ABSTRACT

Previous studies in our laboratory have shown that the sex-differentiated metabolism of 4-androstene-3,17-dione and of several other steroid hormones in adult rat liver is "feminized" following neonatal castration of male rats, due to an influence via the hypothalamic-pituitary-liver axis. The metabolism of many xenobiotics is also sex differentiated, and an important question is whether endocrine ablations might alter hepatic carcinogen metabolism in a way explaining, for example, the decreased tendency of castrated male rats [Y. C. Toh, In: Shanmugarathnam et al. (eds.), Liver Cancer, Cancer Problems in Asian Countries, Proceedings of the Second Asian Cancer Conference, pp. 167-171. Singapore: Singapore Cancer Society, 1976] to form liver tumors following 2-acetylaminofluorene treatment. The results presented in this paper clearly show that neonatal castration of male rats, much more efficiently than adult castration, feminizes the cytochrome P-450-mediated formation of 7-hydroxy-2-acetylaminofluorene, 9-hydroxy-2-acetylaminofluorene, 5-hydroxy-2-acetylaminofluorene, 1-hydroxy-2-acetylaminofluorene, and \( \text{N}-\text{hydroxy-2-acetylaminofluorene} \) from 2-acetylaminofluorene as well as the total microsomal formation of benzo(a)pyrene metabolites (\( \Delta > 9 \)). O-Dethylation of 7-ethoxyresorufin was neither sex differentiated nor affected by castration. The capacity for \( \text{in vitro} \) sulfation of \( \text{N}-\text{hydroxy-2-acetylaminofluorene} \) in the postmicrosomal supernatant, markedly sex differentiated in the rat (\( \Delta > 9 \)), was completely feminized by neonatal but not by adult castration. The results suggest that the influence of endocrine ablations on chemical carcinogenesis in rat liver might be mediated via the hypothalamic-pituitary regulation of certain pathways of hepatic xenobiotic metabolism.

INTRODUCTION

Marked sex differences in the formation of malignant liver tumors have been observed in rats following treatment with several liver carcinogens as, for example, 2-AAF (1) and aflatoxin B\(_1\) (2). The mechanisms behind these sex differences are not known in detail, although the involvement of hormonal factors is well established (3-6). As metabolic activation is a prerequisite for the mutagenic and carcinogenic effects of indirect carcinogens (7, 8), sex differences in metabolic pathways of several carcinogens have been studied. For example, the metabolic activation of both B(a)P (9) and 2-AAF (10, 11) has been shown to be sex differentiated in rat liver.

The importance of the hypothalamic-pituitary-liver axis in the regulation of hepatic steroid metabolism in the rat has been described previously (12). Recent work in our laboratory (10) shows that several cytochrome P-450-mediated xenobiotic re-
Birberg at the Department of Organic Chemistry, University of Stockholm, Sweden. Other reference metabolites of 2-AAF were obtained from the National Cancer Institute, Department of Health, Education, and Welfare, Bethesda, MD. Unlabeled BaP, NADP, NADPH, iso- 
citrate, and isocitrate dehydrogenase were from Sigma Chemical 
Company, St. Louis, MO. Unlabeled androstenedione was bought from the 
Upjohn Company, Kalamazoo, MI. 7-EOR and 7-OHR were purchased 
from Pierce Eurochemie B. V., Rotterdam, Holland. All other che-

micals and solvents were of reagent grade and obtained from common 
commercial sources.

Animals. Inbred male and female rats of the Sprague-Dawley strain 
were obtained from ALAB, Stockholm, Sweden. The rats in Experi-

ment 1 were born in the laboratory, whereas the rats in Experiment 2 
were delivered at the age of 3 wk. All rats were fed a basal diet and 
water ad libitum. The first 105,000 × g supernatant was resuspended 
in 0.25 M sucrose in a volume (ml) equivalent with the original liver weight (g).

Preparation of Microsomes. In order to reduce the hepatic levels of 
glycogen, the rats were starved overnight before decapitation. Microsomes 
were prepared according to Erns ter et al. (23) and were washed 
once in 0.15 M Tris-HCl, pH 8.0. The first 105,000 × g supernatant 
contained about 10 mg of protein per ml, and the assay for 
sulfotransferase activity was performed at 37°C instead of at 31°C.

RESULTS

Influence of Neonatal and Adult Castration on Liver Micro-

somal Cytochrome P-450 Content, AHH Activity, and O-De-

ethylation of 7-EOR. No significant differences in the total 

microsomal content of cytochrome P-450 were observed be-

tween the different groups of rats, either in Experiment 1 or in 

Experiment 2 (Table 1). Neonatal castration of male rats sig-

nificantly decreased the sexually differentiated microsomal 

AHH activity (d > 9) towards the female level, whereas castra-

tion of adult male rats did not alter the reaction rate.

