In Vitro Metabolism and Activation of Carcinogenic Aromatic Amines by Subcellular Fractions of Human Liver1

Erik Dybing, Christer von Bahr, Tore Aune, Hans Glaumann, David S. Levitt, and Snorri S. Thorgeirsson

Department of Environmental Toxicology, National Institute of Public Health, Oslo 1, Norway [E. D., T. A.]; Departments of Clinical Pharmacology [C. von B.] and Morphology [H. G.] Huddinge Hospital, Karolinska Institutet, S-141 86 Huddinge, Sweden; and Laboratory of Chemical Pharmacology, National Cancer Institute, NIH, Bethesda, Maryland 20014 [D. S. L., S. S. T.]

ABSTRACT

In vitro metabolism and metabolic activation of 2-acetylaminofluorene (AAF), 2-aminofluorene, and 2,4-diaminoanisole to mutagenic (Salmonella test system) and covalently protein-bound intermediates were evaluated in subcellular fractions from seven human livers. The cytochrome P-450 content, aryl hydrocarbon hydroxylase activity, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the human liver microsomes were concomitantly studied. AAF was extensively metabolized (both C- and A/-hydroxylated) by all of the human liver microsomal samples, but severalfold variations between the individual metabolites were observed among the different microsomal fractions. Similar variation was also observed for aryl hydrocarbon hydroxylase activity. Electrophoresis of the microsomal fractions revealed several polypeptides with a molecular weight range of 40,000 to 60,000. Two of these polypeptides, with molecular weights of approximately 54,000 and 55,000, correspond to those seen in rat liver microsomes following pretreatment with 3-methylcholanthrene. The in vitro mutagenicity of AAF with the individual samples corresponded well with those of 2-aminofluorene and 2,4-diaminoanisole, as did the degree of AAF /V-hydroxylation. N-Hydroxy-2-acetylaminofluorene was converted to mutagen(s) by both human liver microsomal and cytosol fractions, presumably via deacetylation. A poor association between the extent of covalent binding of AAF to liver microsomal proteins and the degree of mutagenicity in the Salmonella system was observed among the samples, possibly indicating that the reactive metabolite(s) arylating the protein differs from that causing the frame-shift mutation in the Salmonella.

The results from the present study on AAF and benzo(a)pyrene hydroxylation; the metabolic activation of AAF, 2-aminofluorene, and 2,4-diaminoanisole; as well as the electrophoretic characterization of hepatic cytochrome P-450 indicate great qualitative similarities between the subcellular fractions from human liver and those from the rat, the mouse, and the rhesus monkey.

INTRODUCTION

Aromatic amines, and in particular AAF,2 have been used extensively as model compounds in studies on chemical carcinogenesis and mutagenesis (1, 16, 29, 33). From these studies, it is apparent that the carcinogenicity of AAF differs greatly among animal species and even among strains of the same species (17, 33). AAF is a procarcinogen and requires, as do many other chemical carcinogens, metabolic activation before its carcinogenic potential is expressed. The metabolic activation of AAF involves at least 2 steps, the first being a cytochrome P-450-dependent N-hydroxylation resulting in the formation of N-OH-AAF, which becomes a substrate for several enzymes each known to further activate N-OH-AAF to a reactive intermediate(s) capable of covalent interaction with cellular macromolecules. The cytosolic sulfotransferase and N-O-acyltransferase as well as the membrane-bound deacetylase and UDP-glucuronitransferase have all been implicated in forming the ultimate carcinogenic and mutagenic species of AAF (4, 17, 31). Since AAF requires metabolic activation, explanations for the carcinogenicity or lack thereof in the various animal species have been sought, and in some instances found, in the metabolism of AAF in the exposed animals. The resistance to AAF carcinogenesis in guinea pigs appears, for example, to be due to lack of N-hydroxylation (16, 30). However, studies in other resistant animals such as rhesus monkey, cotton rat, and X/Gf mouse indicate a more complex relationship between AAF metabolism and carcinogenesis (7, 11, 16, 24, 28).

In previous studies with subcellular fractions from mouse and rat liver, we have shown that the mutagenic activation of AAF in the Salmonella system (1) requires 2 activation steps, namely, N-hydroxylation and a subsequent deacetylation by either the membrane-bound deacetylase or the soluble N-O-acyltransferase (9, 23–25). In this paper, we have examined the in vitro metabolism and activation of AAF, AF, and 2,4-DAA to mutagenic and covalently protein-bound intermediates in subcellular human liver fractions.

