Elevation of hepatic microsomal epoxide hydrolase activity by 2-acetylaminofluorene: strain and species differences

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Hepatocarcinogens have been shown to cause marked elevation of hepatic microsomal epoxide hydrolyase activity in the rat at short intervals after administration. The present studies were designed to characterize 2-acetylaminofluorene (AAF) mediated epoxide hydrolase elevation and to investigate the relationship between epoxide hydrolase increases, AAF metabolism, and hepatocarcinogenicity. Oral or i.p. administration of AAF to F-344 rats produced log-linear dose-response curves for epoxide hydrolase elevation, measured with either benzo[a]pyrene-4,5-oxide or styrene oxide substrate. Following a single dose of AAF (35 mg/kg), epoxide hydrolase activity was maximally increased (560% of control) within 48 h, and the activity declined slowly, with a half-life of 17.5 days. Co-treatment with actinomycin D effectively blocked the AAF dependent increase in epoxide hydrolase, suggesting that de novo protein synthesis is associated with the increase in enzyme activity. Dose-response curves for epoxide hydrolase induction by AAF, N-hydroxy-2-acetylaminofluorene (N-OH-AAF), and 2-aminofluorene were compared, and the potencies for increasing epoxide hydrolase activity reflected the relative hepatocarcinogenic potentials of these agents. In mice, which are resistant to the hepatocarcinogenic action of AAF and deficient in AAF-N-hydroxylase activity, AAF caused no significant increase in hepatic microsomal epoxide hydrolase activity. Similarly, in Cotton rats and guinea pigs, which are lacking in ability to form the sulfate conjugate of N-OH-AAF, neither i.p. nor dietary administration of AAF elicited increases in epoxide hydrolase activity at doses which were maximally effective in F-344 rats. These results support the hypothesis that the ability of compounds to increase epoxide hydrolase activity is related to their carcinogenic potency. Furthermore, the results suggest that increases in epoxide hydrolase activity are associated with metabolism of AAF to the putative proximate carcinogen N-OH-AAF, and the subsequent conversion of this compound to the N-O-sulfate conjugate.

Introduction

Short-term exposure of rats to hepatocarcinogens has been shown to result in a marked increase in hepatic microsomal epoxide hydrolase (EC 3.3.2.3) activity, while having little effect on cytochrome P-450-dependent mixed-function oxidase activities (1–5). The elevation of epoxide hydrolase activity has been observed following treatment with a wide variety of hepatocarcinogens and is dose-related. Comparison of the slopes of the log-linear dose response curves for increases in epoxide hydrolase elicited by several hepatocarcinogens of diverse structure showed them to be similar, suggesting that the increases in epoxide hydrolase occur through a common mechanism. Furthermore, a correlation has been suggested between heaptocarcinogenic potency and potency in increasing epoxide hydrolase activity (1).

Okita and co-workers (6) demonstrated the presence of an antigenic component, termed preneoplastic or PN-antigen*, in phenotypically altered foci of liver cells induced by chemical carcinogens. Purification of PN-antigen led to its identification as epoxide hydrolase (7,8). More recently, Lin et al. (9) suggested that PN-antigen and epoxide hydrolase are different proteins. While the relationship between PN-antigen and epoxide hydrolase remains to be resolved, it is evident that phenotypically altered foci of hepatocytes induced by chemical carcinogens contain elevated quantities of epoxide hydrolase (8). Elevated quantities of epoxide hydrolase have been demonstrated immunocytochemically in hyperplastic nodules (10,11), and increased epoxide hydrolase activity has been observed in rats subjected to various promoting regimens for induction of hyperplastic nodules (12) and in several hepatomas (8,13).

The metabolic activation and carcinogenicity of 2-acetylaminofluorene (AAF) have been extensively studied (for reviews see 14–16). The putative proximate carcinogenic metabolite N-hydroxy-2-acetylaminofluorene (N-OH-AAF) is formed by the hepatic cytochrome P-450 mixed-function oxidase system (17). Several enzymes are known to further metabolize N-OH-AAF, including microsomal deacetylase and cytosolic N-O acetyltransferase and sulfotransferase (18–20). Species and strain differences in the metabolic activation of AAF have been reported, and are correlated with susceptibilty to its carcinogenic effects (14,21–23).

