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RAPID DECREASE IN *N*-HYDROXY-2-ACETYLAMINOFLUORENE SULFOTRANSFERASE ACTIVITY OF LIVER CYTOSOLS FROM RATS FED CARCINOGEN

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Abstract—Sulfation of *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF) by *N*-OH-AAF sulfotransferase yields a candidate for ultimate carcinogen in hepatocarcinogenesis in rats. We have monitored this pathway during the initial phase(s) of hepatocarcinogenesis produced by feeding male Holtzman rats a diet containing 0.05% 2-acetylaminofluorene (AAF). Our studies revealed an immediate and precipitous decrease in *N*-OH-AAF sulfotransferase activity beginning after 1 day on the AAF diet and decreasing 4- to 5-fold after 5 days on the AAF diet. This decrease in activity remained at low values during continuous administration of AAF throughout 4 weeks but was shown to be both reversible and AAF dose dependent. Parallel monitoring of rat serum glutamic oxaloacetic acid transaminase activity during the administration of AAF indicated that no appreciable hepatocellular toxicity occurred during the period of sulfotransferase activity lowering. Other known carcinogens, i.e. 3'-methyl- and 4'-fluoro-4-dimethylaminoazobenzene, aflatoxin B_1, thioacetamide, ethionine, and diethylnitrosamine, and the hepatotoxin α-naphthylisothiocyanate, also caused decreases in *N*-OH-AAF sulfotransferase activity after 7 and 28 days of administration. In contrast, very weak or non-carcinogens, i.e. *p*-aminoazobenzene, fluorene, and barbital, failed to reduce *N*-OH-AAF sulfotransferase activity during 28 days of feeding. Data from these studies on the short-term chronic administration of xenobiotics suggest (a) reduced likelihood for the direct involvement of the sulfotransferase pathway in providing sufficient cytotoxic AAF metabolites to cause compensatory hyperplasia and its putative promotion-effect for AAF-mediated carcinogenesis, and (b) the possible use of the rapid loss in sulfotransferase activity as an early indicator of hepatocarcinogenesis.

The initial step in metabolic activation of AAF† toward a more potent mutagen or carcinogen involves *N*-hydroxylation by liver microsomal cytochrome P-450-dependent monooxygenases to form *N*-OH-AAF [1–5]. Furthermore, metabolism of *N*-OH-AAF by enzymes such as sulfotransferase [6, 7], deacetylase [8, 9], transacetylase [10, 11], and UDP-glucuronyltransferase [12, 13] has been implicated in the activation of *N*-OH-AAF to its ultimate carcinogenic form. Early studies in rat liver revealed the sulfate ester to be a principal electrophilic metabolite of *N*-OH-AAF [6, 7]. In addition, levels of *N*-OH-AAF sulfotransferase activity in liver cytosols were found to parallel the susceptibility of rats to *N*-OH-AAF hepatic carcinogenesis [14]. Such observations suggested sulfation of *N*-OH-AAF as a mechanism for the production of an ultimate carcinogenic form of AAF capable of initiating liver carcinogenesis. More recently, other reports [15–18] have suggested that sulfotransferase-mediated *N*-*O*-sulfation of *N*-OH-AAF plays an important role in the hepatotoxic action of *N*-OH-AAF in the rat. Since the response of the liver to cytotoxicity is one of compensatory hyperplasia, a condition which may act as a promotor of cells initiated in the process of carcinogenesis [19, 20], sulfotransferase may have a role in the promotion of AAF carcinogenesis.

In spite of indications that *N*-OH-AAF sulfotransferase may play a prominent role in the initiation and promotion stages of AAF hepatocarcinogenesis, few studies have examined the levels of activity of this enzyme during the course of the carcinogenic process. DeBaun *et al.* [14] reported that a fall in the level of liver cytosolic *N*-OH-AAF sulfotransferase activity to one-half the control values occurred in the livers of rats fed diets containing 0.02% *N*-OH-AAF for 2 weeks. Jackson and Irving [21] reported greater losses in *N*-OH-AAF sulfotransferase activity, i.e. to 10–30% of control values, when rats were fed a diet containing 0.04% AAF. Employing a regimen for the production of hepa-