The microsomal O-deethylation of 7-EOR was not signifi-
cantly sex differentiated in these experiments and was not 

affected by castration.

Influence of Neonatal and Adult Castration on Liver Micro-

somal Metabolism of Androstenedione. The 6β- and 16α-

hydroxylations of androstenedione in rat liver microsomes were, 
as previously shown (13), sex differentiated (d > 9) (Table 2). The 

rate of these two reactions was lower in microsomes from 

neonatally castrated male rats than in controls. Castration of 

adult male rats significantly decreased 16α- but not 6β-hydroxy-

lation. On the other hand, 5α-reduction was significantly 
increased following both neonatal and adult castration. Finally, 

the ratio between 5α-reduction and 16α-hydroxylation, used as 
an index for the degree of feminization of hepatic steroid 

metabolism, was increased more than 80-fold by neonatal 

castration and 25-fold by adult castration.

Influence of Neonatal and Adult Castration on the Cytochrome 
P-450-dependent Liver Microsomal Metabolism of 2-AAF. The 

overall rate of formation of hydroxylated 2-AAF metabolites 

was sex differentiated (d > 9) as shown in Table 3. Both neonatal 

and adult castration of male rats significantly decreased the 

sum of hydroxylated metabolites when compared to sham-

operated controls. A significant sex difference was observed 

also with respect to microsomal formation of 7-OH-AAF (d > 

9). The formation of this metabolite was decreased by neonatal 

castration but not by castration during adult life.

Microsomes from control male rats were much more efficient 

than microsomes from females in the capacity to form 9-OH-

AAF. Neonatal castration decreased the activity to the female 

level, whereas adult castration decreased the activity when 

expressed as pmol of product formed per min and mg of protein 

but not when expressed as percentage of the total amount of 

hydroxylated 2-AAF metabolites formed.

The formation of 5-OH-AAF was 5-fold higher in micro-

somes prepared from female rats than in microsomes from 

male rats. Neonatal castration increased the microsomal capaci-

ty to perform the reaction almost 3-fold when compared to 

control males but was still significantly lower than in micro-

somes from female rats. Adult castration did not alter the 5-

hydroxylation.

No significant sex difference in the capacity to perform 3-

hydroxylation of 2-AAF was observed nor did neonatal or adult 

castration affect this reaction.

The 1-hydroxylation of 2-AAF was 3- to 4-fold higher in 

microsomes prepared from adult male rats than in microsomes 

from females. Both neonatal and adult castration significantly 

decreased the microsomal rate of formation of 1-OH-AAF. If, 

however, the rate of formation was expressed as the percentage 

of the total amount of hydroxylated metabolites, the effect 

remained significant only in the neonatally castrated rats.

The capacity of microsomes prepared from female rats to 

form N-OH-AAF was approximately 3-fold higher than in 

microsomes from males. Neonatal castration completely fem-

inized the male rat liver with respect to N-OH-AAF formation, 

whereas castration of adult male rats had no significant effect 

on this reaction.

The ratio between the rate of N-hydroxylation and the sum of 

the 7-, 9-, 5-, 3-, and 1-hydroxylations of 2-AAF (N/C ratio) 

was calculated in order to express cytochrome P-450-mediated 

metabolic activation (N-OH-AAF formation) in relation to 

detoxification (C-hydroxylations) of 2-AAF. The ratio in 

females was significantly higher (6-fold) than the ratio in males. 

Neonatal castration increased the ratio to the female level, 

whereas adult castration had no effect.
**EFFECTS IN RAT LIVER OF NEONATAL AND ADULT CASTRATION**

### Table 1  Influence of neonatal and adult castration on liver microsomal cytochrome P-450 content, AHH activity, and O-deethylation of 7-EOR

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Cytochrome P-450 content (nmol/mg protein)</th>
<th>AHH activity (pmol/min • mg protein)</th>
<th>O-Deethylation of 7-EOR (pmol/min • mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>None</td>
<td>0.63 ± 0.14</td>
<td>54 ± 11</td>
<td>111 ± 33</td>
</tr>
<tr>
<td>δ</td>
<td>Neonatal castration</td>
<td>0.58 ± 0.02</td>
<td>74 ± 19</td>
<td>87 ± 20</td>
</tr>
<tr>
<td>δ</td>
<td>Sham neonatal castration</td>
<td>0.60 ± 0.05</td>
<td>209 ± 8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>92 ± 29</td>
</tr>
<tr>
<td>δ</td>
<td>None</td>
<td>0.67 ± 0.05</td>
<td>249 ± 33&lt;sup&gt;e&lt;/sup&gt;</td>
<td>74 ± 22</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>Adult castration</td>
<td>0.65 ± 0.05</td>
<td>201 ± 76</td>
<td>73 ± 15</td>
</tr>
<tr>
<td>δ</td>
<td>Sham adult castration</td>
<td>0.73 ± 0.05</td>
<td>312 ± 32</td>
<td>117 ± 28</td>
</tr>
</tbody>
</table>

* Mean ± SD. Each group consisted of 4 animals.
* Significantly different from male rats castrated as adults (*p* < 0.05; Wilcoxon rank sum test).
* Significantly different from bilaterally castrated male rats (*p* < 0.05; Wilcoxon rank sum test).

