Effect of Sex Difference on the in Vitro and in Vivo Metabolism of Aflatoxin B₁ by the Rat

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SUMMARY

Hepatic microsome-catalyzed metabolism of aflatoxin B₁ (AFB₁) to aflatoxin M₁ and aflatoxin Q₁ and the "metabolic activation" of AFB₁ to DNA-alkylating metabolite(s) were studied in normal male and female Sprague-Dawley rats, in gonadectomized animals, and in castrated males and normal females treated with testosterone. Microsomes from male animals formed 2 to 5 times more aflatoxin M₁, aflatoxin Q₁, and DNA-alkylating metabolite(s) than those from females. Castration reduced the metabolism of AFB₁ by the microsomes from males by about 50%, whereas ovarioectomy had no significant effect on AFB₁ metabolism by the microsomes from females. Testosterone treatment (4 mg/rat, 3 times/week for about 6 weeks) of castrated immature males and immature females enhanced the metabolism of AFB₁ by their microsomes. A sex difference in the metabolism of AFB₁ by liver microsomes was also seen in other strains of rats tested: Wistar, Long-Evans, and Fischer. The activity of kidney microsomes for metabolic activation was 1 to 4% that of the liver activity and was generally lower in microsomes from male rats as compared to those from female rats of Sprague-Dawley, Wistar, and Long-Evans strains. The in vitro results obtained with hepatic microsomes correlated well with the in vivo metabolism of AFB₁, in that more AFB₁ became bound in vivo to hepatic DNA isolated from male rats and from a female rat treated with testosterone than that isolated from control female rats. These data suggest that the differences in hepatic AFB₁ metabolism may be the underlying cause of the sex difference in toxicity and carcinogenicity of AFB₁ observed in rats.

INTRODUCTION

AFB₁ is a potent hepatotoxic and hepatocarcinogenic agent in a wide range of species (31, 41). It is produced by some strains of the mold Aspergillus flavus and has been reported to contaminate several human foods (4, 32, 35, 36). All the experimental evidence thus far indicates that the AFB₁ metabolite which binds to nucleic acids is a very reactive epoxide and is believed to be the ultimate carcinogen in the induction of hepatocarcinogenesis by AFB₁ (18, 38). AFB₁ metabolized by hepatic microsomes is lethal to some bacteria (9), causes mutations in some bacteria (1, 10), and binds to macromolecules such as DNA, RNA, and proteins (8, 18); the binding of DNA was in excess of the binding to RNA (18). In addition to the metabolism of AFB₁ via the 2,3-oxide pathway (9, 18, 38), MFO mediates oxidation at other sites in the AFB₁ molecule, as shown in Chart 1 (2, 14, 29).

Although AFB₁ is a potent hepatotoxin and a hepatocarcinogen in various animal species, the type and the extent of AFB₁-induced hepatic damage are variable from species to species and are also influenced by strain, sex, and hormonal and dietary factors (40). Carcinogenicity in the rat is greatly decreased by hypophysectomy (11). Strain differences in the carcinogenicity of AFB₁ have been reported for mice (40). Although various rat strains have been used in different studies and found to be sensitive, to our knowledge no systematic and controlled study is available that compares the sensitivity of various rat strains to AFB₁. Male and female rats also differ in their sensitivity to AFB₁, in that it takes a larger cumulative dose and a longer exposure period to produce liver tumors in females than in males of the same strain (3, 42).

Differences in the metabolism of AFB₁, especially its conversion to the putative 2,3-oxide, might be the underlying mechanism for differences observed in responsibility to AFB₁. Because of this, and since sex differences in the metabolism of AFB₁ have not been previously investigated, we studied the metabolism of AFB₁ to DNA-alkylating metabolite ("metabolic activation") and to AFM₁ and AFQ₁ by the microsomes isolated from the livers of male and female rats of different strains, some of which were gonadectomized and/or treated with testosterone. We also studied the metabolic activation of AFB₁ by kidney microsomes to evaluate the ability of non-target organs to metabolize AFB₁. Investigations were also carried out to determine the relationship between the metabolism of AFB₁ in vitro and in vivo. A preliminary report of some of these studies has appeared (12, 19).