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† Abbreviations: AAF, 2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; BAR, barbital; ETH, ethionine; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; 4'-F-DAB, 4'-fluoro-4-dimethylaminoazobenzene; ANIT, α-naphthylisothiocyanate; TA, thioacetamide; AFB_1, aflatoxin B_1; AB, *p*-aminoazobenzene; FLU, fluorene; DEN, diethylnitrosamine; SGOT, serum glutamic-oxaloacetic acid transaminase; PAP, 3',5'-adenosine diphosphate; and PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

tocarcinogenesis in rats by feeding AAF [22], we examined the activity of *N*-OH-AAF sulfotransferase in the livers of rats with respect to (a) changes in its levels during the early stages (0–4 weeks) of carcinogenesis, (b) comparison of AAF-mediated changes in *N*-OH-AAF sulfotransferase activity with AAF hepatotoxicity as measured by changes in SGOT activity, and (c) the influence of other known carcinogens and non-carcinogens upon the level of *N*-OH-AAF sulfotransferase activity.

MATERIALS AND METHODS

Chemicals. The carcinogens AAF, AFB_1, ETH and TA were purchased from the Aldrich Chemical Co. (Milwaukee, WI), the Calbiochem-Behring Corp. (La Jolla, CA), the Sigma Chemical Co. (St. Louis, MO), and the J. T. Baker Chemical Co. (Phillipsburg, NJ) respectively. ANIT and DEN were obtained from the Eastman Kodak Co. (Rochester, NY), while AB and FLU were obtained from ICN Pharmaceuticals, Inc. (Cleveland, OH). The two azo dyes, 3′-Me-DAB and 4′-F-DAB, were synthesized according to the procedure of Giese *et al.* [23]. *p*-Nitrophenylsulfate and PAP were purchased from the Sigma Chemical Co. The *N*-OH-AAF was supplied by Dr. Robert A. Floyd (Associate Member, Oklahoma Medical Research Foundation, Oklahoma City, OK).

Animals and enzyme preparations. Male Holtzman rats (200–250 g) were fed a semi-synthetic diet [24] for 1 week prior to use in experiments. Rats were then fed this diet alone or supplemented with 0.05% AAF, 0.05% DEN, 0.0012% AFB_1, 0.06% AB, 0.3% ETH, 0.06% 4′-F-DAB, 0.06% FLU, 0.06% 3′-Me-DAB, 0.08% ANIT or 0.06% TA; BAR (0.05%) was administered in the drinking water.

For the preparation of sulfotransferase-containing cytosols, rats were killed by cervical dislocation, and their livers were removed and homogenized in a Potter–Elvehjem homogenizer with 3 vol. of 20 mM Tris (pH 7.4), 150 mM KCl. Post-mitochondrial supernatant fractions were obtained by differential sedimentation [25] and microsomes were sedimented from the supernatant fractions by centrifugation at 100,000 *g* for 60 min. The upper two-thirds of the post-microsomal supernatant fractions were removed, and their protein concentrations were determined according to the method of Lowry *et al.* [26] using bovine serum albumin as the standard. Serum samples for the determination of GOT activity were obtained by cardiac puncture from rats following anesthesia with diethyl ether.

Sulfotransferase assay. *N*-OH-AAF sulfotransferase activity was measured as described by Mulder *et al.* [25]. In this assay, *p*-nitrophenylsulfate is used as a sulfate donor in the phenol sulfotransferase catalyzed conversion of PAP to PAPS; the sulfate of PAPS then is transferred by *N*-OH-AAF sulfotransferase to *N*-OH-AAF to form the sulfate ester of *N*-OH-AAF. The rate-limiting step is the formation of AAF sulfate ester, for the rate of PAPS formation is 8- to 10-fold faster than the transfer of sulfate to *N*-OH-AAF [25]. The rate of sulfation was monitored spectrophotometrically by following the accumulation of *p*-nitrophenol (450 nm) that paralleled the formation of the *N*-OH-AAF sulfate ester. A typical reaction mixture contained in 0.5 ml: 100 mM Tris (pH 8.0), 10 mM *p*-nitrophenylsulfate, 20 μM PAP, 0.5 mM *N*-OH-AAF, 5% (v/v) ethanol, and 300 μg protein from post-microsomal supernatant fraction. Reactions were initiated by the addition of protein. Absorbancy at 405 nm was continuously monitored with a Gilford 250 spectrophotometer during a 10-min incubation at 31°. The rate of formation of *p*-nitrophenol in control incubation mixtures, which did not contain *N*-OH-AAF, was subtracted from the values of the experimental mixtures, thus making the remaining *p*-nitrophenol formation an *N*-OH-AAF-dependent accumulation. Sulfotransferase activity was expressed as nmoles of *p*-nitrophenol released per min per mg post-microsomal supernatant protein. The molar extinction coefficient used to *p*-nitrophenol was 17,500 M^{-1} cm^{-1} [25].

