major metabolites. The production of 16α-HT and 7α-HT correlated well with the gene expression of CYP2B1/2 and CYP2A1/2, respectively. When compared with microsomal membranes of liver, production of 16α-HT and 7α-HT was 3% and 5%, respectively, in thymus tissue (see Table 2 and Fig. 3b).

Discussion

We show rat thymus to express several cytochrome P450 and other steroid-metabolizing enzymes and demonstrate tissue-specific metabolism of testosterone. We also demonstrate an age-dependent trun-

![Fig. 1. Gene expression of P450 monooxygenases, 17β-HSD1–4, 3β-HSD1, 5α-reductase, estrogen receptor α (ERα), and the androgen receptor (AR) in thymus tissue of adult and fetal rats.](image1)

![Fig. 2. Western immunoblotting of CYP1A1, CYP2A, CYP2B, and CYP2E1 in Supersomes (positive control) and microsomal extracts (100 μg) of adult thymus tissue.](image2)
script expression of isoforms of P450 and 17β-HSDH, as well as the androgen receptor, in thymus tissue of fetal and adult rats. We thus extend earlier findings of a time-dependent P450 monoxygenase gene expression during liver organogenesis to the thymus (Borlakoglu et al., 1993; Yang et al., 1995; Juchau et al., 1998).

Importantly, steroid hormones are versatile signaling molecules and are metabolized via several enzyme systems, including P450 monoxygenases, hydroxysteroid dehydrogenases/reductases, and aromatases, and are usually bound reversibly to carrier proteins in the blood. Upon receptor-mediated endocytosis (Porto et al., 1995), they interact specifically with steroid-hormone-receptor proteins in the cytoplasm and nucleus (Becker et al., 1986). Binding of the hormone activates the receptor, enabling high affinity to specific DNA sequences to act as transcriptional enhancers. It is known that thymus size and weight are very sensitive to changes in the androgen status, and more recently, testosterone was shown to participate in apoptosis (Olsen et al., 1998), which suggests the androgen receptor to be an important modulator in the control of thymus development.

17β-HSDH3 is predominantly expressed in the testes and catalyzes the reaction from androstenedione to testosterone, whereas 17β-HSD1 is highly expressed in the placenta, catalyzing the reactions from estradiol to estrone, testosterone to androstenedione, and androst-5-ene-3β and 17β-diol to dehydroepiandrosterone (Labrie et al., 2000). Notably, expression of 17β-HSDH2, 3, and 4 was detected in fetal thymus tissue, whereas in adult thymus, expression was confined to the isoforms 17β-HSDH1 and 4. This difference in the expression of 17β-HSDHs between fetal and adult thymic tissue is likely to play an important role in the maintenance of tissue-specific steroid level and, by implication, in the onset and progression of hormone-sensitive thymus atrophy.

Interestingly, the lowest enzyme activities were associated with poorly transcribed genes in thymic tissue, whereas we found good correlation between CYP2A and CYP2B protein expression and production of the testosterone metabolites 16α-HT and 7α-HT. However, there is a need for in-depth experiments to establish enzyme kinetics of cytochromes P450 in thymic tissue. It is well known that the above-named cytochrome P450 monoxygenases play a major role in the metabolism of testosterone but are also competent in drug oxidation. In view of the expression of CYP2A and CYP2B, the thymus may have, additionally, tissue-specific drug metabolism capacity. For instance, CYP2A-catalyzed N-demethylation of cocaine leads to a hepatotoxic compound (Aoki et al., 2000) and is reported to have immunosuppressive and toxic effects on the thymus (Xu et al., 1998). It is reasonable to speculate that expression of CYP2A in the thymus is, at least in part, responsible for the production of a toxic metabolite to result in metabolically induced toxicity. Interestingly, expression of 5α-reductase was 3-fold higher in fetal animals. This is suggestive for enhanced local production of dihydrotestosterone (DHT) to be higher in the fetal thymus. The effects of testosterone and DHT on thymus are controversial. For instance, Greenstein et al. (1988) reported testosterone to inhibit thymus growth in adult animals, whereas no effects were observed with DHT.

We used a long incubation time (4 h) to gain a maximum of testosterone metabolite production, especially from thymic tissue. We did not aim to perform a time line of metabolite production but, rather, focused on the type of metabolites that are produced in thymic tissue. It is noteworthy that thymic tissue consists of several cell types, including lymphoid thymocytes, nonlymphoid epithelial cells, endothelial cells, fibroblasts, macrophages, dendritic cells, and others. Further studies are therefore needed to understand in detail the role of each cell type and its metabolic competence for a better understanding of steroid metabolism and organ function. Moreover, metabolism experiments with testosterone were only done with microsomal ex-

<table>
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<th>Metabolites</th>
<th>Thymus</th>
<th>Liver</th>
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<tbody>
<tr>
<td>6α-HT</td>
<td>0.014</td>
<td>0.096</td>
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</tr>
<tr>
<td>7α-HT</td>
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<tr>
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<td>0.20</td>
</tr>
<tr>
<td>Androstenedione</td>
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<td>2.053</td>
<td>0.61</td>
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</table>
tracts from adult thymus tissue, because the thymus tissue harvest from fetal animals was too low.

In conclusion, we demonstrate thymus tissue to be metabolically competent and to express several steroid-metabolizing enzymes. We speculate that the observed differences in steroid metabolism are important signals for thymus growth and function.

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References