MATERIALS AND METHODS

Chemicals. AFB₁, obtained from Calbiochem, San Diego, Calif., was titrated by New England Nuclear, Boston.
Mass., and purified repeatedly by TLC as described previously (15), to a single fluorescent spot in 2 solvent systems (chloroform:acetone, 80:20 and chloroform:methanol, 95:5). The labeled AFB, thus obtained was diluted with unlabeled AFB, in DMSO, as described previously (15).

Testosterone propionate was obtained from Schering Corp., Bloomfield, N. J. Silica gel TLC plates (5 x 20 cm), 0.25-mm thickness without fluorescent indicator, were obtained from Merck (EM Laboratories), Elmsford, N. Y. Aquous counting scintillant was obtained from Amer sham/Searl, Arlington Heights, Ill. 4-Aminosalicylic acid and 8-hydroxyquinoline were obtained from Aldrich Chemical Co., Milwaukee, Wis. Sodium dodecyl sulfate was supplied by Fisher Scientific Co., Fairlawn, N. J. Other chemicals not reported previously (15, 18) were reagent grade. Authentic samples of AFB, metabolites, namely, AFM, AFQ, and aflatoxin P, were a generous gift from Dr. G. N. Wogan of Massachusetts Institute of Technology, Cambridge, Mass.

Gonadectomy and Testosterone Treatment. For portions of this study, male and female Sprague-Dawley rats were gonadectomized. Male rats, either immature (about 30 days old) or mature (about 70 days old), were castrated via a scrotal incision or via a ventral abdominal incision, and the controls were sham operated. Mature female rats (about 70 days old) were ovariectomized via a ventral abdominal incision, and the controls were sham operated. Following surgery, all animals were caged individually and allowed to recover for 1 week; at the termination of this period, 2 or 3 rats were caged together, and mature animals were used 7 days later. Immature female (about 30 days old) and immature normal and gonadectomized male rats were treated s.c. with corn oil or testosterone propionate in corn oil (0.1 ml volume) for about 6 weeks, 3 times weekly, at a dose of 4 mg/rat. At the termination of the treatment period, rats were sacrificed for the isolation of hepatic microsomes.

Preparation of Microsomes. All animals received food and water ad libitum until the day of sacrifice and were maintained under identical conditions. Animals were decapitated, and the liver was excised from each animal, perfused with cold 0.85% sodium chloride solution, and homogenized in ice cold sucrose solution containing 1 mM EDTA. The homogenate was centrifuged at 15,000 x g for 15 min, and the resulting supernatant was collected and centrifuged at 105,000 x g for 90 min to obtain the microsomal pellet which was suspended in 0.1 M potassium phosphate buffer, pH 7.4. All operations were carried out at 4°. Protein in the microsomal suspension was determined by the method of Lowry et al. (25).

Microsomes were isolated individually from the livers of control, gonadectomized, and testosterone-treated Sprague-Dawley rats and from the livers and kidneys of male and female rats of all the strains used in our studies: Wistar, Sprague-Dawley, Fischer, and Long-Evans. For the isolation of kidney microsomes, kidneys from 2 rats of each sex were pooled, rinsed in 0.9% NaCl solution, chopped with scissors and rinsed again, and then used for the preparation of microsomes by the method outlined above for the liver.

Determination of Cytochrome P-450. Cytochrome P-450 was determined by the previously described procedure (28). A solution of microsomal protein (1.5 to 2 mg/ml) was divided equally between 2 cuvets, and the base line of equal light absorbance was recorded. The contents of both cuvets were then reduced with dithionite, and CO was bubbled for 30 sec through the contents of the sample cuvet only. The spectrum between 390 and 500 nm was recorded with an Aminco DW-2 recording spectrophotometer. A mm extinction coefficient of 91 was used to calculate the concentration of cytochrome P-450 (28).