SGOT assay. The GOT activity in rat serum was determined using a Sigma GOT kit, by the spectrophotometric method of Karmen [27]. Measurements were made with a Gilford 250 spectrophotometer equipped with a set of four matched quartz cuvettes. SGOT activity was expressed in I.U. (25°). Intubation of rats with 1.6 mg CCl_4/kg body weight produced a 20-fold elevation in SGOT activity within 24 hr.

RESULTS

N-OH-AAF sulfotransferase activities of liver cytosols derived from rats fed control or AAF diet. *N*-OH-AAF sulfotransferase activities were determined spectrophotometrically for post-microsomal supernatant fractions from the livers of male Holtzman rats fed continuously for up to 4 weeks a semi-synthetic diet containing 0.05% AAF. As shown in Fig. 1, there was an immediate and rapid decrease in sulfotransferase activity in the supernatant fractions from AAF-fed animals relative to those from control-fed animals. After 1 day on AAF-containing diet, a significant ($P < 0.05$) decrease in activity of 25% occurred; this was fol-

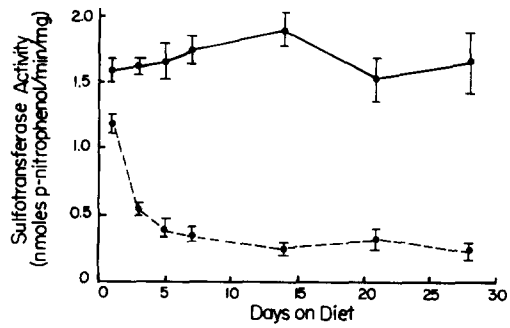

Fig. 1. *N*-OH-AAF sulfotransferase activities of liver cytosols derived from rats fed control or 0.05% AAF diets. Rats were killed at the times indicated above, and the *N*-OH-AAF sulfotransferase activitiy was determined as described in Materials and Methods. Each point is the mean (±S.E.M.) of four experiments and each experiment consisted of a three-rat pool. Key: (●——●) control diet; and (●----●) 0.05% AAF diet.

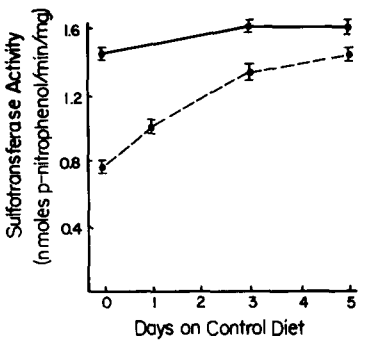

Fig. 2. Recovery of *N*-OH-AAF sulfotransferase activity in liver cytosols of rats pre-conditioned on AAF diet and then fed control diet. Rats were pre-conditioned for 3 days on either a control diet (●——●) or AAF diet (●----●), and then on control diet over a 5-day period. Rats were killed at various times during the 5-day period, and sulfotransferase activities were determined as described in Materials and Methods. Each point is the mean (± S.E.M.) of three experiments, and each experiment consisted of a two- or three-rat pool.