The objectives of the studies described here were to utilize the known species and strain differences in metabolism of AAF and sensitivity to AAF-induced hepatocarcinogenicity to investigate the mechanism of hepatic microsomal epoxide hydrolase elevation by this compound.

Materials and methods

F.Oesch, Institute of Pharmacology, Mainz, FRG. All other chemicals were the best grades commercially available.

Animals and treatments

CDF® (F-344)/CrlBR rats, DBA/2NCr1BR and C57BL/6NCr1BR mice, and Hartley Crl:(HA)BR guinea pigs were purchased from Charles River Breeding Laboratories, Inc., Kingston, NY. Cotton rats originated from a breeding pair obtained from the National Institutes of Health, Bethesda, MD. Sera from sentinel animals of the same age, sex, and strain, and maintained in the same rooms were tested weekly (Microbiological Associates, Bethesda, MD) and found negative to titers to a range of viruses. [Rats were screened for Reovirus type 3, pneumonia virus of mice, mouse encephalomyelitis, Kilham rat virus, Toolan's H-1, Sendai, mouse adenovirus, lymphocytic choriomeningitis, mouse hepatitis virus, and rat corona virus; guinea pigs for simian virus-5, Reovirus type 3, Sendai, pneumonia virus of mice, and lymphocytic choriomeningitis; mice for Reovirus type 3, mouse encephalomyelitis, K virus, polyoma virus, Sendai, ectromelia, pneumonia virus of mice, minute virus of mice, mouse adenovirus, mouse hepatitis virus, and lymphocytic choriomeningitis.] The animals were housed in temperature (21 ± 3°C) and humidity (50 + 10%) controlled rooms, with a 12 h light cycle, and with free access to food (certified NIH-07 open-formula diet, Zeigler Bros. Inc., Gardners, PA (rats and mice) or Purina guinea pig chow, Ralston Purina Co., St. Louis, MO) and tap water.

All animals were acclimated for 2 weeks prior to use, by which time the mice, guinea pigs, and F-344 rats were 9—10 weeks of age. Due to the difficulties involved in raising Cotton rats, these animals were between 8 and 30 weeks of age when treatments began. Older and younger rats were mixed within treatment groups, and no age-related variations in enzymatic activity were noted in control or treated animals.

The majority of treatments were administered by i.p. injection (1 or 2 ml/kg in DMSO) or by gavage (10 ml/kg in corn oil). Control animals received the vehicle alone. Cotton rats were lightly anesthetized with CO$_2$ before injection. For dietary administration, animals were fed a semisynthetic diet (24) containing 0.02% AAF, purchased from Bio-Serve, Inc., Frenchtown, NJ. Control animals received the semisynthetic basal diet.

Preparation of liver subcellular fractions and enzyme assays

Animals were killed by cervical dislocation or by exsanguination under methoxyflurane anesthesia. Livers were quickly removed and washed in ice-cold 1.15% KCl. After weighing, the livers were coarsely chopped, washed in ice-cold 1.15% KCl, 20 mM Tris-HCl, pH 7.4, and homogenized in 3 volumes of this buffer in a teflon-glass Potter-Elvehjem homogenizer. Post-mitochondrial supernatant (PMS) was prepared by centrifugation of the liver homogenate at 10 000 g for 20 min. In some studies, microsomes were isolated by centrifugation of the PMS for 100 000 g-h, followed by resuspension in 20 mM Tris-HCl (pH 7.4) containing 250 mM sucrose and 5.4 mM EDTA. Subcellular fractions were stored at −80°C.

Epoxide hydrolase activity was measured using BPO or SO substrate. After incubation, the reaction products were separated by extraction (mice) (25,26) or by t.l.c. (all other experiments) (27). DT-diaphorase (EC 1.6.99.2) activity was assayed by measuring the rate of reduction of methyl red (28). Protein concentrations were determined by the biuret method (29) using American Monitors Total Protein Kit (American Monitor Co., Indianapolis, IN) with bovine serum albumin as a standard.

Statistical analysis

Where appropriate, data were evaluated by a one-way analysis of variance. Where significant F values were obtained, tests for differences between treatment means were performed using the Student-Newman-Keuls procedure (30).

Results

Time course and dose-response relationship for epoxide hydrolase elevation

Following administration of a single oral dose of AAF (35 mg/kg) to male F-344 rats, hepatic microsomal epoxide hydrolase activity reached a peak level (560% of control) at 48 h (Figure 1). The enzyme activity declined very slowly, in a log-linear fashion, with a half-life of 17.5 days. At 32 days following treatment, the epoxide hydrolase activity was still nearly twice control levels.