Metabolism In Vitro of AFB, to DNA-binding Metabolite(s). The composition of the incubation mixture used to demonstrate DNA-binding was as follows: 0.08 M potassium phosphate buffer, pH 7.4: 3.3 mM MgCl2; 0.2 mM EDTA; NADPH-generating system containing 0.81 mM NADP, 17 mM DL-isocitrate, and isocitrate dehydrogenase Sigma type IV (200 μg protein); 4 mg native calf thymus DNA; hepatic microsomes (2 mg protein) or kidney microsomes (2 mg protein unless stated otherwise in the legend); and 0.44 mM [3H]AFB, in 50 μl DMSO. (Specific activity, 0.33 to 2.5 μCi/mmole). The buffer mixture containing the NADPH-generating system was incubated at 37° for 15 min to ensure the presence of an adequate amount of NADPH. The reaction was started with the addition of [3H]AFB, and the incubation was carried out for 1 hr. Total volume of the incubation mixture was 4.5 ml. At the termination of the incubation, DNA was extracted by repeated extraction with an equal volume of phenol (550 ml):m-cresol (50 ml):8-hydroxyquinoline (50 mg) mixture; aqueous extract was washed several times with diethyl ether. The DNA was then precipitated, redissolved, and subsequently deproteinized by repeated extraction with chloroform:isoamyl alcohol (19:1) mixture. The final DNA preparation had a 260:280 nm value of 1.9 and contained less than 3% protein or RNA (18, 22). Details of this method have been reported previously (18). The amount of the metabolite bound to DNA in the absence of NADPH in the incubation mixture ranged from 0.015 to
Distribution of 3H label and the percentages of 3H ax
known, and the metabolism of AFBI is, as yet, incompletely
described previously (15, 17, 18). [3H]AFBI used in these
be applied at this time. Therefore, the results on metabolism
are corrected for 3H exchange due to NIH shift (7) because the
sent when incubation was carried out in the presence of CO
were not produced in the absence of NADPH or microsomas
or in the presence of boiled microsomas in the incubation
studies received an additional TLC purification, in addition
cochromome P-450. This was due to a significantly lower
dependency on these aspects of AFB, chemistry and metabolism is available, correction cannot
be applied at this time. Therefore, the results on metabolism
represent minimal values, since 3H lost due to NIH shift has not been taken into account in specific activity calculations.

Binding in vivo of [3H]AFB, to Macromolecules. Two
experiments were conducted. In 1 experiment, a male and a
female rat (about 60 days of age) of Sprague-Dawley strain
each received an i.p. injection of 5 μCi 3H label in 124 μg AFB,
dissolved in 0.1 ml DMSO. Twelve hr later the animals
were killed, livers were removed, and DNA was isolated
from the liver homogenate essentially by the method reported
by Murthy et al. (30).

In another experiment, an immature male and 2 immature
females rats (all about 30 days old) of Sprague-Dawley strain
were used. The male was treated with 0.1 ml corn oil s.c., 3
times weekly for 6 weeks, and 1 female rat received testoster-
onate propionate, 4 mg in 0.1 ml oil, 3 times weekly for 6
weeks; the other female rat received only corn oil. At the
termination of the treatment period each animal received
124 μg of [3H]AFB, (5 μCi), as above, and was killed 12 hr
later for the isolation of liver. Liver was homogenized in a
buffer mixture containing 0.25 M sucrose and 1 mM magnesium acetate, and portions were used for the isolation of DNA and proteins essentially by the methods reported by
Murthy et al. (30). Briefly, DNA was precipitated with
phenol:m-cresol:8-hydroxyquinoline, as described earlier.
The aqueous phase was extracted 4 to 6 times with 2 volumes
diethyl ether, and DNA in the aqueous phase was precipitated
at 20 to 30 mg of sodium acetate and a 1.5 volume of 2-ethoxyethanol (99% pure), followed by freezing
overnight at −20°C. The DNA was redissolved in about 10 ml
of 10 mM Tris-HCl buffer, pH 7.4, and then deproteinized by
extracting it 3 times with 20 ml of CHCl₃:isopropyl alcohol
(19:1); DNA was then reprecipitated as before, but ethanol
was used instead of 2-ethoxy ethanol. The DNA was rediss-
solved in Tris-HCl buffer, pH 7.4, and then treated with heat-
treated RNase (100 μg/ml) (18) at 37°C for 1 hr. This treatment
results in >90% digestion of the rat liver RNA (18, 23), and
the resulting DNA after repeated extraction with
CHCl₃:isoamyl alcohol (19:1) had a 260:280 nm ratio identi-
cal to that of native calf thymus DNA.