lowed by equally significant losses in activity of 65% by day 3, 80% by day 7, and 85% by day 14 where it remained during an additional 2 weeks of AAF feeding. Since Jackson and Irving [21], using a method of sulfotransferase analysis which trapped the unstable sulfate ester of *N*-OH-AAF in the form of a methionine adduct, observed a 50–70% decrease in sulfotransferase activity after 1 week on 0.04% AAF, it would appear that the spectrophotometric method of analysis used in our studies mimics the adduct-forming method with regard to assessing sulfotransferase activity. Furthermore, decreases observed in *N*-OH-AAF sulfotransferase activities would not appear to be accounted for by losses in the relative ability of supernatant fraction from control versus AAF-fed rats to provide PAPS for the sulfation reaction with *N*-OH-AAF. Substitution of phenol for *N*-OH-AAF in the assay of sulfotransferase activity in supernatant fractions derived from rats fed for 1 week on either control or AAF diets gave rates of sulfation which were similarly elevated (7.4 pmoles *p*-nitrophenol per min per mg) for both supernatant fractions.

Reversibility and dose responsiveness of AAF-mediated losses in N-*OH-AAF sulfotransferase activity*. The dependence of the loss in *N*-OH-AAF sulfotransferase activity upon the continuous presence of AAF in the diet was examined by monitoring the sulfotransferase activities of rats initially fed the AAF diet for 3 days and then switched to the control diet for 5 days (Fig. 2). Following a reduction of sulfotransferase activity to an intermediate level after 3 days of feeding the AAF-containing diet, there was a return of activity to near control levels during 5 days of feeding the control diet. Evidence of a direct relationship between the amount of AAF in diets and the loss of sulfotransferase activity was indicated by dose–response experiments (Fig. 3). When sulfotransferase activities were determined for rats fed 1 week on diets containing either 0.0125, 0.025, or 0.05% AAF, a linear dose–response was observed for the three concentrations. Extrapolation of this line to zero AAF present yielded a smaller sulfotransferase activity than was actually found, suggesting a more precipitous dose–response at AAF levels below 0.0125%. Together, these experiments indicate that the AAF-mediated lowering of sulfotransferase activity was dose dependent and required the continuous presence of AAF in the diet.

SGOT estimation of liver damage during AAF administration. It was possible that reductions in sulfotransferase activity reflect a more general cytotoxic response of the liver to AAF administration. For instance, rats fed a choline-devoid diet containing 0.05% AAF had SGOT activities that were 3- to 4-fold higher than control after 1 week of feeding [28], while rats analyzed 24 hr after intravenous administration of 120 μmoles/kg *N*-OH-AAF demonstrated 40-fold elevations in SGOT activity [18]. As shown in Fig. 4, animals fed AAF over a 2-week period had a slight (averaging 36%) but statistically significant ($P < 0.05$) increase in SGOT activity when compared to those fed control diet. On the other

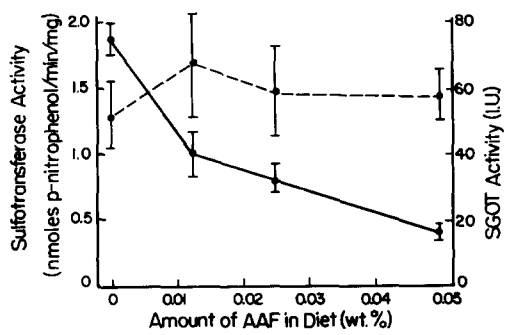

Fig. 3. *N*-OH-AAF sulfotransferase activities and SGOT activities of rats maintained for 1 week on diets containing various levels of AAF. Sulfotransferase (●——●) and SGOT (●----●) activities were determined as described in Materials and Methods. Each point is the mean (± S.E.M.) of six individual rats.

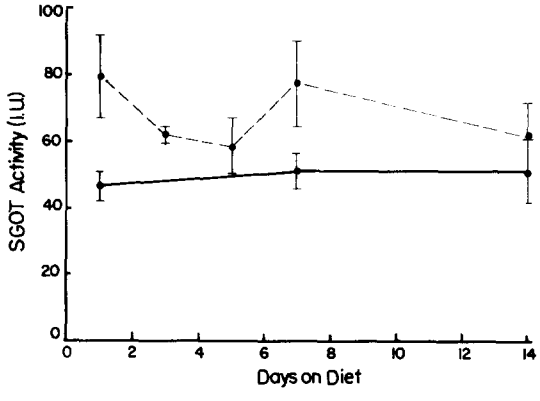

Fig. 4. GOT activities of serums derived from rats fed control or 0.05% AAF diets. Rats were killed at the times indicated above, and SGOT activities were determined as described in Materials and Methods. Each point is the mean (±S.E.M.) of six to eight individual rats. Key: (●——●) control diet; and (●----●) 0.05% AAF diet.

