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SEX HORMONE-MEDIATED EFFECTS ON THE PHASE I AND PHASE II METABOLISM OF *N*-2-FLUORENYLACETAMIDE

MODULATION OF 9-HYDROXYLATION

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Abstract—Sex differences in the phase I (cytochrome P-450-catalyzed hydroxylations) and phase II (conjugations) metabolism of *N*-2-fluorenylacetamide (2-FAA) by the livers of 50-day-old Sprague–Dawley rats and effects of gonadectomy were determined. The higher level (1.4 times) of cytochrome P-450 in the microsomes of male rats correlated with their 8 and 1.3 times greater capacities to form 9-hydroxy(OH)-2-FAA and 7-OH-2-FAA respectively. One week after gonadectomy, the formation of 9-OH-2-FAA, the major metabolite in the male, was decreased by 70%, whereas in the female it was increased 1.3 times. Treatment of male rats with β-naphthoflavone (β-NF) increased the formation of phenolic metabolites and *N*-OH-2-FAA, but decreased that of 9-OH-2-FAA. The amounts of 9-OH-2-FAA were increased, however, in β-NF-treated female and gonadectomized male rats. These sex hormone- and β-NF-mediated differences in the extent of 9-hydroxylation of 2-FAA are discussed in relation to the fluctuations in the levels of specific cytochrome P-450 isozymes. In contrast to the phenolic metabolites and *N*-OH-2-FAA, 9-OH-2-FAA was a poor substrate for UDP-glucuronyltransferase; this conjugation was not induced by treatment of male rats with β-NF. Hence, in the presence of male hormones, relatively large amounts of 9-OH-2-FAA were formed and possibly retained in the liver. A role of this alcohol as a potential promoter in hepatocarcinogenesis by 2-FAA is suggested.

A greater susceptibility of male than female rats to hepatocarcinogenesis by *N*-2-fluorenylacetamide (2-FAA) and modulation of this process by sex hormones resulting in the protection of the male and sensitization of the female have been established [reviewed in Refs. 1 and 2]. The mechanism of action of sex hormones in hepatocarcinogenesis has not been elucidated yet although the evidence supporting their role in both initiation and promotion of the process has been presented [reviewed in Refs. 2 and 3]. The role of sex hormones in the initiation of hepatocarcinogenesis may be associated with hormonal regulation of enzymes activating carcinogens. 2-FAA requires a two-step metabolic activation: N-hydroxylation to *N*-hydroxy(OH)-2-FAA, and sulfation of *N*-OH-2-FAA to the sulfate ester, an electrophile capable of covalent binding to DNA [4]. A sex difference in the N-hydroxylation of 2-FAA has been shown in the greater capacity of hepatic microsomes of female rats to form *N*-OH-2-FAA [5–7]. Moreover, greater amounts of the glucuronide of *N*-OH-2-FAA are excreted in the bile of female rats [8, 9] and urine of castrated male rats [10]. On the other hand, the activity of hepatic sulfotransferase catalyzing the sulfation of *N*-OH-2-FAA is greater in the presence of male or the absence of female hormones [8, 11–13] and correlates with the susceptibility of the male or testosterone-primed female rat to hepatocarcinogenesis by 2-FAA or *N*-OH-2-FAA [1, 2, 14, 15]. Katayama *et al.* [16] reported that castration of 8-week-old male rats of Fischer 344 strain 2 weeks before the dietary intake of 2-FAA reduces neoplastic nodules in the liver. This result was correlated with the reduction of hepatic microsomal cytochrome P-450 level and aryl hydrocarbon hydroxylase and UDP-glucuronyltransferase (GT) activities. However, the effect of gonadectomy-induced decreases in these microsomal enzymes on the metabolism of 2-FAA has not been determined.

The present study was undertaken to determine the effects of sex hormones on the phase I (cytochrome P-450-catalyzed N- and ring-hydroxylations) and phase II (UDP-GT-catalyzed conjugations) metabolism of 2-FAA by the livers of Sprague–Dawley (SD) rats. Thus, the metabolites of 2-FAA formed by hepatic microsomal fractions of normal and gonadectomized male and female rats were determined. Because in normal rat liver the overall metabolism of 2-FAA, particularly its N-hydroxylation, is low [5, 9, 17], treatment of normal and gonadectomized rats with β-naphthoflavone (β-NF), an inducer of metabolism of 2-FAA [18, 19], was included in this study.

MATERIALS AND METHODS

Chemicals, columns and solvents. Fluorenyl compounds were synthesized and tested for purity as described previously [18]. 2-FAA and *p*-hydroxybiphephyl were purchased (Aldrich Chemical Co.