RESULTS

Sex Difference and the Effect of Gonadectomy on the in
Vitro Metabolic Activation of AFB, Castration of male rats
depresses their MFO activity (6, 27). Furthermore, castra-
tion of male rats also decreases the toxicity of AFB, (34). To
determine whether this decrease in sensitivity is related to
the reduced formation of the DNA- and protein-binding
metabolites of AFB, male and female Sprague-Dawley rats
were gonadectomized or sham operated, and 2 weeks later
hepatic microsomes were isolated and then assayed (data
not shown). In DNA-binding studies, hepatic microsomes
from male rats were 3 times more active in metabolizing
AFB, than those from female rats. Castration had a feminiz-
ing effect, in that it decreased the activity of microsomes
isolated from males to that found in microsomes isolated
from females; on the other hand, ovariectomy did not in-
crease or decrease the activity. The sex difference was
reduced by about 30% when the activity was related to
cytochrome P-450. This was due to a significantly lower
content of cytochrome P-450 in microsomes isolated from

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females as compared to those from males; castration decreased the cytochrome P-450 level in males, whereas ovariectomy resulted in a cytochrome P-450 level similar to that seen in castrated males. The effects of sex difference and gonadectomy on the hepatic cytochrome P-450 level are consistent with our recent studies on aryl hydrocarbon hydroxylase (20) and with those reported by others (27). When binding of AFB, metabolites to microsomal proteins was studied by the previously described procedure (15, 19) the sex difference was less marked than that observed with DNA-binding; if control male activity was set at 100%, the relative activity in females, ovariectomized females, and castrated males ranged from 60 to 64%. The results obtained in the present studies on the effects of sex difference and gonadectomy on the binding in vitro of AFB, metabolites to DNA and to microsomal proteins were similar to those reported earlier by us (19) and those illustrated in Table 1. Because of a larger sex-related difference in DNA-binding than in protein-binding, further studies were carried out on the binding of AFB, metabolite(s) to DNA, using hepatic microsomes isolated from Sprague-Dawley rats.

Effect of Testosterone Treatment on the in Vitro Metabolic Activation of AFB,

Since the studies outlined above suggested that testosterone was involved in the regulation of AFB, metabolic activation, investigations were extended to study the effect of testosterone treatment on the activities of hepatic microsomes isolated from female and castrated male rats. Preliminary studies indicated that the treatment of mature castrated male rats (about 65 days old) with testosterone for 3 weeks, with 4 mg s.c./rat given twice weekly, did not increase the metabolism of AFB,. This is consistent with the effect of testosterone treatment of mature castrated rats on the metabolism of aminopyrine and ethylmorphine (5). However, when castrated immature male and normal immature female rats (about 30 days old) received s.c. injections 3 times weekly for about 6 weeks, a significant effect of testosterone treatment was noticed (Table 1). Hepatic microsomes isolated from castrated males, as expected, had less than half the activity of microsomes from normal males, but the activity in microsomes from testosterone-treated castrated rats was slightly greater than that in microsomes from corresponding control male rats. Hepatic microsomes from female rats, as expected, were one-third as active as those from male rats; however, the activity in microsomes from testosterone-treated female rats was equivalent to that in microsomes from the control male rats. When the activity was related to cytochrome P-450, the difference between male and female rats was narrowed. This was due to a lower content of cytochrome P-450 in females and castrated males as compared to other animals (control males, testosterone-treated females, and castrated males).

### Table 1

<table>
<thead>
<tr>
<th>Sex and treatment</th>
<th>nmoles cytochrome P-450/mg microsomal protein</th>
<th>Activity/mg microsomal protein</th>
<th>Mean as % of male activity</th>
<th>Activity/nmole cytochrome P-450</th>
<th>Mean as % of male activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1.56 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.28 ± 0.01</td>
<td>100</td>
<td>0.82 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>Castrated</td>
<td>1.30 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.48 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37</td>
<td>0.37 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>45</td>
</tr>
<tr>
<td>Castrated-testosterone</td>
<td>1.50 ± 0.04</td>
<td>1.49 ± 0.10</td>
<td>117</td>
<td>1.00 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>122</td>
</tr>
<tr>
<td>Female</td>
<td>0.90 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.49 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38</td>
<td>0.54 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>66</td>
</tr>
<tr>
<td>Female-testosterone</td>
<td>1.10 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.21 ± 0.24</td>
<td>94</td>
<td>1.10 ± 0.12</td>
<td>134</td>
</tr>
</tbody>
</table>

<sup>a</sup> Four rats were used in each group, except in the "Female-testosterone" group which had 5 animals.

<sup>b</sup> Mean ± S.E.