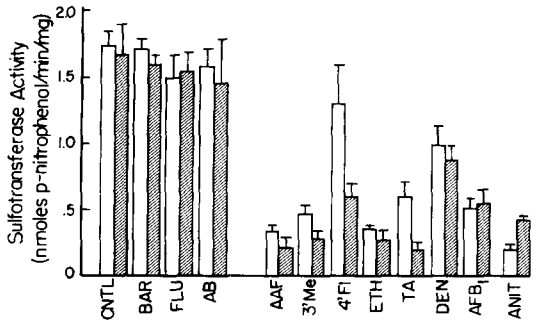

Fig. 5. *N*-OH-AAF sulfotransferase activities of liver cytosols derived from rats administered various xenobiotics. Rats were fed for 1 week (open bars) or 4 weeks (shaded bars) on control diet (CNTL) or diets containing 0.05% BAR, 0.06% FLU, 0.06% AB, 0.05% AAF, 0.06% 3′-Me-DAB, 0.06% 4′-F-DAB, 0.3% ETH, 0.06% TA, 0.05% DEN, 0.0012% AFB_1, or 0.08% ANIT, and sulfotransferase activities were determined as described in Materials and Methods. Each bar is the mean of two experiments, and each experiment consisted of a three-rat pool. The S.E.M. has been indicated on the top of the bars.

hand, when serum which had been isolated from rats in the AAF dose–response experiments was examined for changes in GOT activity, no significant elevations were observed (see Fig. 3). The degree and pattern of changes in SGOT observed in these studies suggest that there was little, if any, correlation between decreases in sulfotransferase activity observed in response to feeding AAF diets and a general cytotoxic response of the liver to AAF.

Effects of other xenobiotics on N-OH-AAF sulfotransferase activity. To assess the specificity of the AAF-mediated loss in *N*-OH-AAF sulfotransferase activity, two groups of xenobiotics were examined over a 4-week period of continuous administration for their abilities to mimic the effect of AAF. Figure 5 shows levels of sulfotransferase activity found at 1 and 4 weeks. Values at 2 and 3 weeks were intermediate between 1- and 4-week values and are not shown. The first group of xenobiotics consisted of compounds which are known to be either non- or weakly carcinogenic, i.e. BAR [29, 30], FLU [31], and AB [32, 33], and, as shown, caused no loss in *N*-OH-AAF sulfotransferase activity when compared to controls. A second group of compounds known to be hepatocarcinogenic or hepatotoxic, i.e. AAF [34], 3′-Me- and 4′-F-DAB [35], ETH [36], TA [37], DEN [38], AFB_1 [39], and ANIT [40], produced significant ($P < 0.05$) decreases in sulfotransferase activity after 1 week of administration, except for 4′-F-DAB, which did not show a significant decrease in activity until week 2. These data indicate that, while the reductions in sulfotransferase activity are not specific for AAF, they appear well-correlated with the carcinogenicity of the xenobiotics studied.