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Inc., Milwaukee, WI) and recrystallized [18]. Acetohydroxamic acid, albumin (bovine serum), chloroacetic acid, corn oil, cytochrome *c* (type III from horse heart), dithionite, NADH, NADPH, β-NF, *p*-nitrophenyl sulfate, Trizma hydrochloride and uridine 5′-diphosphoglucuronic acid (UDPGA) (sodium-salt) were obtained from the Sigma Chemical Co., St. Louis, MO. Dithiothreitol and *p*-nitrophenol (*p*-NP) (A grade) were from Calbiochem, San Diego, CA. UDPGA [*glucuronyl*-U-^{14}C] was from ICN Radiochemicals, Irvine, CA. Acetone and glycerol, A.C.S. grade, were from the Fisher Scientific Co., Fair Lawn, NJ. All other reagents were A.C.S. grade from EM Science, Cherry Hill, NJ. C_{18} (Baker-10-SPE) extraction columns were from the J. T. Baker Chemical Co., Phillipsburg, NJ. Perisorb RP-2, 30–40 μm (E. Merck), for guard column was from Altech Associates, Inc., Deerfield, IL, and Zorbax C_8 column was from the DuPont Co., Wilmington, DE. All solvents used for chromatography were glass distilled, HPLC grade, from the Mallinckrodt Chemical Works, St. Louis, MO.

Maintenance and treatment of rats. Male and female SD rats (Holtzman Co., Madison, WI), three groups of each sex (twelve rats/group), 36 ± 1 days old upon arrival, were maintained on regular Purina Chow pellets and water *ad lib.* At 43 ± 1 days of age, bilateral orchidectomy or ovariectomy or sham-surgery was performed. Rats were injected i.p. for 3 consecutive days with corn oil (2.5 ml/kg of body weight) or β-NF in corn oil (40 mg/kg of body weight) with the last injection 24 hr before sacrifice at 50 ± 1 days of age. Food was withdrawn from all rats 12 hr before decapitation. One group of twelve male rats was killed 5 weeks after gonadectomy. Each group consisted of four subgroups according to the treatment: corn oil-treated/sham operated (CRL/SH), corn oil-treated/gonadectomized (CRL/GND), β-NF-treated/sham operated (β-NF/SH), β-NF-treated/gonadectomized (β-NF/GND) with two to three rats per treatment group.

Preparation of microsomal fractions. All buffers were cooled in ice, and all procedures were carried out at 4°. Livers were perfused with 0.154 M KCl, 0.05 M Tris–HCl buffer, pH 7.4, excised and immersed in the same buffer. The homogenates and microsomal fractions were prepared from individual livers as described previously [18]. The protein content of the microsomal fractions was determined by the method of Lowry *et al.* [20] with a bovine serum albumin standard.

Microsomal enzyme assays. The microsomal suspensions (0.1 ml) were diluted with 0.3 M sodium phosphate buffer, pH 7.5, containing 50% glycerol to approximately 1 mg/ml protein concentration. Cytochrome P-450 and cytochrome b_5 contents were determined by the classical spectrophotometric method [21]. The activities of NADH- and NADPH-dependent cytochrome *c* reductases were determined essentially as described [22] using microsomal suspensions diluted with 0.05 M sodium phosphate buffer, pH 7.5, to protein concentration of 0.02 mg/ml. The assays were carried out in 0.3 M sodium phosphate buffer, pH 7.5, containing 0.10 mM EDTA, 0.07 mM cytochrome *c* and 0.10 mM NADPH or 0.12 mM NADH in a 1-ml volume. An Hitachi 110A computerized double beam ratio recording spectrophotometer was used.

Microsomal UDP-GT activity with *p*-NP was assayed according to the standardized colorimetric method [23]. Determinations of UDP-GT activities with 5-OH-2-FAA, *N*-OH-2-FAA and 9-OH-2-FAA were as described [19] with some modifications. The microsomal suspension was pre-incubated with the substrate (in a total volume of 1 ml) for 5 min at 37° and, after addition of [^{14}C]UDPGA (approximately 2×10^5 dpm), 0.2-ml aliquots were taken at 0, 5, 10 and 30 min. After heat inactivation of the aliquots (immersion in a boiling water bath for 2 min), they were applied on 1-ml Baker C_{18} extraction columns which were prewashed with methanol followed by 1 mM ammonium acetate [24]. The [^{14}C]glucuronides were eluted with 1 ml of 60% (v/v) methanol. The radioactivity of these eluates was measured in scintillation fluid [25] using a Packard liquid scintillation spectrometer (TRI-CARB model 3255). The average counting efficiency was 78%.