A single i.p. injection of F-344 rats with AAF resulted in a log-linear dose-related increase in epoxide hydrolase activity measured 48 h later, to a maximum of 450% control at a dose of 100 mg/kg (Figure 2). The pattern of increased en-
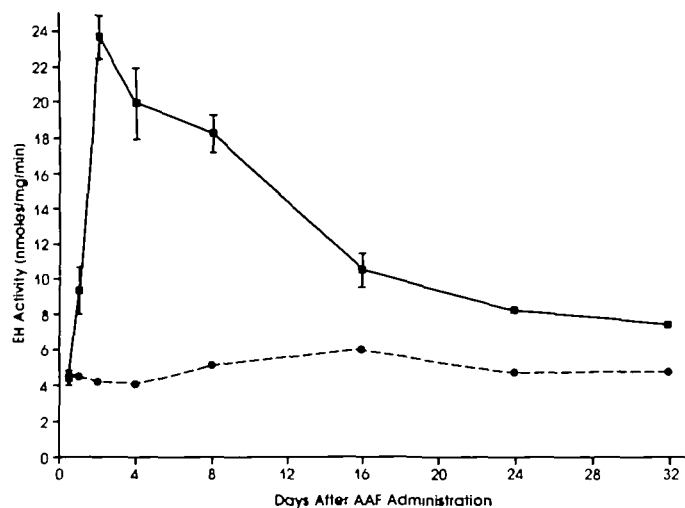

Fig. 1. Time course for epoxide hydrolase elevation following AAF treatment. EH activity was measured with BPO substrate in microsomes prepared from livers of male F-344 rats killed at indicated times after a single oral dose of 35 mg/kg AAF in corn oil (■). Control animals received corn oil alone (●). Points represent mean ± SEM, n = 3.
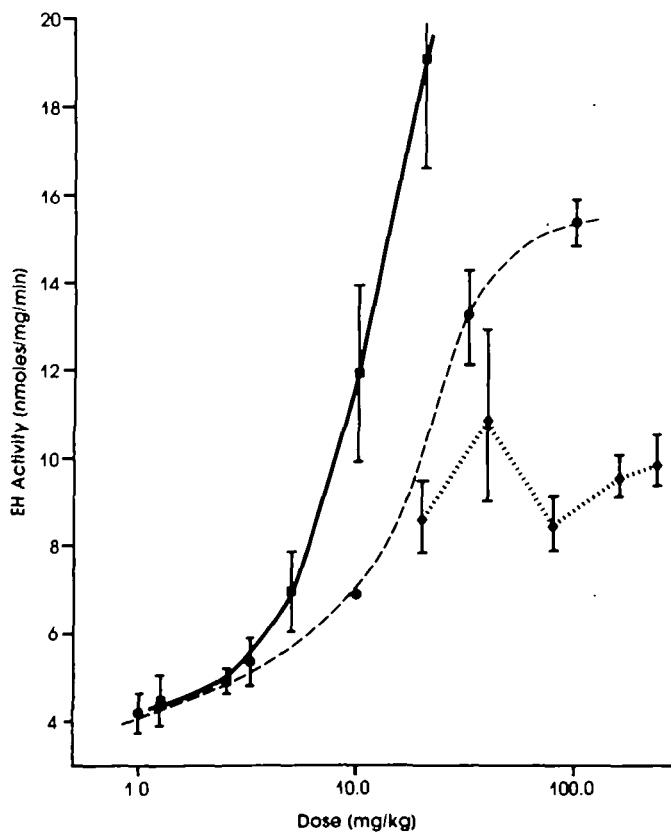

Fig. 2. Dose-response for epoxide hydrolase elevation by AAF, NOHAAF and AF. Male F-344 rats were treated with a single i.p. injection of AAF (●) in corn oil, or N-OH-AAF (■) or AF (♦) in DMSO, and were killed 48 h later. Microsomal epoxide hydrolase was measured with BPO substrate. Points represent mean ± SEM, n = 3 animals. Control activity was 3.38 ± 0.50 nmol diol produced/mg/min for AAF; 4.13 ± 0.36 nmol/mg/min for N-OH-AAF and AF.

zyme activity was identical whether measured with BPO or SO substrate (data not shown); the ED[50] for epoxide hydrolase elevation following a single dose was 20 mg/kg.