### Table 2  Influence of neonatal and adult castration on liver microsomal metabolism of 4-androstenedione-3,17-dione

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>Hydroxylations (nmol/min • mg protein)</th>
<th>5α-Reductase (nmol/min • mg protein)</th>
<th>5α/16α Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>None</td>
<td>0.21 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.02 ± 0.01&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>23.8 ± 3.6</td>
</tr>
<tr>
<td>δ</td>
<td>Neonatal castration</td>
<td>0.17 ± 0.03</td>
<td>0.07 ± 0.06&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>18.8 ± 1.8</td>
</tr>
<tr>
<td>δ</td>
<td>Sham neonatal castration</td>
<td>0.39 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.52 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6 ± 0.9&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>δ</td>
<td>None</td>
<td>0.76 ± 0.29</td>
<td>0.70 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.4 ± 0.3&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>Adult castration</td>
<td>1.6 ± 0.6</td>
<td>0.66 ± 0.32</td>
<td>9.9 ± 2.2</td>
</tr>
<tr>
<td>δ</td>
<td>Sham adult castration</td>
<td>1.8 ± 0.5</td>
<td>2.10 ± 0.27&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>1.6 ± 0.6</td>
</tr>
</tbody>
</table>

* Mean ± SD. Each group consisted of 4 animals.
* Significantly different from females (*p* < 0.05; Wilcoxon rank sum test).
* Significantly different from neonatally castrated male rats (*p* < 0.05; Wilcoxon rank sum test).
* Significantly different from male rats castrated as adults (*p* < 0.05; Wilcoxon rank sum test).

### Table 3  Influence of neonatal and adult castration on the microsomal hydroxylations of 2-AAF in rat liver (pmol/min • mg protein)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment</th>
<th>7-OH (nmol/min • mg protein)</th>
<th>9-OH (nmol/min • mg protein)</th>
<th>5-OH (nmol/min • mg protein)</th>
<th>3-OH (nmol/min • mg protein)</th>
<th>1-OH (nmol/min • mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>None</td>
<td>68.9 ± 10.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.9 ± 1.5</td>
<td>4.4 ± 1.1</td>
<td>2.4 ± 0.9</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>δ</td>
<td>Neonatal castration</td>
<td>66.3 ± 2.0</td>
<td>4.9 ± 0.2</td>
<td>2.4 ± 0.4</td>
<td>2.0 ± 0.3</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>δ</td>
<td>Sham neonatal castration</td>
<td>113.1 ± 13.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.8 ± 4.1&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.9 ± 0.4&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>2.5 ± 0.4</td>
<td>6.7 ± 0.8&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>δ</td>
<td>None</td>
<td>129.1 ± 14.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30.8 ± 3.6&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.9 ± 0.2&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>2.2 ± 0.1</td>
<td>10.7 ± 1.2&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>Adult castration</td>
<td>101.3 ± 9.0</td>
<td>20.4 ± 6.3</td>
<td>0.9 ± 0.2</td>
<td>2.0 ± 0.5</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>δ</td>
<td>Sham adult castration</td>
<td>162.9 ± 7.4</td>
<td>37.6 ± 4.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 ± 0.4</td>
<td>2.9 ± 0.5</td>
<td>12.3 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Mean ± SD. Each group consisted of 4 animals.
* Numbers in parentheses, relative amount (percentage) that each metabolite constitutes of the sum of all hydroxylated metabolites.
* Significant different from female rats (*p* < 0.05; Wilcoxon rank sum test).
* Significantly different from neonatally castrated male rats (*p* < 0.05; Wilcoxon rank sum test).
* Significantly different from sham-castrated male rats (*p* < 0.05; Wilcoxon rank sum test).
* Significantly different from male rats castrated as adults (*p* < 0.05; Wilcoxon rank sum test).

**Influence of Neonatal and Adult Castration on the in Vitro Sulfation of N-OH-AAF in the 105,000 × g Supernatant from Rat Liver**

A marked sex difference was found with respect to the capacity for N-O-sulfation of N-OH-AAF in the 105,000 × g supernatant, male rats exhibiting a much higher sulfotransferase activity than the females (Table 4). Neonatal castration of male rats efficiently decreased (i.e., feminized) the capacity for N-OH-AAF sulfation, whereas castration of adult males was without effect. Interestingly the anesthetics and the surgical trauma of sham castration of neonatal male rats also significantly decreased the sulfotransferase activity, although not to the female level.