Determination of ring- and N-hydroxylations of 2-FAA by hepatic microsomes. The composition of the incubation mixtures was described previously [18]. The incubation mixtures were terminated by immersing flasks in a boiling water bath for 5 min and then cooled in ice. *p*-Hydroxybiphenyl (10 nmol in 0.05 ml) was added as internal standard (IS). The method of extraction of metabolites was modified in that a solid phase extraction was used. The C_{18} extraction columns were activated by applications of the following: 0.5 ml of methanol, 1.0 ml of 2.5% (w/v) acetohydroxamic acid in 0.1 M potassium phosphate buffer, pH 7.4, and 0.5 ml of buffer. The mixtures were then applied on the columns and washed with 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4. 2-FAA, its metabolites and IS were eluted with 0.5 ml methanol:isopropanol (1:1, v/v) and dried with Na_2SO_4, and the solvent was evaporated to dryness under N_2. The residues were then dissolved in methanol for HPLC analysis. The mobile phase [18] and chromatographic equipment [26] were described previously. The metabolites were identified by UV spectroscopy of individual peaks during chromatographic run and quantified from peak areas relative to standard curves. The peak areas were integrated at 280 nm except for 9-OH-2-FAA and IS which were integrated at 300 and 260 nm respectively. Small amounts of UV absorbing substances from the extracts of the incubation mixtures lacking 2-FAA coeluted with 5-OH-2-FAA and 2-FAA, and were subtracted from their respective peak areas. Corrections for losses during extraction were calculated using recoveries of IS from individual analyses and ratios of recovery of each standard compound to that of IS determined with the mixtures of standard compounds as described previously [18]. For ring-hydroxylated metabolites of 2-FAA and 9-oxo-2-FAA, the ratios were 1.0; for 2-FAA and *N*-OH-2-FAA the ratios were 0.9 and 0.42 respectively.

Data analysis. Statistical analysis was performed and the P values were obtained by analysis of variance according to the published method [27].

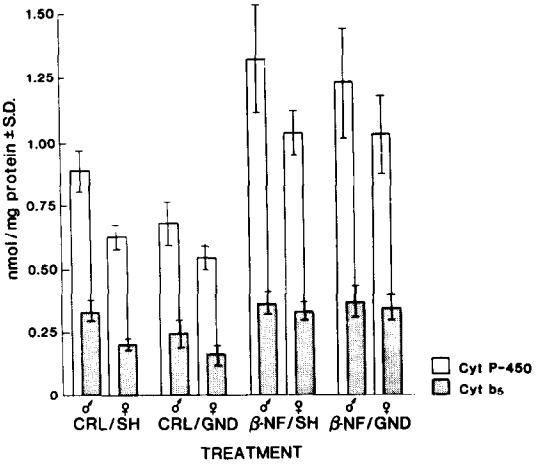

Fig. 1. Effect of gonadectomy on hepatic microsomal cytochrome P-450 (cyt P-450) and cytochrome b_5 (cyt b_5) in corn oil (CRL)- and β-naphthoflavone (β-NF)-treated rats. Treatment of rats, preparation of microsomes, and determination of cytochromes are described in Materials and Methods. Three separate experiments with each sex were carried out, each consisting of four treatment groups with two to three rats per group. Values are the means ± SD from six to nine individual livers. Differences in the amounts of cyt P-450 and cyt b_5 between male and female livers of CRL/SH groups were significant ($P < 0.001$). Differences in the amounts of cyt P-450 between CRL/SH and CRL/GND ($P < 0.01$), and CRL- and β-NF-treated groups (CRL/SH vs β-NF/SH and CRL/GND vs β-NF/GND) ($P < 0.001$) were significant in both sexes. Differences in the amounts of cyt b_5 between CRL/SH and CRL/GND in the male ($P < 0.01$) and female ($P < 0.05$) groups, and between CRL/SH and β-NF/SH in the female groups ($P < 0.001$), and between CRL/GND and β-NF/GND in the male ($P < 0.01$) or female ($P < 0.001$) groups, were significant.

RESULTS

Effect of gonadectomy on the hepatic microsomal mixed-function oxidase and related enzymes of male and female rats. The levels of hepatic microsomal cytochrome P-450 and cytochrome b_5 were 1.4 and 1.7 times higher, respectively, in male than female rats (Fig. 1, CRL/SH groups). One week after gonadectomy, 10–30% decreases in the cytochrome levels were determined in hepatic microsomes of rats of both sexes (CRL/GND vs CRL/SH groups). Treatment of rats of both sexes with β-NF increased the levels of cytochrome P-450 in hepatic microsomes of CRL/SH (~1.6 times) and CRL/GND (~1.9 times) groups. Induction of cytochrome b_5 by β-NF was somewhat greater in female (1.6 and 2.1 times in CRL/SH and CRL/GND groups respectively) than male rats. The activities of NADPH- and NADH-dependent cytochrome *c* reductases were similar in hepatic microsomes of both sexes and essentially unaffected by treatment of rats with β-NF (data not shown). One week after gonadectomy, marked decreases (~30%) were determined in the activities of NADPH–cytochrome *c* reductase (male liver) and NADH–cytochrome *c* reductase (female liver). The hepatic microsomal cytochromes were also determined in 78-day-old male rats 5 weeks after gonadectomy (Table 1). The β-NF- and gonadectomy-induced fluctuations in the cytochrome P-450 levels were similar to those determined 1 week after gonadectomy.

