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

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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 hypothalamo-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-dependent, sex-differentiated, liver microsomal formation of 7-hydroxy-2-acetylaminofluorene, 9-hydroxy-2-acetylaminofluorene, 5-hydroxy-2-acetylaminofluorene, 1-hydroxy-2-acetylanaflolefin, and N-hydroxy-2-acetylaminofluorene from 2-acetylaminofluorene as well as the total microsomal formation of benzo(a)pyrene metabolites (d > 9). Deethylation of 7-ethoxyresorufin was neither sex differentiated nor affected by castration. The capacity for in vitro sulfation of N\(\rightarrow\)hydroxy-2-acetylaminofluorene in the postmitochondrial supernatant, markedly sex differentiated in the rat (d > 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 hypothalamo-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 hypothalamo-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-actions are under hypothalamo-pituitary control. The major determinant of the sexual dimorphism in rat liver metabolism is the secretory pattern of GH (13). A prerequisite for a male pattern of steroid metabolism in the adult rat is exposure of the neonatal male to testicular androgens (14). This exposure leads to an “imprinting” at the hypothalamic level. Sex differences in GH secretion occur following pituitary maturation at puberty (15). In the adult male the pituitary secretion of GH is under a cyclic hypothalamic influence, which seems to be mediated by the combination of somatostatin and a GH-releasing factor (16). The male GH pattern is characterized by a low basal hormone level with marked regular peaks every 3–4 h and is maintained by circulating androgens (13, 15). Female rats exhibit higher basal hormone levels and less marked, more irregular peaks (15).

It has been shown that neonatal castration of male rats “feminizes” the adult male liver with regard to steroid hormone metabolism and makes it unresponsive to testosterone treatment later in life (17). Castration during adult life, on the other hand, results in a partial feminization of liver metabolism which can be reversed by testosterone substitution (18). Estrogen treatment of adult male rats also feminizes steroid metabolism (19), and this effect (as well as the effects of testosterone) seems to be mediated via a regulation at the hypothalamo-pituitary level through the secretory pattern of GH (13). The male rat liver can also be feminized by osmotic minipumps which continuously secrete GH and thereby mimic the GH pattern in the female rat (20).

Toh (6) showed that castration of male rats before the administration of 2-AAF markedly decreased their tendency to form malignant liver tumors. Castration of neonatal rats inhibited tumor formation more efficiently than castration at 2½ mo of age, when the rats were fed 2-AAF at the age of 3 mo. These findings and results discussed above raise the question whether an altered hepatic metabolism of 2-AAF might be responsible for the decreased tendency of castrated male rats to form liver tumors, and, if so, which pathways of 2-AAF metabolism are important with respect to the carcinogenic response.

With the aim to shed some light on these issues we have investigated if neonatal and adult castration of male rats feminizes the metabolism of some well-known xenobiotics, including cytochrome P-450-mediated hydroxylations of 2-AAF as well as the sex-differentiated sulfation of N-OH-AAF (11), in a similar way as shown for rat liver metabolism of steroid hormones.

MATERIALS AND METHODS

Chemicals. 4\(\text{[\(1^4\text{C}\)]Androstene-3,17-dione (androstenedione) (59 mCi/mmol), 2-[\(1^2\text{H}\)]BAF (55.3 mCi/mmol), and \[\text{25 Ci/mmol}\]were purchased from the Radiochemical Centre (Amersham, England). The \(1^4\text{C}\)-labeled androstenedione was purified by silica gel thin-layer chromatography using chloroform:methanol (4:1, v/v) as solvent system. The \(1^4\text{C}\)-labeled 2-AAF was purified as previously described (21), and the \(1^2\text{H}\)-labeled B(a)P, according to Van Cantfort et al. (22). N-OH-AAF was synthesized by Dr. Åke Pilotti and Dr. Winni

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3 The abbreviations used are: 2-AAF, 2-acetylaminofluorene; 7-OH-AAF, 7-hydroxy-2-acetylaminofluorene; 9-OH-AAF, 9-hydroxy-2-acetylaminofluorene; 5-OH-AAF, 5-hydroxy-2-acetylaminofluorene; 3-OH-AAF, 3-hydroxy-2-acetylaminofluorene; 9-OH-AAF, 9-hydroxy-2-acetylaminofluorene; N/C ratio, the ratio between the amount of N-OH-AAF and the amount of ring-hydroxylated metabolites of 2-AAF; AHH, aryl hydrocarbon hydroxylase; B(a)P, benzo[a]pyrene; 7-EOR, 7-ethoxresorufin; 7-OHR, 7-hydroxyresorufin; GH, growth hormone.