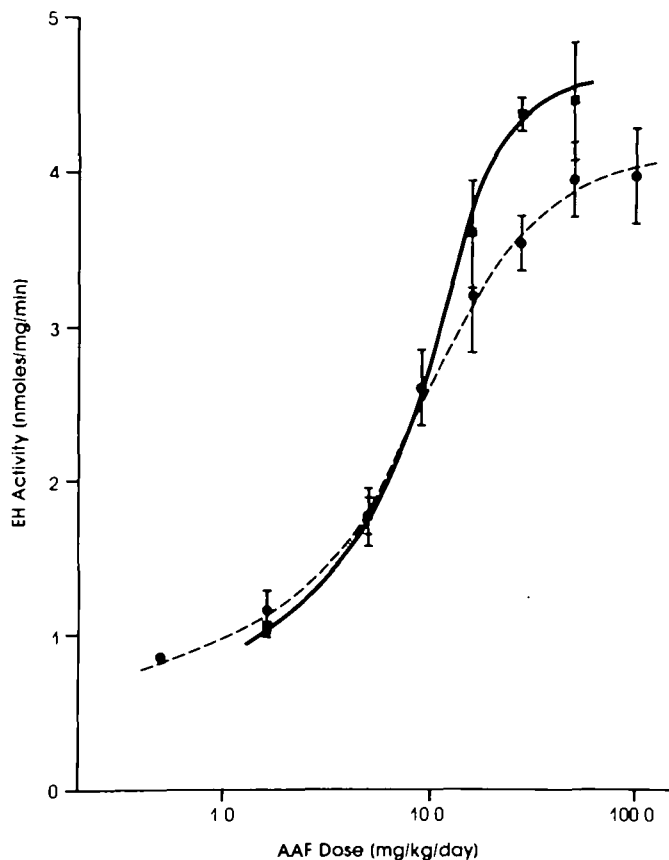When rats were treated with three consecutive daily doses

Fig. 3. Dose-response for hepatic epoxide hydrolase activity. Male F-344 rats received 3 consecutive daily doses of AAF, either i.p. (1 ml/kg in DMSO) (●), or p.o. (10 ml/kg in corn oil) (■). Animals were killed 48 h after the last dose and EH activity was measured in PMS with BPO substrate. Points represent mean ± SEM, n = 3. Control values were: i.p., 1.056 ± 0.057; p.o., 0.779 ± 0.042 nmol diol produced/mg protein/min.
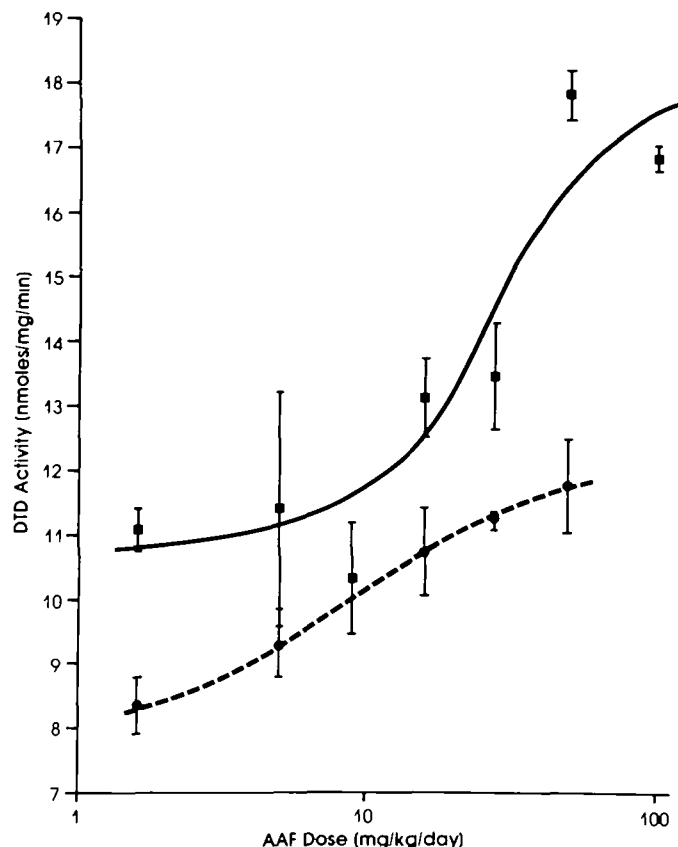