Effect of gonadectomy of male and female rats on the hepatic microsomal ring- and N-hydroxylations of 2-FAA. The higher level of cytochrome P-450 in the hepatic microsomes of male rats (Fig. 1, male vs female CRL/SH groups) correlated with their greater capacities to form 9-OH-2-FAA (8 times) and 7-OH-2-FAA (1.3 times) (Fig. 2, male vs female CRL/SH groups). The amounts of 9-OH-2-FAA were affected by gonadectomy in both sexes in that they were decreased by 70% in the male liver and increased 1.3 times in the female liver (CRL/SH vs CRL/GND groups). Thus, 9-OH-2-FAA was the major metabolite formed by hepatic microsomes of male rats and was the only metabolite significantly affected in both sexes by gonadectomy. Small amounts of 9-oxo-2-FAA, 1.60 ± 0.42 and 0.60 ± 0.30 nmol/6 mg protein/10 min, were also formed by hepatic microsomes of CRL/SH and CRL/GND male rats respectively (data not shown). The incubations of hepatic microsomes from CRL/SH rats with 9-OH-2-FAA (100 nmol) yielded 9-oxo-2-FAA (5 or 2 nmol with or without NADPH respectively). Sex differences in the formation of 9-OH-2-FAA were also determined in response to the treatment of rats with β-NF, an inducer of phase I metabolism of 2-FAA [18, 25]. In the β-NF/SH male rats formation of 9-OH-2-FAA was decreased by 42%, whereas in the β-NF/SH female or β-NF/GND male rats it was increased 2.9 and 1.3 times respectively (Fig. 2). The sex hormone- and β-NF-mediated differences in the levels of specific cytochrome P-450 isozymes [28] may explain the fluctuations in 9-hydroxylation of 2-FAA [29].

The amounts of *N*-OH-2-FAA, a proximate carcinogenic metabolite of 2-FAA, formed by hepatic microsomes of male and female rats were similar and not affected significantly by gonadectomy (Fig. 2, CRL/SH vs CRL/GND groups). Treatment of rats with β-NF induced N-hydroxylation of 2-FAA in hepatic microsomes of male (4.1 times) and female (3.4 times) rats (β-NF/SH vs CRL/SH groups). A greater increase in N-hydroxylation of 2-FAA (7.2 times) was shown in hepatic microsomes of β-NF-treated ovariectomized rats (β-NF/GND vs CRL/GND groups) which suggested the induction of N-hydroxylating cytochrome P-450 isozyme(s).

Treatment of sham-operated or gonadectomized rats with β-NF increased to a similar extent the capacities of hepatic microsomes of both sexes to form the phenols of 2-FAA: 5- or 3-OH-2-FAA (18 times), 7-OH-2-FAA (~7 times) and 1-OH-2-FAA (~3 times) (Fig. 2, β-NF/SH vs CRL/SH and β-NF/GND vs CRL/GND groups). Metabolic capacity of hepatic microsomes of 50-day-old male rats to form the phenols of 2-FAA was similar to that of 78-day-old male rats (Table 1). However, the younger rats had 1.4 and 1.9 times greater capacities to form 9- and *N*-OH-2-FAA, respectively, than the older rats. In the latter, 5 weeks after orchidectomy or treatment with β-NF, the formation of 9-OH-2-FAA was

Table 1. Effect of gonadectomy on activities of hepatic microsomal enzymes and metabolism of 2-FAA of 78-day-old male rats

Enzymes and metabolites	Treatment groups			
	CRL/SH	CRL/GND	β-NF/SH	β-NF/GND
Cytochrome P-450*	0.89 ± 0.04	0.74 ± 0.01†	1.23 ± 0.23	1.18 ± 0.34
Cytochrome b_5*	0.36 ± 0.06	0.28 ± 0.04	0.39 ± 0.05	0.49 ± 0.22
Metabolites of 2-FAA‡				
9-OH-2-FAA	10.1 ± 1.19	3.92 ± 2.20§	7.45 ± 1.74‖	4.21 ± 0.60¶
7-OH-2-FAA	5.82 ± 0.99	5.29 ± 1.18	29.6 ± 4.74**	32.0 ± 5.31††
5-OH-2-FAA	1.34 ± 0.21	1.18 ± 0.12	23.34 ± 2.47**	28.5 ± 3.13††
3-OH-2-FAA	1.28 ± 0.22	1.13 ± 0.08	21.1 ± 2.20**	23.3 ± 0.97††
1-OH-2-FAA	0.43 ± 0.08	0.43 ± 0.10	0.85 ± 0.13**	0.85 ± 0.18††
N-OH-2-FAA	0.85 ± 0.12	0.72 ± 0.05‖	1.20 ± 0.41†	1.51 ± 0.32††
UDP-GT-p-NP‡‡	16.7 ± 1.07	9.41 ± 1.55†	21.9 ± 2.32	15.7 ± 6.40
UDP-GT-5-OH-2-FAA‡‡	1.42 ± 0.25	1.42 ± 0.51	3.44 ± 0.38‖	3.43 ± 0.75
UDP-GT-N-OH-2-FAA‡‡	1.62 ± 0.52	1.09 ± 0.22	1.21 ± 0.43	1.28 ± 0.29