<sup>c</sup> Significantly different from the male at p < 0.05.
Metabolism of AFB₁ in the Rat

Sex difference in the metabolism of AFB₁ to DNA-alkylating metabolite(s) by hepatic microsomes isolated from rats of various strains

Native calf thymus DNA was incubated for 1 hr at 37°C with [³H]AFB₁, NADPH, and hepatic microsomes from male and female rats of various strains. The DNA was isolated as in Table 1 and then counted.

<table>
<thead>
<tr>
<th>Sex and strain</th>
<th>nmoles cytochrome P-450/µg microsomal protein</th>
<th>DNA-binding activity (nmoles metabolite bound/µmoles DNA-P)</th>
<th>Activity/mg microsomal protein</th>
<th>Mean as % male activity</th>
<th>Activity/µmol cytochrome P-450</th>
<th>Mean as % male activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.04 ± 0.05</td>
<td>1.13 ± 0.06</td>
<td>100</td>
<td>1.09 ± 0.09</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.73 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>34</td>
<td>0.53 ± 0.06</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Wistar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.09 ± 0.04</td>
<td>0.74 ± 0.03</td>
<td>100</td>
<td>0.68 ± 0.02</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.99 ± 0.04</td>
<td>0.23 ± 0.02</td>
<td>44</td>
<td>0.23 ± 0.02</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Fischer</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.09 ± 0.02</td>
<td>0.51 ± 0.01</td>
<td>100</td>
<td>0.47 ± 0.02</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.00 ± 0.04</td>
<td>0.23 ± 0.00</td>
<td>44</td>
<td>0.23 ± 0.00</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Long-Evans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.22 ± 0.02</td>
<td>0.54 ± 0.05</td>
<td>100</td>
<td>0.44 ± 0.03</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.96 ± 0.04</td>
<td>0.23 ± 0.01</td>
<td>42</td>
<td>0.24 ± 0.02</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

* Four rats were used in each group, except in the groups of females of Sprague-Dawley and Fischer strains which had only 3 animals each.

a Mean ± S.E.

Significantly different from the male at p < 0.05.

Sex Difference and the Effects of Gonadectomy and Testosterone Treatment on the Metabolism of AFB₁ to AFM₁ and AFQ₁. In addition to the formation of a reactive metabolite(s) that binds to macromolecules and may be involved in the development of toxicity and carcinogenesis, AFB₁ is also metabolized to several other metabolites, such as AFM₁ and AFQ₁, which do not involve the intermediate formation of the putative 2,3-oxide. To ascertain whether metabolism of AFB₁ to AFM₁ and AFQ₁, catalyzed by hepatic microsomes, was also affected by the sex of the rat, we studied the effect of gonadectomy and testosterone treatment of immature castrated male and immature female rats (Table 3). Although specific differences were noted, results were qualitatively similar to those obtained in the metabolic activation studies. When related to microsomal proteins, male rats had higher activity than females: 2 times higher for AFM₁ and 5 times higher for AFQ₁. Castration had a feminizing effect, whereas testosterone treatment of the immature females or castrated immature males had a masculinizing effect, increasing the activity of hepatic microsomes isolated from female rats toward that found in microsomes from control male rats. Because the sex difference was less pronounced for the formation of AFM₁ than for that of AFQ₁, adjustment of the activity to cytochrome P-450 content resulted in a near elimination of the sex difference for AFM₁. The formation of AFQ₁ was by far the most sensitive pathway affected by the sex of the animal.

Sex Difference in the Metabolism of AFB₁ to AFM₁ and AFQ₁ in Various Strains of Rats and in C57BL/6 Mice. A sex difference was also observed in the formation of both AFM₁ and AFQ₁ by the hepatic microsomes isolated from other strains of rats (Table 4). In the formation of AFM₁, the magnitude of the difference was 2-fold in Sprague-Dawley, Long-Evans, and Wistar rats and about 1.4-fold in Fischer rats. In the formation of AFQ₁, the difference between male and female rats ranged from 5-fold in Sprague-Dawley to 2-fold in Long-Evans rats. When the activities were related to cytochrome P-450 content, the sex difference was reduced for both pathways. However, this reduction was more pronounced for the formation of AFM₁, and only a marginal difference between male and female rats derived from Sprague-Dawley strain was seen after the activity was related to cytochrome P-450 content.

On the other hand, when the hepatic microsomes isolated from male and female mice of C57BL/6 strain were compared, no sex difference was observed, either in the conversion of AFB₁ to AFM₁ or AFB₁ to AFQ₁ (Table 4).