Feminization of 2-AAF Hydroxylations in Comparison to Feminization of Androstenedione Metabolism. The ratio between 5α-reduction (♀ > ♂) and 16α-hydroxylation (♂ > ♀) of androstenedione has been used when estimating the degree of feminization of steroid metabolism in rat liver (13). A similar concept might be useful also with respect to the metabolism of 2-AAF. We have therefore calculated both the previously mentioned N/C ratio (♀ > ♂) and the ratio between N-OH-AAF (♀ > ♂) and 9-OH-AAF (♀ > ♂) (N-OH/9-OH ratio). Both of these ratios are, like the 5α/16α ratio of androstenedione, higher in female than in male rat liver.

The correlation coefficients between the N/C and the N-OH/9-OH ratios in 2-AAF metabolism in relation to the 5α/16α ratio in androstenedione metabolism were calculated. Since neonatal castration feminized both the 2-AAF ratios very well, a good correlation with the 5α/16α ratio was obtained in Experiment 1. The linear regression coefficient between the N/C and 5α/16α ratios was 0.92 when mean values for each of.
cytochrome P-450-mediated microsomal metabolism of steroid androstenedione was good in both experiments, and this result strengthens earlier presented data indicating that one and the same species of cytochrome P-450 is the partial feminization of 5α-reduction and 16α-hydroxylation of 2-AAF metabolism as a positive control for sex differences and effects of neonatal castration. Accordingly, the correlation between the N-OH/9-OH ratio of 2-AAF and the 5α/16α ratio of androstenedione was good in Experiment 1. The negative correlation in Experiment 2 could partly be explained by the fact that adult castration decreased instead of increased N-hydroxylation. The correlation between the 9-hydroxylation of 2-AAF and the 16α-hydroxylation of androstenedione was good in both experiments, and this result strengthens earlier presented data indicating that one and the same species of cytochrome P-450 is capable of catalyzing both these reactions.

The feminizing effect of adult castration on 2-AAF metabolism was very weak when compared to the effects of neonatal castration. When, consequently, the 2-AAF ratios mentioned above were used to investigate the feminizing effect of adult castration (Experiment 2), a slightly negative correlation with the 5α/16α ratio was found. It could, however, be noted that, e.g., the 9-hydroxylation of 2-AAF and the 16α-hydroxylation of androstenedione correlated well in Experiment 1 as well as in Experiment 2 (r = 0.86, n = 16, and r = 0.95, n = 8).

DISCUSSION

Our previous studies on the influence of pituitary hormones on rat liver metabolism have shown that continuous infusion of human GH to intact and hypophysectomized Wistar rats partly feminizes in vitro microsomal metabolism of 2-AAF (10). These studies also suggest that multiple cytochrome P-450 species are involved in the sex-differentiated hydroxylations of 2-AAF. The results obtained in the present study clearly show that neonatal castration is an efficient way to feminize not only cytochrome P-450-mediated microsomal metabolism of steroid (androstenedione) and xenobiotic [2-AAF, B(a)P] substrates but also the capacity for N-OH-AAF sulfation in male rat liver. In fact, neonatal castration is even more efficient than GH administration for achieving full feminization. Furthermore, the basic sex differences in 2-AAF metabolism in the Sprague-Dawley rats used in this study are comparable to the sex differences previously observed in Wistar rats (10). The hypothalamo-pituitary regulation of androstenedione metabolism has been extensively studied in our laboratory (10, 12–14, 17, 19, 20). Consequently, in this study, we have used androstenedione metabolism as a positive control for sex differences and effects of neonatal and adult castration.

The partial feminization of 5α-reduction and 16α-hydroxylation of androstenedione following castration of neonatal as well as of adult animals was as expected in view of previously presented results (13, 14, 17). Neonatal castration was 3 times more efficient than adult castration in feminizing the 5α/16α ratio. Results concerning xenobiotic metabolism also showed that neonatal castration was significantly more efficient than adult castration in feminizing cytochrome P-450-dependent metabolism of 2-AAF as well as of B(a)P.
as a determinant of sex differences in chemical carcinogenesis of rat liver.

ACKNOWLEDGMENTS

The authors wish to thank Allan Asp and Stefan Westin for skillful technical assistance.

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Effects of Neonatal and Adult Castration on the in Vitro Metabolism of Steroids and Xenobiotics in Rat Liver

Agneta Blanck, Anders Åström and Tiiu Hansson


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