MATERIALS AND METHODS

Materials. [9-14C]AAF (46.16 mCi/mmol) was obtained from New England Nuclear Chemicals (Dreieichenhain, Federal Republic of Germany). The [9-14C]AAF was more than 99.9% pure [thin-layer chromatography with silica gel and chloroform: methanol (97:3)]. AAF and AF were purchased from Koch-Light Laboratories (Colnbrook, England); 2,4-DAA was from ICN Pharmaceuticals (Plainview, N. Y.); benzo(a)pyrene was from Eastman Organic Chemicals (Rochester, N. Y.). N-OH-AAF and 9-hydroxy-2-2-acetylaminofluorene were generous gifts of Dr. Elizabeth K. Weisburger, National Cancer Institute (Bethesda, Md.), and Dr. Duane D. Miller, Ohio State University (Columbus, Ohio), respectively. S. typhimurium TA98 was kindly provided by Dr. Bruce N. Ames, University of California (Berkeley, Calif.).

Human Liver Samples. Samples of human liver were obtained from 7 patients with total cerebral infarction who served...
as kidney transplant donors at the Huddinge University Hospital, Huddinge, Sweden. Consent to the experimental use of the livers was given by the Swedish National Board of Health and Welfare. Information on the donors is presented in Table 1, but regrettably no information on smoking habits has been available. The patients were given 250 mg chlorpromazine and 25,000 IU heparin i.v. 15 min before liver extirpation. The livers were perfused with cold Perfadex for about 5 min. Thereafter, the livers were cut into small pieces and immediately placed in liquid nitrogen. About 2 hr later, the pieces were transferred to a freezer and stored at —80° until used.

Preparation of Liver Subfractions. Ten-g pieces of frozen liver were thawed, minced in 2 volumes of sterile, ice-cold 20 mM Tris buffer, pH 7.4, containing 1.15% KCl, with an Ultra-Turrax homogenizer for 2 sec and subsequently homogenized with 5 strokes of a Teflon:glass homogenizer. Liver 9,000 x g supernatant fractions, washed microsomes, and 105,000 x g supernatant fractions were prepared as previously described (5, 25).

Sodium Dodecyl Sulfate:Polyacrylamide Gel Electrophoresis. Slab gels (1.5 mm thick) with 1-cm tracks were prepared as described by Laemmli (14), and 30 ¿g of microsomal protein were applied per track. The mixture of standards run alongside, with their subunit molecular weight, included aldolase (40,000), ovalbumin (43,000), glutamate dehydrogenase (53,000), and bovine serum albumin (68,000). Gels were stained for 1 hr in methanol:glacial acetic acid:water (5:1:4) containing 2 g of Coomassie Brilliant Blue R per liter and then destained overnight in methanol:glacial acetic acid:water (10:3:27).

Assays. The in vitro mutagenesis was assayed in the Salmonella test system of Ames et al. (1) as described previously (6, 25). Total microsomal cytochrome P-450 content was measured according to the method of Omura and Sato (20), and protein concentrations were measured by the method of Lowry et al. (15). AHH activity was determined fluorometrically by reference to the fluorescence of recrystallized 3-hydroxybenzo(a)pyrene as standard (19) and using the benzo(a)-pyrene and microsomal protein concentrations of 80 ¿g and 0.6 mg/ml in the incubation mixture, respectively. The NADPH-dependent covalent binding of 0.6 mM [14C]AAF to microsomal protein was determined as previously described (5). The oxidative metabolism of AAF was assayed, and the separation of metabolites was achieved using high-pressure liquid chromatography according to the method of Thorgerisson and Nelson.