Sex Difference in the Kidney Microsome-catalyzed Metabolic Activation of AFB₁ in Rats of Various Strains. Although liver is the target organ for aflatoxin carcinogenicity, the appearance of tumors in the kidney following aflatoxin treatment has been reported (3, 40). Despite these observations, no reports, to our knowledge, are in the literature concerning the metabolism of AFB₁ by the kidney. Furthermore, the metabolism of AFB₁ by extrahepatic tissues has not been studied. We therefore examined the effect of sex difference in 4 rat strains on the metabolic activation of AFB₁, by kidney microsome (Table 5). The striking obser-
Sex difference and the effects of castration and testosterone treatment on the metabolism of AFB₁, to AFM, and AFQ₁ by hepatic microsomes isolated from Sprague-Dawley rats

Hepatic microsomes from male, female, and testosterone-treated rats were incubated with [³H]AFB₁ and NADPH for 1 hr at 37°. AFM, and AFQ₁, which are the microsome-mediated metabolites of AFB₁, were isolated by TLC on silica gel, scraped, and then counted. Details of the procedure are given in the text.

Activity (pmoles metabolite formed)*

<table>
<thead>
<tr>
<th>Sex and treatment</th>
<th>AFM₁</th>
<th>AFQ₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity/mg microsomal protein</td>
<td>Mean as % male activity</td>
</tr>
<tr>
<td>Male</td>
<td>3168 ± 508b</td>
<td>100</td>
</tr>
<tr>
<td>Castrated</td>
<td>1173 ± 187c</td>
<td>37</td>
</tr>
<tr>
<td>Castrated-testos-</td>
<td>2412 ± 99</td>
<td>76</td>
</tr>
<tr>
<td>Female</td>
<td>1684 ± 163d</td>
<td>53</td>
</tr>
<tr>
<td>Female-testos-</td>
<td>3280 ± 171</td>
<td>103</td>
</tr>
</tbody>
</table>

* Four rats were used in each group.  
** Mean ± SE.  
† Significantly different from the male at p < 0.05.

Table 3

Sex difference in the metabolism of AFB₁ to AFM, and AFQ₁ by hepatic microsomes isolated from various strains of rats and from C57BL/6 mouse strain

Hepatic microsomes from male and female rats of different strains were incubated at 37° for 1 hr, and those from male and female mice of C57BL/6 strain were incubated for only 30 min, with [³H]AFB₁ and NADPH. Metabolites, AFM, and AFQ₁, were isolated by TLC on silica gel, scraped, and then counted. Details of the procedure are given in the text.

Activity (pmoles metabolite formed)*

<table>
<thead>
<tr>
<th>Species, strain, and sex</th>
<th>AFM₁</th>
<th>AFQ₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity/mg microsomal protein</td>
<td>Mean as % male activity</td>
</tr>
<tr>
<td>Sprague-Dawley rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3168 ± 508b</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>1684 ± 163d</td>
<td>50</td>
</tr>
<tr>
<td>Long-Evans Rats</td>
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<td>Male</td>
<td>1537 ± 108</td>
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<tr>
<td>Female</td>
<td>634 ± 86b</td>
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<tr>
<td>Wistar rats</td>
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<tr>
<td>Male</td>
<td>1669 ± 159</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>887 ± 101d</td>
<td>53</td>
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<tr>
<td>Fischer rats</td>
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</tr>
<tr>
<td>Male</td>
<td>1768 ± 69</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>1400 ± 201</td>
<td>79</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4732 ± 238</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>4605 ± 685</td>
<td>97</td>
</tr>
</tbody>
</table>

* Incubations with rat liver microsomes were carried out for 1 hr at 37°, whereas incubations with mouse liver microsomes were carried out for only 30 min at the same temperature.  
** Four animals were used in each group.  
† Significantly different from the male at p < 0.05.