Treatment of rats included gonadectomy (GND) or sham operation (SH) at 43 days of age. Rats were treated with corn oil (CRL) or β-naphthoflavone (β-NF) as described in Materials and Methods; injections were given on 3 consecutive days prior to sacrifice at 78 days of age, i.e. 5 weeks after gonadectomy. Preparation of microsomal fractions and enzyme and metabolite assays are described in Materials and Methods. Values are the means ± SD from three individual livers, each in duplicate.

* Expressed as nmol/mg protein.
† Different from CRL/SH group at P = 0.05.
‡ Expressed as nmol/6 mg protein/10 min.
§ Different from CRL/SH group at P = 0.005.
‖ Different from CRL/SH group at P = 0.025.
¶ Different from β-NF/SH group at P < 0.001.
** Different from CRL/SH group at P < 0.001.
†† Different from CRL/GND group at P < 0.001.
‡‡ Expressed as nmol/mg protein/min.

decreased by 61 or 26% respectively. These data indicated that 9-OH-2-FAA was the major hepatic microsomal metabolite of 2-FAA in male rats ranging from 50 to 78 days of age, and that the early effect of gonadectomy resulting in the marked decrease in the formation of 9-OH-2-FAA was not potentiated by a prolonged period of sex hormone deprivation.

Effect of gonadectomy of male and female rats on the hepatic microsomal UDP-GT activities. UDP-GT activities of male hepatic microsomes toward *p*-NP, 5- and *N*-OH-2-FAA were 2.6, 2.5 and 1.8 times greater, respectively, than those of female rats (Fig. 3, CRL/SH groups). One week after orchidectomy these UDP-GT activities were decreased by 63, 32 and 40% respectively (CRL/GND vs CRL/SH male groups). Although fluctuations in UDP-GT activity toward 9-OH-2-FAA were similar to the above, these differences were not significant ($P \leq 0.09$), probably because of very low rates of glucuronidation of 9-OH-2-FAA, and thus an increased error of the assay. In the female, ovariectomy-induced effects varied with the substrate: UDP-GT activity toward *p*-NP was decreased by 45%, whereas the rates of glucuronidation of 9- and *N*-OH-2-FAA were increased 4.4 and 1.4 times, respectively, and the rate of glucuronidation of 5-OH-2-FAA was unchanged (Fig. 3, CRL/GND vs CRL/SH female groups). These data suggested a different composition of UDP-GT isozyme(s) in male and female rat liver. The sex differences in the rates of glucuronidation of the phenols, but not of *N*-OH-2-FAA, were also determined in response to the treatment of rats with β-NF, an inducer of UDP-GT activities [19, 30]. Thus, UDP-GT activities toward *p*-NP and 5-OH-2-FAA were increased 1.5 and 2.2 times, respectively, in the male liver, and 3.4 and 11 times, respectively, in the female liver (Fig. 3, β-NF/SH vs CRL/SH groups). Even with the increased rate of glucuronidation of 9-OH-2-FAA in the hepatic microsomes of β-NF-treated female rats, this compound remained a poor substrate for hepatic UDP-GT(s). When the accumulation of the glucuronide was measured at 30 min, very small amounts of this conjugate (~1 nmol) were determined and were not increased significantly by treatment of rats with β-NF (data not shown). Since 9-OH-2-FAA was the major metabolite of 2-FAA formed by hepatic microsomes of male rats (Fig. 2, CRL/SH group) and was a poor substrate for conjugation by UDP-GT, this alcohol may accumulate in the liver or be metabolized prior to the conjugation.