### Table 1

**Individual patient data**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Cause of death</th>
<th>Known drug intake last week before death</th>
<th>Regular drug intake and smoking and drinking habits</th>
<th>Other relevant information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>22</td>
<td>Drug intoxication.</td>
<td>Intoxication with Doleron natt (acetylsalicylic acid, dextropropoxyfen, antipyrine, diethylaminocarbamylpenothiazine, vinbarbital). After admission to hospital, single doses of epinephrine and atropine at admission; a few days treatment with dexamethasone, furosemide, ampicillin.</td>
<td>Alcohol intake at intoxication. Drugs and smoking habits unknown.</td>
<td>Admission to hospital with cardiac and respiratory arrest (5 days before death). 5 days in coma, aspiration pneumonia. Recurrent backaches, sporadic drug intake (Doleron natt). Slight fatty infiltration in liver.</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>68</td>
<td>Cerebral aneurysm.</td>
<td>Mannitol, dextran, a few doses of betamethasone and furosemide. At operation 1–2 days before death; atropine, succinylcholine, droperidol, fentanyl, tubocurarine.</td>
<td>Unknown</td>
<td>2 days in hospital, intracranial infarction.</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>28</td>
<td>Cerebral contusion, road accident.</td>
<td>Mannitol, dextran, furosemide, a few doses of betamethasone, benzylpenicillin, guaifenesin.</td>
<td>Unknown.</td>
<td>1 day in hospital, orthopedic operation the day before death. Low blood pressure a few hr before death.</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>69</td>
<td>Cerebral infarction.</td>
<td>One dose of dexamethasone and meperidine.</td>
<td>Probably O-methyldopa and hydrochlorothiazide. Smoking and drinking habits unknown.</td>
<td>3 days in hospital, amputation of leg after severe burn. Hypertension.</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>52</td>
<td>Meningioma. Postoperative infarction.</td>
<td>Barbiturate, fentanyl, pancuronium, tubocurarine at operation 5 and 3 days before death. Betamethasone, a few doses of furosemide, paracetamol, dihydroergotamine, metoclopramide.</td>
<td>Unknown.</td>
<td>11 days in hospital, brain surgery 11, 5 and 3 days before death. Headaches 2 yr.</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>59</td>
<td>Cerebral aneurysm.</td>
<td>None</td>
<td>Unknown.</td>
<td>2 days in hospital.</td>
</tr>
</tbody>
</table>
E. Dybing et al.

(27). N-OH-AAF sulfotransferase activity was determined spectrophotometrically with p-nitrophenyl sulfate as the sulfate donor (18).

RESULTS

Microsomal Cytochrome P-450 Content and AHH Activity. The microsomal cytochrome P-450 content in the 7 livers ranged from 0.16 to 0.60 nmol/mg protein with a mean ± S.D. of 0.36 ± 0.15 nmol/mg protein (Chart 1A). AHH activity averaged 175 ± 138 pmol per mg protein per min (Chart 1B). Liver 1 had considerably higher AHH activity, 483 pmol per mg protein per min, than did the other livers. When AHH activities were expressed per nmol cytochrome P-450, a range of 285 to 805 pmol per nmol P-450 per min was found, i.e., a 2.8-fold variation. AHH activities in human microsomes were of the same order of magnitude as in liver microsomes from untreated mice and rats (19, 26, 35).

[14C]AAF Covalent Binding to Microsomal Protein. As a measure of NADPH-dependent formation of reactive metabolites from AAF in vitro, covalent binding to microsomal proteins was determined (Chart 1C). Microsomes from Liver 1 had much higher activity (86 pmol per mg protein per min) than did the other microsomal preparations. These ranged from 13 to 35 pmol per mg protein per min. When expressed per nmol cytochrome P-450, the activities varied 2.8-fold.

Sodium Dodecyl Sulfate:Polyacrylamide Gel Electrophoresis. Electrophoresis of the human liver microsomal fractions revealed several polypeptides in the molecular weight range of 40,000 to 60,000, corresponding to the various forms of cytochrome P-450 (Fig. 1; Refs. 26 and 34). Two of these polypeptides, with molecular weights of approximately 54,000 and 55,000, correspond to those seen in rat liver microsomes following pretreatment with 3-methylcholanthrene (Fig. 1).