Fig. 4. Dose-response for hepatic DT-diaphorase activity. Animals and treatments were as described in legend to Figure 3. (■) i.p.; (●) p.o. administration. Control values (mean ± SEM, n = 3) were: i.p., 11.40 ± 0.94; p.o., 8.15 ± 0.21 nmol methyl red reduced/mg PMS protein/min.

of AAF, epoxide hydrolase activity measured 48 h after the last dose was again increased in a log-linear, dose-related manner (Figure 3). Oral administration of AAF produced a slightly greater maximal increase in epoxide hydrolase than intraperitoneal administration (570% and 380% control, respectively); however the slope and shape of the dose-response curves were independent of the route of administration. The ED[50] for epoxide hydrolase elevation following three oral or i.p. doses was 12 mg/kg/day.

Cytosolic DT-diaphorase activity was also increased in a dose-related manner after three treatments with AAF, but the maximal activity attained was only 156% of control at a dose of 100 mg/kg (Figure 4).

Effect of actinomycin D on AAF-dependent epoxide hydrolase elevation

Co-administration of AD with AAF inhibited the AAF dependent increase in epoxide hydrolase activity in F-344 rats (Table I). A single dose of 35 mg/kg AAF elicited a 250% increase in epoxide hydrolase activity, but in animals treated with AAF plus AD, epoxide hydrolase activity was not significantly different from control. AD alone had no significant effect on hepatic epoxide hydrolase levels.

Epoxide hydrolase elevation by compounds structurally related to AAF

Both N-OH-AAF and AF increased hepatic epoxide

Table I. Effect of AD on the elevation of epoxide hydrolase activity by AAF. AAF (35 mg/kg) was administered to F-344 rats as a single oral dose dissolved in corn oil. AD (1 mg/kg in 1 ml/kg water containing 5% DMSO, i.p.) was administered 0.5 h before AAF treatment and repeated 10 h later. Control animals received corn oil alone. Rats were killed 24 h after AAF treatment.

Treatment	Epoxide hydrolase activity[a]
Control	0.826 ± 0.049
AD	0.858 ± 0.042
AAF	2.169 ± 0.194[b]
AD + AAF	0.970 ± 0.146

[a]nmol benzo[a]pyrene-4,5-diol formed/mg PMS protein/min. Mean ± SEM, n = 3. [b]Significantly different from control, $p < 0.05$.

hydrolase activity in F-344 rats (Figure 2). N-OH-AAF was a more potent inducer than AAF, with a single dose ED[50] of 12 mg/kg, assuming a maximal response equal to that obtained with AAF. The maximal response to N-OH-AAF could not be determined as doses above 20 mg/kg were lethal. In contrast, AF at a dose of 240 mg/kg produced only a 2-fold increase in epoxide hydrolase activity.

Species and strain comparisons in the response to AAF

Treatment of guinea pigs with three daily doses of AAF as high as 240 mg/kg/day, or with 10 mg/kg N-OH-AAF failed to elicit any significant changes in epoxide hydrolase activity

Table II. Effect of AAF or N-OH-AAF treatment on hepatic epoxide hydrolase activity in guinea pigs. Male Hartley guinea pigs received 3 consecutive daily i.p. injections of AAF or N-OH-AAF dissolved in DMSO (2 ml/kg), and were killed 48 h after the last dose.

Treatment	Epoxide hydrolase activity[a]
Control	22.16 ± 0.68
AAF, 40 mg/kg	24.79 ± 1.24
AAF, 80 mg/kg	24.67 ± 2.71
AAF, 160 mg/kg	27.56 ± 1.45
AAF, 240 mg/kg	23.69 ± 2.72
N-OH-AAF, 10 mg/kg	20.99 ± 2.86

[a]nmol benzo[a]pyrene-4,5-diol formed/mg microsomal protein/min. Mean ± SEM, n = 3.

Table III. Effect of AAF treatment on hepatic activity in mice. Male C57BL6 and DBA2 mice received 0.02% AAF in the diet for 5 days and were sacrificed immediately thereafter. Epoxide hydrolase activity was measured in liver microsomes. Data are presented as mean + SEM, n = 4 animals.