Comparison of UDP-GT activities in hepatic microsomes of 50-day- and 78-day-old male rats showed 2.5 times greater activity toward 5-OH-2-FAA in the younger rats (3.59 ± 0.89 vs 1.42 ± 0.25 nmol/mg protein/min at $P < 0.05$) (Table 1). This activity in the older rats was unchanged 5 weeks after orchidectomy, whereas the activities toward *p*-NP and *N*-OH-2-FAA were decreased by 40 and 30% respectively. The effect of treatment of older rats with β-NF on UDP-GT activities was similar to that determined in younger rats. Hence, the data on phase II metabolism sup-

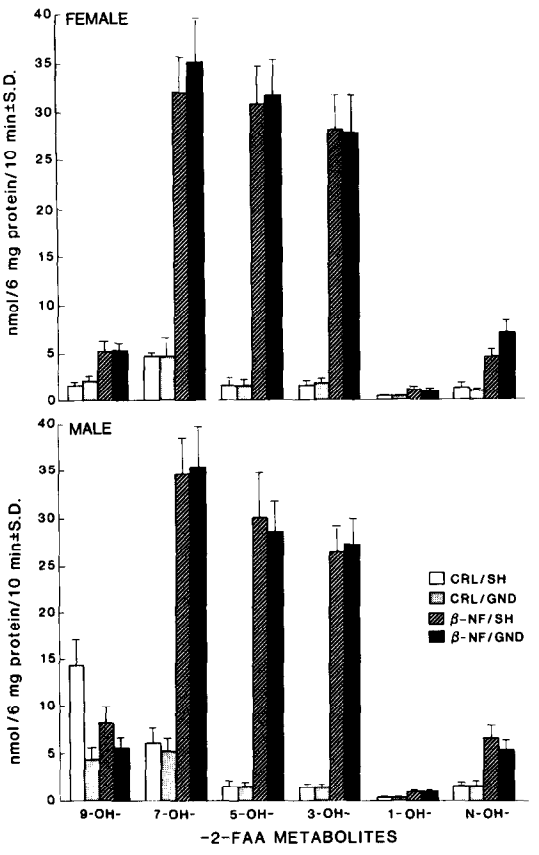

Fig. 2. Effect of gonadectomy on ring- and N-hydroxylations of 2-FAA by hepatic microsomes of corn oil (CRL)- and β-naphthoflavone (β-NF)-treated rats. Treatment of rats, preparation of microsomes, composition of the incubation mixtures, extraction, and determination of metabolites of 2-FAA are described in Materials and Methods. Three separate experiments with each sex were carried out, each consisting of four treatment groups, with two to three rats per group. Values are the means ± SD from six to nine individual livers, each in duplicate incubations. Differences in the amounts of 9-OH-2-FAA between male and female livers of CRL/SH groups ($P < 0.001$), and between CRL/SH and CRL/GND male ($P < 0.001$) or female ($P = 0.005$) groups, and between CRL/SH and β-NF/SH groups of both sexes ($P < 0.001$), and between CRL/GND and β-NF/GND male ($P = 0.01$) or female ($P < 0.001$) groups, were significant. Differences in the amounts of 7-, 5-, 3-, 1- and *N*-OH-2-FAA between CRL/SH and β-NF/SH, and between CRL/GND and β-NF/GND groups of both sexes, were significant ($P < 0.001$).

ported the findings on the phase I metabolism in that the effects of sex hormones were already evident 1 week after gonadectomy and not potentiated by a longer period of sex hormone deprivation.

DISCUSSION

It has been established that the levels of hepatic microsomal enzymes related to the activities of mixed-function oxidase and UDP-GT are higher in male than female rats and are decreased by gonadectomy in both sexes [8, 16, 28, 31–34]. The results of our studies are consistent with these reports; in 50-day-old SD rats the levels of cytochrome P-450, cytochrome b_5 and UDP-GT activity toward *p*-NP were significantly higher in male than female hepatic microsomes (Figs. 1 and 3). One week after orchidectomy, the decreases in the cytochrome levels and *p*-NP-UDP-GT activity were somewhat greater (20–60%) than after ovariectomy (10–40%). The sex differences and gonadectomy-induced decreases in the enzymes related to phase I and phase II metabolism of xenobiotics above were reflected in the metabolism of 2-FAA (Figs. 2 and 3). The sum of ring- and N-hydroxylated metabolites of 2-FAA formed by hepatic microsomes was 2.2 times greater in the male (25.6 nmol) than in the female (11.4 nmol.) This difference was due to 8 and 1.3 times greater amounts of 9-OH-2-FAA and 7-OH-2-FAA, respectively, formed by the hepatic microsomes of male rats. Thus, 9-OH-2-FAA emerged as a major metabolite of 2-FAA formed in 2.3 times greater amounts than 7-OH-2-FAA by hepatic microsomes of male rats. In Wistar rats, the sex difference in the rate of formation of 9-OH-2-FAA (5.4 times greater in the male) was also shown [7]; however, the initial rates of formation of 7-OH-2-FAA were greater than of 9-OH-2-FAA. Substantial amounts of 9-OH-2-FAA formed by hepatic microsomes of male SD rats have been also reported by earlier investigators [35–37]. In addition, small amounts of 9-oxo-2-FAA, most likely derived from 9-OH-2-FAA, were found among metabolites of 2-FAA [35, 36].