Oxidative AAF Metabolism. The relative pattern of the 5 ring-hydroxylated and the N-hydroxylated metabolites of AAF formed by human liver microsomal NADPH-dependent metabolism is shown in Chart 2. The rates of formation of 9-hydroxy-2-acetylamino-fluorene, 7-OH-AAF, 5-hydroxy-2-acetylamino-fluorene, 3-hydroxy-2-acetylamino-fluorene, and N-OH-AAF were 67 ± 11, 423 ± 183, 82 ± 26, 79 ± 62, 26 ± 21, and 58 ± 41 pmol per mg protein per min, respectively. Again, there was considerable variation among the different microsomal preparations, with Liver 1 containing the highest activities. With microsomes from Livers 1 and 6, the highest N-hydroxylase activities were found, 118 and 105 pmol per mg protein per min, respectively. When expressed per nmol cytochrome P-450, there was a 2.4-fold variation in N-hydroxylase activities. The N-hydroxylase activities were of the same order of magnitude as the activities observed in liver microsomes from untreated mice and rats (24, 26, 35).

In Vitro Mutagenic Activation. The in vitro mutagenicity of AAF with human liver 9,000 x g supernatant fractions was compared to that of the carcinogenic amines, AF and 2,4-DAA (Chart 3). The mutagenicity of AAF varied 10-fold, from 460 to 4430 revertants per plate, whereas those of AF and of 2,4-DAA varied approximately 5-fold. The mutagenicity of the 3 substrates in the individual samples seemed to correlate, in that the highest activities were found with Livers 1 and 6 and the lowest activities were found with Livers 2 and 3. N-OH-AAF is converted to potent mutagenic derivatives by deacetylation via microsomal and cytosolic liver enzymes of laboratory animals (23-25, 31). Similarly, N-OH-AAF was converted to mutagen(s) by both human liver microsomes (without NADPH-generating cofactors) and cytosol (Chart 4). These activities also showed interindividual variations. However, they did not seem to correlate with the metabolism or mutagenic
Chart 2. Oxidative metabolism of AAF in human liver microsomes. Metabolites A to F were measured by high-pressure liquid chromatography as indicated in "Materials and Methods." Each column represents means of duplicate estimations from individual microsomal preparations. 9-OH-AAF, 9-hydroxy-2-acetylaminofluorene; 5-OH-AAF, 5-hydroxy-2-acetylaminofluorene; 3-OH-AAF, 3-hydroxy-2-acetylaminofluorene; 1-OH-AAF, 1-hydroxy-2-acetylaminofluorene; N-OH-AAF, N-hydroxy-2-acetylaminofluorene.

One of the most important factors in determining whether a chemical compound has the potential for becoming a carcinogen is the capacity of the exposed organism to metabolically activate the compound into a reactive metabolite(s) which can covalently interact with cellular macromolecules. Comparison of data obtained with human tissues to existing data from animal tissues should therefore provide useful information on whether animal tissues are suitable models for evaluation of metabolism and activation of chemical compounds in humans.

In this report, we have shown that the carcinogenic aromatic amine AAF is extensively metabolized and that the aromatic amines AAF, AF, and 2,4-DAA are metabolically activated by subcellular fractions from human livers.

The liver microsomal cytochrome P-450 content and the AHH activity are in good agreement with values reported by others from studies with human liver microsomes (12, 13, 21, 22). The interindividual variations in AHH activities, when expressed per nmol of cytochrome P-450, are between 2- and 3-fold, indicating that the relative amount of the different forms of cytochrome P-450 differs among individuals. This is further illustrated by the electrophoretogram of the human liver microsomes (Fig. 1), showing differences in staining intensity of the polypeptide bands, in particular those with molecular weights of approximately 54,000 and 55,000. Recent electrophoretic evidence indicates that at least 2 cytochrome P-450 subunits (m.w. ~54,000 and 55,000) are induced by 3-methylcholanganthrene in genetically aromatic hydrocarbon-responsive mice and rats (3, 24, 26).

Studies on in vivo and in vitro metabolism of AAF in humans by analysis of urinary metabolites and in incubations with subcellular liver fractions show that humans are able to ring- as well as N-hydroxylate AAF and that the major urinary metabolite is 7-OH-AAF (8, 32). In the present study, we found AAF to be extensively metabolized by human liver microsomes (Chart 2). 7-OH-AAF is the major hydroxylated metabolite, but N-OH-AAF is also formed to a substantial degree, ranging from activation of AAF, nor was there an apparent correlation between the mutagenic activation of N-OH-AAF in the microsomal and cytosolic fractions. No N-OH-AAF sulfotransferase activity could be detected in the cytosolic fractions (data not shown).