DISCUSSION

The ability of rat liver *N*-OH-AAF sulfotransferase to produce a chemically active intermediate of *N*-OH-AAF capable of modifying cellular macromolecules has been amply demonstrated. The actual role of the metabolic pathway in AAF-mediated carcinogenesis, however, remains open to debate [41–43]. As an initial step toward evaluation of this pathway, we examined the level of sulfotransferase activity during the early phase(s) of liver carcinogenesis produced by feeding chronic levels (0.05%) of AAF. We observed a rapid decrease in sulfotransferase activity during the first week of feeding to levels equalling 20% of control-fed animals. These findings confirm previous reports [14, 21] that *N*-OH-AAF sulfotransferase activity was decreased in the livers of male rats fed AAF. We also demonstrated that the decrease observed during the first week was found to be dependent on the dose of AAF and upon its continuous administration. Since the initiation events of chemical carcinogenesis are generally thought not to require prolonged exposure to carcinogen, arguments for the participation of sulfotransferase during the initiation events of AAF-mediated carcinogenesis may be little affected by an early decrease in sulfotransferase activity. On the other hand, its possible role as a promoter through cytotoxicity and compensatory hyperplasia [18, 19, 41] would seem to be weakened by the rapid lowering of sulfotransferase activity observed during continuous AAF administration. In support of this conclusion, marginal elevations of SGOT activity observed during 2 weeks of feeding AAF (Fig. 1) and its failure to increase during AAF dose–response studies (Fig. 3) suggest a lack of appreciable hepatotoxicity during the period corresponding to losses in sulfotransferase activity. These low levels of SGOT activity were in marked contrast to more cytotoxic responses previously reported in the literature. Increases of 40-fold in SGOT activity were observed 24 hr after the intravenous injection of *N*-OH-AAF into rats [18] and a 3- to 4-fold elevation in SGOT activity was observed for rats maintained 1 week on a choline-devoid diet containing 0.05% AAF [28]. Differences in SGOT levels among the three studies may be associated with differences in *modus operandi*, viz. the form of AAF used, dose, route of administration, and diet. Thus, our results do not appear to support the putative participation of the sulfotransferase pathway in producing a promotion-effect during the early stages of carcinogenesis induced by feeding 0.05% AAF.

Although sulfotransferase may not directly participate in the production of an AAF-mediated cytotoxic stress, it may participate indirectly, acting as a forerunner of such a response. An initial AAF-mediated reduction of sulfotransferase activity could result in an influx of *N*-OH-AAF to alternative and more cytotoxic pathways. Recently, studies employing a selective inhibitor of rat liver sulfotransferase have indicated that shifts in *N*-OH-AAF to an alternative metabolic route can occur as a result of lowered sulfotransferase activity [17]. In addition, since the first indications of compensatory hyperplasia in response to AAF feeding do not appear prior to 4 weeks on AAF diet (D. E. Kizer, B. Cox, D. P. Ringer and B. A. Howell, manuscript in preparation), our studies, limited to the first 4 weeks, may have been precluded from observing the promotion effects of cytotoxic metabolites.

Studies examining the ability of other xenobiotics to mimic the AAF-mediated decrease in sulfotransferase activity revealed that (a) other carcinogens lowered sulfotransferase activity, (b) carcinogens other than those reported to be activated by the sulfotransferase pathway lowered sulfotransferase activity, and (c) non-hepatocarcinogens, with the exception of ANIT, did not lower activity. The observation that other carcinogens caused a lowering of sulfotransferase activity poses the question of whether it may be useful either as a specific indicator of sulfotransferase involvement in carcinogenesis or as a more general indicator of the early impact of carcinogen presence in rat liver. The finding that carcinogens which reportedly can utilize the sulfotransferase pathway for activation, i.e. AAF [14], 3′-Me- and 4′-F-DAB [44], as well as carcinogens believed to use other pathways, i.e. TA [45, 46], AFB_1 [47], DEN [38], and ETH [48], can lower sulfotransferase activity reduces the likelihood of its utility as a specific indicator for sulfotransferase involvement in carcinogenesis. On the other hand, the observation that xenobiotics reported to be very weakly or non-carcinogenic in rat liver, i.e. BAR, AB, and FLU, failed to decrease sulfotransferase activity suggests its possible use in detecting the onset of carcinogen-mediated changes in liver metabolism. An exception to the above pattern for very weak or non-carcinogens was the reduction of sulfotransferase activity caused by ANIT. Administration of ANIT is well-known for its ability to induce bile duct hyperplasia [49] and, more recently, has been reported to undergo metabolic activation by the liver to produce a metabolite which can bind irreversibly with microsomal proteins [50]. Hence, the fact that ANIT, a non-carcinogen, also caused a reduction in sulfotransferase activity may reflect certain features of its pharmacology that are similar to those of hepatocarcinogens.

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