In the adult male rat liver, four cytochrome P-450 isozymes (2c(♂)/UT-A > PB-1/PB-C > 3/UT-F > PB-4/PB-B)* (listed in the order of the amounts present) [28] are capable of 9-hydroxylation of 2-FAA [29]. The latter three isozymes are also present in the female liver. After orchidectomy the amounts of 2c(♂)/UT-A and PB-1/PB-C are decreased [28], which may account for the marked decrease in 9-hydroxylation of 2-FAA by hepatic microsomes of the orchidectomized rats in this study (Fig. 2). In ovariectomized rats, an increase in the 2c(♂)/UT-A isozyme [28] would explain the increased amounts of 9-OH-2-FAA. As with gonadectomy, treatment of rats with β-NF resulted in sex opposite effects on 9-hydroxylation of 2-FAA: a decrease in the male and an increase in the female (Fig. 2). This can be explained by the effect of β-NF on cytochrome P-450 isozymes. Although β-NF induces 9-hydroxylating isozyme βNF-B in the livers of both sexes, it affects differently the native 9-hydroxylating isozymes in the male (decrease in 2c(♂)/UT-A and PB-1/PB-C) and in the female (increase in 3-UT-F) [28]. The decrease in 9-hydroxylation of 2-FAA in the male rat effected by β-NF was consistent with that by 3-methylcholanthrene (3-MC) [35, 36], an inducer of cytochrome P-450 isozymes analogous to β-NF [29].

In the rat liver, N-hydroxylation of 2-FAA was catalyzed by PB-2a/PCN-E cytochrome P-450 isozyme [29]. Although this isozyme is present in 20 times greater amounts in the male than female adult

* The nomenclature for the cytochrome P-450 isozymes is that of Ref. 28.

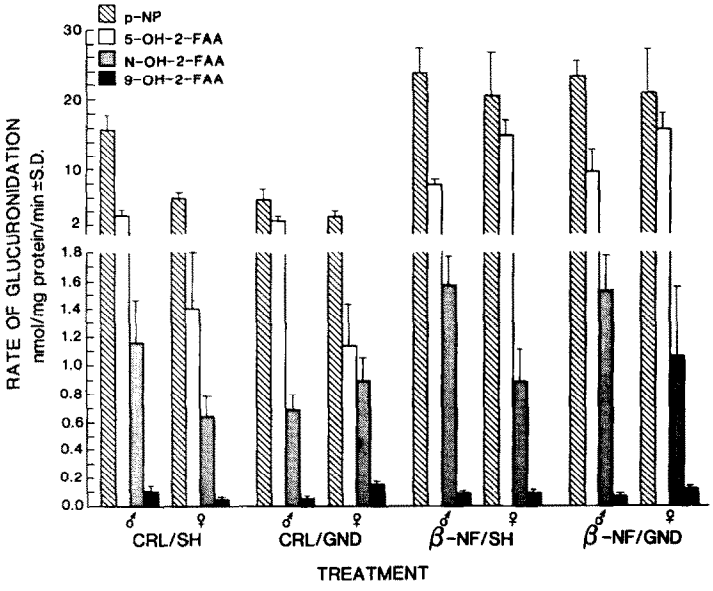

Fig. 3. Effect of gonadectomy on hepatic microsomal UDP-GT activities in corn oil (CRL)- and β-naphthoflavone (β-NF)-treated rats. Treatment of rats, preparation of microsomes and UDP-GT assays with *p*-NP, 5-OH-2-FAA, *N*-OH-2-FAA and 9-OH-2-FAA are described in Materials and Methods. Three separate experiments with each sex were carried out, each consisting of four treatment groups with two to three rats per group. Values are the means ± SD from six to nine livers, each carried out in duplicate. Differences in UDP-GT activities toward *p*-NP and 5-OH-2-FAA ($P < 0.001$), and *N*-OH-2-FAA ($P = 0.005$) between male and female livers of CRL/SH groups were significant. Differences in UDP-GT activities toward *p*-NP between groups CRL/SH and CRL/GND, CRL/SH and β-NF/SH, and CRL/GND and β-NF/GND of both sexes were significant ($P < 0.001$). Differences in UDP-GT activities toward 5-OH-2-FAA between CRL/SH and CRL/GND male groups ($P < 0.05$) and between CRL/SH and β-NF/SH or CRL/GND and β-NF/GND groups of both sexes ($P < 0.001$) were significant. Differences in UDP-GT activities toward *N*-OH-2-FAA between groups CRL/SH and CRL/GND of both sexes ($P < 0.01$), and between CRL/SH and β-NF/SH of the male ($P < 0.01$) and female ($P < 0.05$) groups, and between CRL/GND and β-NF/GND of the male groups ($P < 0.001$), were significant. Differences in UDP-GT activities toward 9-OH-2-FAA between CRL/SH and CRL/GND ($P < 0.01$) and between CRL/SH and β-NF/SH ($P < 0.05$) of the female groups were significant.