Strain	Treatment	Epoxide hydrolase (BPO)[a]	Epoxide hydrolase (SO)[b]
C57BL6	Control	0.331 ± 0.066	2.53 ± 0.17
	AAF	0.356 ± 0.021	3.09 ± 0.28
DBA2	Control	0.284 ± 0.011	2.77 ± 0.52
	AAF	0.319 ± 0.012	2.59 ± 0.06

[a]nmol benzo[a]pyrene-4,5-diol formed/mg protein/min. [b]nmol styrene glycol formed/mg protein/min.
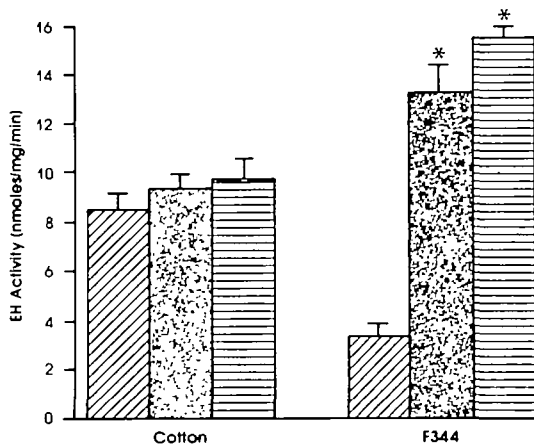

Fig. 5. AAF-dependent effects on EH activity in Cotton and F-344 rats. Male rats were treated with a single i.p. injection of AAF dissolved in DMSO (1 ml/kg), 48 h before sacrifice. Doses were: ▨, vehicle control; ▣, 32mg/kg; ▤, 100 mg/kg. Microsomal EH activity was measured with BPO substrate. Bars represent mean ± SEM, n = 3, except for the Cotton 100 mg/kg group for which n = 2 and the range is displayed. Data for F-344 rats are reproduced from Figure 2 to facilitate comparisons. *Significantly different from corresponding control, p < 0.05.

Fig. 6. Effects of dietary administration of AAF on EH activity in Cotton and F-344 rats. Animals received 0.02% AAF in the diet for 2 weeks. EH activity was measured in PMS with BPO substrate. Control (□); AAF (▨). Bars represent mean ± SEM, n = 3 or 4 animals, except in the case of the Cotton rat controls, for which n = 2 and the range is indicated. *Significantly different from corresponding control, p < 0.05.

epoxide hydrolase in these two strains (data not shown).

The epoxide hydrolase activity in Cotton rats was not increased in response to three daily treatments (Figure 5) or two weeks of dietary administration (Figure 6) of AAF at doses that caused 4- to 7-fold increases in F-344 rats. AFB and PB were also administered to Cotton rats as positive controls for epoxide hydrolase increase. Three daily doses of 80 mg/kg PB caused a 230% elevation of epoxide hydrolase activity (data not shown). However, it is of interest to note that AFB did not elicit any significant changes in epoxide hydrolase activity in this strain either when measured 24–96 h after a single dose of 0.32 mg/kg, or when measured 48 h after three daily doses of 0.16 or 0.32 mg/kg (data not shown). By comparison, the ED[50] for AFB-dependent epoxide hydrolase elevation in F-344 rats measured 48 h after three daily doses, is 0.18 mg/kg/day (1).

Discussion

Treatment of male F-344 rats with AAF elicited a dramatic and dose-related increase in hepatic microsomal epoxide hydrolase activity. The 5- to 6-fold increases measured after only 1 or 3 doses are similar in magnitude to those reported by others (1–4).

The pattern of elevation observed was independent of the substrate used to assay the enzyme or the route of administration. The same pattern of activity was evident when epoxide hydrolase was measured in microsomes or PMS. A cytosolic epoxide hydrolase (31) and a membrane-bound form of the cytosolic enzyme (32) have recently been reported; however these enzymes display no detectable activity with BPO or SO substrate under the assay conditions (pH 9.0) employed in this study (31,32), and thus the epoxide hydrolase activities reported in this study are solely due to the microsomal enzyme.