References
Influence of Neonatal and Adult Castration on Liver Microsomal Metabolism of Androstenedione. The 6β- and 16α-hydroxylations of androstenedione in rat liver microsomes were, as previously shown (13), sex differentiated (δ > 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-hydroxylation. 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 (δ > 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 (δ > 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 microsomes prepared from female rats than in microsomes from male rats. Neonatal castration increased the microsomal capacity to perform the reaction almost 3-fold when compared to control males but was still significantly lower than in microsomes 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 feminized 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.

RESULTS

Influence of Neonatal and Adult Castration on Liver Microsomal Cytochrome P-450 Content, AHH Activity, and O-Deethylation of 7-EOR. No significant differences in the total microsomal content of cytochrome P-450 were observed between the different groups of rats, either in Experiment 1 or in Experiment 2 (Table 1). Neonatal castration of male rats significantly decreased the sexually differentiated microsomal AHH activity (δ > 9) towards the female level, whereas castration of adult male rats did not alter the reaction rate.

The microsomal O-deethylation of 7-EOR was not significantly sex differentiated in these experiments and was not affected by castration.

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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-OH-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 anesthesia 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 the four groups were used. When only one group was used, it was 0.87 (♂).
The four groups in Experiment 1 were used and 0.87 when the same calculation was made with individual values for the 16 rats. Corresponding values for the N-OH/9-OH and 5α/16α ratios were r = 0.90 and r = 0.82.

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.

Also the sulfotransferase activity towards N-OH-AAF was completely feminized by neonatal castration. No significant effect was, however, seen following adult castration, which confirms the data previously presented by De Baun et al. (11).

We have previously suggested that the ratio between N-OH-AAF and 9-OH-AAF formation could be used as a tool to measure the degree of feminization of 2-AAF metabolism (10). In these terms the neonatally castrated males were completely feminized, whereas no feminization was achieved following adult 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 (10).

N-Hydroxylation is generally considered as an important step in the formation of carcinogenic metabolites from 2-AAF (8). It was therefore interesting to notice that the ratio between metabolic activation and detoxification of 2-AAF (N/C ratio) was higher in female than in male rats. The N/C ratio was well feminized (i.e., increased) in neonatally castrated rats, whereas no significant alteration was seen following adult castration. As male rats develop hepatomas both earlier and more frequently than female rats following 2-AAF treatment, these observations are surprising.

It is reasonable to assume that the effects of neonatal castration on the metabolism of 2-AAF and B(a)P are due to the lack of imprinting by testicular androgens at the hypothalamic level during the neonatal period, as suggested for androstenedione metabolism (14, 17). It remains to be investigated whether the feminization of xenobiocic metabolism following castration during neonatal or adult life is reversible by testosterone substitution. The previously mentioned reports (10, 13), indicating the secretory pattern of GH as an important determinant of sex differences in androstenedione as well as in 2-AAF and B(a)P metabolism, however, strongly support the concept that similar mechanisms are involved in the hypothalomo-pituitary regulation of steroid and xenobiotic metabolism in rat liver.

In view of the previously mentioned experiments by Toh (6) it is particularly interesting to notice that neonatal castration of male rats, much more efficiently than castration of adult males, feminizes several pathways of 2-AAF metabolism. The high N/C ratio of 2-AAF found in female and in neonatally castrated male rats seemingly is difficult to reconcile with the fact that male rats are generally more susceptible to 2-AAF carcinogenesis. However, the marked sex differences in and effects of neonatal castration on the N-OH-AAF sulfotransferase levels provide a plausible biochemical explanation to the obvious sexual dimorphism of rats in the tendency to form hepatocellular carcinomas following 2-AAF treatment. The very toxic and reactive sulfate ester of N-OH-AAF has recently been postulated to be an important promotor in 2-AAF carcinogenesis (29, 30), which further strengthens the view that this specific pathway of 2-AAF metabolism might be a major determinant of the hepatocarcinogenic response. A detailed study concerning the pituitary regulation of the N-OH-AAF sulfotransferase is presently being performed in our laboratory in order to further corroborate the concept of the hypothalomo-pituitary control of xenobiocetic-metabolizing hepatic enzymes.
as a determinant of sex differences in chemical carcinogenesis of rat liver.

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