liver [28], no significant sex difference in the level of N-hydroxylation of 2-FAA was determined (Fig. 2). This suggested that yet another cytochrome P-450 isozyme(s) in the female rat liver was capable of N-hydroxylation of 2-FAA. Greater 2-FAA N-hydroxylating capacities of hepatic microsomes of female rats reported previously [5–7] may have been due to different conditions of incubations and strain or age of rats. It has been noted that the level of N-hydroxylation varied with the substrate concentration [37]. At 40 μM 2-FAA used in our study, *N*-OH-2-FAA constituted 6.4 and 11.5% of the total hydroxylated metabolites of 2-FAA in the male and female liver respectively. Gonadectomy of adult rats does not alter the level of PB-2a/PCN-E isozyme [28], which is consistent with the lack of effect of gonadectomy on the 2-FAA N-hydroxylating activity herein (Fig. 2). Treatment of rats with β-NF induces two N-hydroxylating cytochrome P-450 isozymes, βNF-B and ISF-G, which have much higher specific activities (>1 nmol *N*-OH-2-FAA/min/nmol P-450) than the native PB-2a/PCN-E isozyme (0.006 nmol *N*-OH-2-FAA/min/nmol P-450) [29]. Hence, in spite of the decrease of the latter by β-NF [28], the overall increase in N-hydroxylation of 2-FAA due to β-NF was unaffected.

Our earlier studies on the glucuronidation of ring- and N-hydroxylated metabolites of 2-FAA by hepatic microsomes of female rats showed that the phenols and *N*-OH-2-FAA shared chiefly the characteristics of substrates for group 1 UDP-GT activities, i.e. those inducible with β-NF or 3-MC [19]. Glucuronidations of 5-OH-2-FAA (representative phenol) and *N*-OH-2-FAA determined herein confirmed the above findings and showed that UDP-GT activities toward these substrates were higher in the male than female rat liver (Fig. 3). Whereas 1 week after orchidectomy these UDP-GT activities were decreased, ovariectomy increased the rate of glucuronidation of *N*-OH-2-FAA and did not change that of 5-OH-2-FAA. This pattern suggested that male UDP-GT isozyme(s) is increased in the liver of the ovariectomized rat. Our earlier reports [19, 24] and this study (Fig. 3) indicated that 9-OH-2-FAA was a poor substrate for conjugation by UDP-GT. The low levels of glucuronidation of 9-OH-2-FAA *in vitro* were consistent with the results of the studies *in vivo*, which showed that an extremely small frac-

tion of 2-FAA is excreted in rat urine as 9-OH-2-FAA conjugate [38].

Depending on the dose and regimen of administration, 2-FAA has been shown to be an initiator or a promoter or a complete carcinogen [14, 39–44]. Each function may be affected by sex hormones. Initiation is a domain of genotoxic carcinogens, i.e. those capable of damaging or chemically modifying DNA [4, 45–47]. 2-FAA acquires its genotoxicity via a two-step metabolic activation: N-hydroxylation followed by sulfation of the N-hydroxy compound [4, 47]. In the adult male SD rat, N-hydroxylation of 2-FAA, catalyzed by PB-2a/PCN-E cytochrome P-450 isozyme [29], may be partially dependent on androgens [28], and sulfotransferase-catalyzed sulfation of *N*-OH-2-FAA is most likely dependent on the continuous presence of androgens [34, 48]. Promotion may be a domain of epigenetic carcinogens which are nongenotoxic [45, 46]. On prolonged administration to rats, such compounds are weakly hepatocarcinogenic, e.g. 9-OH-2-FAA and 9-oxo-2-FAA [49]. Our data as well as the results of other studies led us to suggest that these metabolites function as promoters in hepatocarcinogenesis by 2-FAA. After initiation of hepatocarcinogenesis, the conjugation of *N*-OH-2-FAA to the sulfate, the presumed initiator, is decreased [13, 50], whereas relatively large amounts of 9-OH-2-FAA and its glucuronide are accumulated in the liver [51]. Furthermore, 9-OH-2-FAA was the major phase I metabolite in the male rat liver and was decreased by orchidectomy or treatment of rats with β-NF (Fig. 2) or 3-MC [35, 36]. Thus, under the conditions that protected male rats from hepatocarcinogenesis by 2-FAA [1, 2, 52], possibly through the decrease in the formation of the initiator, the formation of 9-OH-2-FAA, a potential promoter, would also be decreased. Further studies including bioassay to assess a promoting activity of 9-OH-2-FAA are needed.

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