A dose-related increase in cytosolic DT-diaphorase activity was observed in F-344 rats following AAF treatment, but the maximal increase seen was relatively slight (156% of control). This is in accord with previous observations on the changes in DT-diaphorase activity elicited by other hepatocarcinogens (1). Some workers, however, have noted larger AAF-

(Table II). As noted by other workers (31), control epoxide hydrolase activity was extremely high in the guinea pig. Similarly, five days' dietary administration of 0.02% AAF to C57Bl6 or DBA2 mice caused no significant changes in epoxide hydrolase activity (Table III). Three daily i.p. injections of AAF (35 mg/kg) also failed to elicit an increase in hepatic
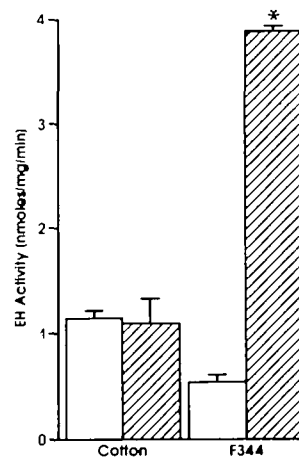
dependent increases in DT-diaphorase in Sprague-Dawley rats (3,33), possibly indicating a strain difference in response to this compound.

Following a single oral dose of AAF, epoxide hydrolase activity increased rapidly and then declined slowly, with a half-life of 17.5 days. The persistence of the altered enzyme activity suggests that this change is not the result of enzyme activation. These findings are at variance with those of Astrom and DePierre (3), who reported a decline of elevated epoxide hydrolase activity to approximately twice control level by 7 days after the last of 5 daily doses of AAF in Sprague-Dawley rats. This discrepancy may again be a reflection of a strain difference.

Co-treatment of rats with AD, an inhibitor of RNA synthesis (34), effectively blocked the epoxide hydrolase elevation in response to AAF. This result is in accord with the work of Gonzalez et al. (35), who demonstrated that both AAF and N-OH-AAF cause an increase in intracellular levels of mRNA coding for epoxide hydrolase in rats. This finding, considered together with the magnitude and persistence of the elevation observed, strongly suggests that the increase in epoxide hydrolase activity is the result of enhanced enzyme synthesis.

The potencies of the structurally related compounds AAF, N-OH-AAF, and AF for inducing epoxide hydrolase activity reflect their hepatocarcinogenic potencies. N-OH-AAF, which is more hepatocarcinogenic than AAF in the rat (36) yielded an ED[50] of $\sim$ 12 mg/kg versus 20 mg/kg for AF. The less potent hepatocarcinogen AF (37,38) elicited a relatively small increase in epoxide hydrolase activity (240% of control), and no dose-response relationship over the range of doses employed.

Hepatic epoxide hydrolase was not inducible by AAF in guinea pigs, mice, or Cotton rats, or by N-OH-AAF in guinea pigs. This reflects the hepatocarcinogenic potency of these compounds in these species (21,23). The strain and species differences in susceptibility to AAF-induced hepatocarcinogenesis have been attributed to variations in the metabolic activation of this compound. Guinea pigs are lacking in sulfotransferase activity, and also readily convert N-OH-AAF to 7-OH-2-acetylaminofluorene, which is inactive as a carcinogen (39). Cotton rats possess 5 times the AAF-N-hydroxylase activity, 3 times the N-OH-AAF-deacetylase activity, but only 1/3 the N-OH-AAF-sulfotransferase activity of Sprague-Dawley rats (23). It is probable that the lack of AAF-dependent epoxide hydrolase induction observed in these animals is related to this altered pattern of AAF metabolism. The results are consistent with the hypothesis that formation of the N-O-sulfate conjugate of N-OH-AAF is necessary for epoxide hydrolase elevation by this compound. Mice possess very low levels of AAF-N-hydroxylase activity (22,40). The lack of epoxide hydrolase increase in response to AAF in this species is likely a reflection of the low rate of formation of N-OH-AAF.

The results presented support the hypothesis that AAF dependent increases in epoxide hydrolase activity are the result of enzyme induction. The relative potencies of AAF, N-OH-AAF, and AF for inducing epoxide hydrolase reflect the hepatocarcinogenicity of these compounds. The extent of epoxide hydrolase elevation by AAF is related to its hepatocarcinogenic potency in several species and strains. These observations provide further support for the hypothesis that a relationship exists between hepatocarcinogenic potency and

increases in epoxide hydrolase. Furthermore, these data, considered together with subsequent studies involving modulation of AAF metabolism (Graichen and Dent, manuscript in preparation), support the suggestion that metabolic activation of AAF to N-OH-AAF and the subsequent conjugation of N-OH-AAF with sulfate are required for AAF to elicit increases in epoxide hydrolase activity.

Acknowledgements

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