DISCUSSION

One of the most important factors in determining whether a chemical compound has the potential for becoming a carcinogen is the capacity of the exposed organism to metabolically activate the compound into a reactive metabolite(s) which can covalently interact with cellular macromolecules. Comparison of data obtained with human tissues to existing data from animal tissues should therefore provide useful information on whether animal tissues are suitable models for evaluation of metabolism and activation of chemical compounds in humans.
Chart 4. Mutagenicity of N-OH-AAF with human liver microsomes (A) and cytosol fractions (B). Each plate contained 2 μg of N-OH-AAF and 0.4 mg of microsomal protein or 3 mg of cytosolic protein. Each column represents the means of duplicate estimations from individual subcellular fractions. Spontaneous revertants, 27 ± 7 in the presence of microsomes or 13 ± 4 in the presence of cytosol, have been subtracted.

4 to 10% of the total metabolites in the 7 liver samples. The rate of N-hydroxylation by human liver microsomes corresponds well with that in untreated mouse and rat liver microsomes, but it is substantially higher than that found in liver microsomes from untreated rhesus monkeys (24, 26, 28). Whether this relatively high activity of AAF N-hydroxylase in the human liver microsomes is a reflection of monooxygenase induction is uncertain. However, cytochrome P-450 content, AHH activity, AAF covalent protein binding, and 7-OH-AAF formation are higher in the microsomes from Patient 1, who died from drug intoxication (Table 1), than in those from the other patients. Patient 6 had received barbiturates i.v. at surgery 5 and 3 days before death (Table 1). The relative degree of in vitro mutagenic activation of AAF, AF, and 2,4-DAA among the liver samples was similar to the variations in AAF N-hydroxylase activities (Chart 3 versus Chart 2F), suggesting that N-hydroxylation may be the rate-limiting step in the mutagenic activation process for all 3 compounds. Both microsomal and cytosol fractions from human livers are, in agreement with data from other species, capable of activating N-OH-AAF to a mutagen, most probably via deacetylation by the membrane-bound carboxyesterase-amidase and/or the soluble N-O-acyltransferase (Chart 4; Refs. 23 to 25 and 31). No sulfotransferase activity is found in the cytosol fractions of the human livers, suggesting that this enzyme is not important in the formation of the mutagenic species from N-OH-AAF.

The in vivo and in vitro covalent binding of foreign compounds to cellular macromolecules has been extensively used as a measurement of the formation of reactive metabolites implicated in cell death, mutations, and cancer (see Ref. 10 for review). The extent of covalent binding of AAF to microsomal proteins and the degree of mutagenicity of AAF in the Salmonella test system do not correlate (Charts 1C and 3A). The possibility therefore exists that the reactive metabolite(s) arylating microsomal proteins is different from the ultimate mutagen(s) causing the frameshift mutation in the Salmonella test system. This interpretation is supported by our earlier data showing a possible difference between the ultimate mutagenic species of N-OH-AAF in the Salmonella test system and the arylating species of N-OH-AAF that binds to nucleic acids and proteins in isolated rat and mouse liver cell nuclei (23). Similarly, the cytochrome P-450-dependent reactions leading to the formation of mutagenic and covalently protein-bound intermediates from 2,4-DAA appear to be different (5, 6).

The results from the present study on AAF and benzo(a)pyrene hydroxylation, the metabolic activation of AAF, AF, and 2,4-DAA as well as the electrophoretic characterization of hepatic cytochrome P-450 indicate great qualitative similarities between the subcellular fractions from human liver and those from the rat, the mouse, and the rhesus monkey. It therefore seems that studies on the mechanism of carcinogenesis and mutagenesis of these aromatic amines in rodents and subhuman primates can reasonably be extrapolated to humans, although more studies with human tissues are clearly warranted.

ACKNOWLEDGMENTS

The expert technical assistance of Lise Timm Haug is highly appreciated.

REFERENCES

3. Boobis, A. R., Nebert, D. W., and Falton, J. S. Comparison of β-naphtho-


In Vitro Metabolism and Activation of Carcinogenic Aromatic Amines by Subcellular Fractions of Human Liver

Erik Dybing, Christer von Bahr, Tore Aune, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/10/4206

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/39/10/4206.
Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.