Table 4

Sex difference in the metabolism of AFB₁, to AFM, and AFQ₁, by hepatic microsomes isolated from various strains of rats and from C57BL/6 mouse strain

The sex difference was reversed in favor of females, who generally showed higher activity than the corresponding males of Sprague-Dawley, Long-Evans, and Wistar strains and about the same activity as the males of Fischer strain. Kidney activity was comparatively lower, representing 1 to 4% of the liver activity.
Sex difference in the metabolism of AFB, to DNA-alkylating metabolite(s) by kidney microsomes isolated from various strains of rats

Kidney microsomes from male and female rats of different strains were incubated for 1 hr at 37°C with native calf thymus DNA, [3H]AFB, and NADPH. DNA from the incubation mixture was isolated and counted. In Experiments 1 and 2, each incubation mixture contained 2 and 4 mg, respectively, of the microsomal protein. Details of the procedure are described in the text.

<table>
<thead>
<tr>
<th>Activity (pmoles AFB1 metabolite/μmole DNA-P)</th>
<th>Mean activity/mg microsomal protein</th>
<th>Mean as % male activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex and strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Expt. 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long Evans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7.1 (5-9)</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>20.1 (18-21)</td>
<td>282</td>
</tr>
<tr>
<td>Wistar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8.8 (6-11)</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>22.9 (15-31)</td>
<td>260</td>
</tr>
<tr>
<td>Fischer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16.0 (13-18)</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>16.4 (16-17)</td>
<td>102</td>
</tr>
<tr>
<td><strong>Expt. 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13.5 (12-14)</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>39.2 (34-44)</td>
<td>290</td>
</tr>
<tr>
<td>Long-Evans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15.3 (14-16)</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>32.5 (25-39)</td>
<td>213</td>
</tr>
</tbody>
</table>

* Two experiments were carried out in each case. In each experiment kidneys from 2 rats of each sex were separately pooled for the isolation of microsomes which were assayed in duplicate.

In comparing the microsomal metabolism of AFB, to DNA-binding metabolite(s) [presumably AFB1-2,3-oxide (18, 38)], we observed significant differences in the activities of hepatic microsomes isolated from male and female rats of 4 different strains: Sprague-Dawley, Wistar, Long-Evans, and Fischer. Microsomes from male rats were 2 to 3 times more active than those from females; castration of males had a feminizing effect, but testosterone treatment of the castrated immature males and immature females enhanced their activities to the level seen in males. Highest activity was found in the Sprague-Dawley strain, and it decreased in the following order: Sprague-Dawley > Wistar > Long-Evans = Fischer. These data are consistent with the reported effects of sexuality and testosterone treatment on the carcinogenicity or toxicity of AFB1 in the rat. Female rats were less susceptible to acute and subacute AFB1 toxicity and developed tumors more slowly than did the male rats (3, 42). In male rats dosed with a total of 400 μg AFB1, in 10 equal doses, 4 of 24 animals developed liver carcinomas 35 to 82 weeks after treatment, but a similar dose did not produce carcinomas in female rats within the 82-week period, although preneoplastic liver lesions were observed (42). In rats fed an aflatoxin-containing diet (0.1 ppm) during their lifetime with an estimated total consumption of about 1 mg AFB1, the incidence of liver tumor in males and females was 50 and 16%, respectively (3). In a toxicity study (34), male Fischer rats fed aflatoxins in the diet showed a mortality of 75 to 100% within 2 weeks; under the same conditions castration prevented the death of animals when performed before 10 weeks of age but had no beneficial effect after that time. Testosterone treatment resulted in 100% mortality in aflatoxin-fed castrated rats, whereas no fatalities occurred in castrated rats fed aflatoxin only.

Sex differences in humans exposed to aflatoxin-contaminated foods are known. Various reports relating the incidence of human liver cancer to aflatoxin contamination of food have shown a 2.5- to 5-fold higher incidence in males as compared to females (32, 35, 36). The extrapolation of rat data presented here to explain the sex-related differences in human susceptibility could be premature at this point and would require investigations with human tissue or at least human cell culture systems (13). As is also shown by our data (Table 4) on the metabolism of AFB1 to AFM, and AFQ, by the mouse hepatic microsomes, sex differences in other species of animals for the metabolism of drugs and carcinogens might not exist (6); this is another reason why the extension of the data obtained from rats to explain the higher incidence of aflatoxin-associated liver cancer in humans remains uncertain.
male humans as compared to females should be made with caution, and detailed studies are needed to establish sex-related metabolic differences in humans.

The sex difference observed for the in vitro metabolic activation (formation of DNA-binding metabolite) of AFB, was not surprising in the light of the male rat’s ability to metabolize type I drugs faster than the female rat (27). The male rat has a higher activity for aminopyrine and ethylmorphine demethylation, yet it hydroxylates aniline at the same rate and steroid sulfate at a thousandfold lesser rate than the female (6, 21, 26, 27).

In order to ascertain whether the pattern of sex differences in aflatoxin metabolism was common to some other pathways involved in the metabolism of AFB, we also studied the metabolism of AFB, to AFM, and AFQ, (see Chart 1). A sex difference existed for both metabolites, and activity in hepatic microsomes isolated from male rats was about 2- to 5-times greater than that in the female rat liver microsomes; castration had a feminizing effect, whereas testosterone treatment of the immature females and the immature castrated males produced a masculinizing effect. The extent to which various metabolic pathways, other than the one involved in the formation of the putative 2,3-oxide, contribute to the overall toxicity and carcinogenicity of AFB, is unknown at this time, although in studies with ducklings AFM, was as toxic as AFB, (33). It is conceivable that some of these metabolites are essential to the biological effects of AFB,. It is possible that they are recycled metabolically to produce more reactive derivatives than AFB, in a manner similar to that reported for some polycyclic aromatic hydrocarbons (37).

Under the experimental conditions, hepatic microsomes isolated from Sprague-Dawley rats, in addition to metabolically activating AFB, to a greater extent that those from the other strains, also metabolized AFB, to AFM, and AFQ, at a faster rate. Whether this superiority over other strains reflects in vivo metabolism and, by implication, increased sensitivity to AFB, remains to be investigated in controlled studies in which these strains are tested for AFB, toxicity and carcinogenesis.

A sex difference in the binding of the metabolite(s) to microsomal proteins was also observed, but, as reported earlier (19), it was much smaller than that observed for the DNA binding (2- to 3-fold versus 1.7-fold). This would suggest that either microsomal proteins and DNA did not bind the same metabolites or microsomal proteins from control female, ovariectomized, and castrated rats were more efficient in trapping the reactive metabolite(s). There is now ample experimental evidence to support the former suggestion. Thus, we have previously reported (15) that aflatoxin B1a and related metabolite(s), because of their hemiacetel nature, cleave at a neutral pH and above to form dialdehyde derivative(s) which bind to microsomal proteins (see Chart 1), probably via the formation of Schiff bases with free amino groups in the proteins. The spectral properties of such a complex isolated from aflatoxin B1a-treated rat liver microsomes have been reported (16). Subsequently, we also reported that aflatoxin B1a, while binding to microsomal proteins by the above postulated mechanism, did not bind to native calf thymus DNA (18). Using a model compound (2,3-dichloride of AFB,) for the putative 2,3-AFB, oxide, Swenson et al. (39) recently confirmed our reports by showing that their hydrated (hydroxylated) model compound did not bind to DNA but became largely bound to proteins; these investigators, supported by their spectral data, have invoked the Schiff base formation to explain the greater binding of their hydrated (hydroxylated) model compound to proteins.

Although liver is the target organ, aflatoxins also induce kidney neoplasms (3, 40). As would be expected from studies on the metabolism of drugs by MFO (24), kidney microsomes had very low activities, ranging in different strains from 1 to 4% of the hepatic activity for the metabolic activation of AFB,. The most striking and interesting observation was that the sex difference was reversed in favor of the female, who had greater activity than the male in all strains, except in the Fischer strain where no sex difference was observed. These data are in accord with the reported sex difference in the metabolism of benzo(a)pyrene by kidney microsomes isolated from male and female Sprague-Dawley rats (20); in these studies higher activity for benzo(a)pyrene hydroxylase was observed for kidney microsomes isolated from female rats. However, the significance of the sex difference in the activity of kidney microsomes, as related to the induction of kidney neoplasms by AFB,1 remains to be investigated.

Preliminary results obtained for the in vivo binding of AFB, to hepatic DNA and proteins of hepatic subfractions correlated well with the in vitro results: relative to female, more AFB, was bound to DNA in the male and testosterone-treated female. Similar differences for protein binding between male versus female, and testosterone-treated female versus control female also existed but were considerably attenuated, again suggesting that in vivo metabolism correlated fairly well with the in vitro metabolism.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the valuable criticisms provided by Dr. P. Creaven, Dr. B. Paigen, Dr. C. Porter, and Dr. F. Rosen during the preparation of this manuscript. They also wish to express their appreciation to Dr. E. Milich for his interest and encouragement.

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