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Binding of Aromatic Amines to the Rat Hepatic Ah Receptor *In Vitro* and *In Vivo* and to the 8S and 4S Estrogen Receptor of Rat Uterus and Rat Liver

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Studies on structurally related aromatic amines with different carcinogenic properties have shown that 2-acetylaminofluorene (2-AAF) and 2-acetylaminophenanthrene (AAP) inhibit the binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the Ah receptor *in vitro*. The apparent inhibitor constants (K_i) are 2.3 μM for 2-AAF and 2.7 μM for AAP. In contrast, 4-acetylaminofluorene, an isomer of 2-AAF, and *trans*-4-acetylaminostilbene do not bind to the rat hepatic cytosolic Ah receptor. Pretreating female Wistar rats with 2-AAF or AAP leads to the induction of the P-450 isoenzymes that are under the control of the Ah receptor. Ornithine decarboxylase activity is induced by all aromatic amines tested irrespective of their Ah receptor affinity. The aromatic amines used as model compounds do not inhibit the binding of 17-β-estradiol to the 8S and 4S estrogen receptor of rat uterus or rat liver in a competition assay analyzed using sucrose density gradient centrifugation. On the other hand, the aromatic amines bind to varying extents to another estrogen-binding protein of rat liver whose function and identity is still unknown. Our study demonstrates that structurally related aromatic amines in their unmetabolized form interact differentially with a cellular target protein, the Ah receptor, *in vitro* as well as *in vivo*. However, a relationship between these effects and the postulated promoting properties of 2-AAF remains to be established.

Introduction

Aromatic amines are widely used in the production of azo dyes, drugs, pesticides, and plastics. The carcinogenic potential of these compounds was already established at the beginning of industrial dye production (*1*). The most important aromatic amines of industrial origin that are responsible for human bladder cancer are 2-naphthylamine, benzidine, and 4-aminobiphenyl. Another aromatic amine,‡ 2-acetylaminofluorene (2-AAF), which was originally intended for use as an insecticide, has gained great importance as a model compound for mechanistic studies of chemical carcinogenesis. 2-AAF is a potent carcinogen in at least eight different species (*2*). The rat is the species most susceptible to the carcinogenic effect of 2-AAF and shows a marked sex-related difference. The incidence of liver tumors is higher in males, whereas females develop mammary tumors (*3*). In contrast to 2-AAF, the isomer 4-acetylaminofluorene (4-AAF) is regarded as a noncarcinogen, or at least a significantly less potent carcinogen than 2-AAF (*4*). 4-AAF is a weak mutagen in the Ames test.

In addition to the two fluorene derivatives, we have used 2-acetylaminophenanthrene (AAP) and *trans*-4-acetylaminostilbene (AAS) as model compounds. AAP produces no tumors in the liver, but is a potent mammary carcinogen in the female rat and induces tumors in other organs in both males and females (*5*). A typical target tissue for the carcinogenic effect of AAS is the Zymbal's gland (*6*). For rat liver, AAS is not a complete carcinogen, but has strong tumor-initiating properties. The extent of DNA binding simply could not explain the different carcinogenic activity of 2-AAF and AAS, and it was proposed that these chemicals may differ in their promoting properties (*7*). 2-AAF is a well-known inducer of drug-metabolizing enzymes; the other aromatic amines have not been studied thoroughly in this respect. 2-AAF is a tumor promoter in rat liver. The mechanism of tumor promotion in general is not well understood and it seems unlikely that only one mechanism is responsible. For a number of diverse chemical tumor promoters, it is hypothesized that cellular receptors may be involved. The strong tumor-promoting effect of the environmental contaminant 2,3,7,8-tetra-

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‡According to common nomenclature, aromatic amines and aromatic amides will be called collectively "aromatic amines."

chlorodibenzo-*p*-dioxin (TCDD) seems to be mediated by the aromatic hydrocarbon (Ah) receptor (*8*). Plasma membrane receptors may play a role in the promoting effect of phorbol esters (*9*). In the case of synthetic steroid hormones, it has been suggested that the estrogen receptor participates in the process of tumor promotion (*10*).

The objective of the present study was to determine the interaction of structurally related aromatic amines with cellular receptors and to correlate these effects with the tumor-promoting properties of aromatic amines.

Methods

Analysis of Cytosolic Receptors, Animals, and Treatment

The binding of the aromatic amines to the rat hepatic cytosolic Ah receptor was measured by determining their capacity to compete with ^{3}H-TCDD as a ligand. Rat hepatic cytosol, with a protein concentration of 7 mg/mL, was incubated with 3 nM ^{3}H-TCDD and varying amounts of the aromatic amines, ranging from a 5- to a 50,000-fold excess, at 4°C for 2 hr. The Ah receptor was analyzed using sucrose density gradient (SDG) centrifugation according to Tsui and Okey (*11*). Control incubations were performed with a 500-fold excess of 2,3,7,8-tetrachlorodibenzofuran (TCDF) instead of unlabeled TCDD because of its greater solubility in water and its similar affinity for the Ah receptor.

For the analysis of the estrogen receptor (ER), cytosol was prepared in the presence of 20 mM sodium molybdate (8S ER) or in the presence of 400 mM NaCl (4S ER). Cytosol was incubated with 10 nM ^{3}H-17-β-estradiol (E_2) for 18 hr at 4°C. The nonspecific binding was determined by the addition of 10 μM unlabeled diethylstilbestrol (DES) to the complete mixture. Protein-bound radioactivity was analyzed by means of SDG centrifugation in a vertical tube rotor at 65,000 rpm for 150 min (8S ER) and for 165 min (4S ER) at 2°C using gradients from 10 to 30% sucrose (8S ER) containing 20 mM sodium molybdate or from 5 to 25% sucrose (4S ER) containing 400 mM NaCl. High performance gel filtration was carried out on Superose 12 (Pharmacia, Uppsala, Sweden). Proteins were eluted with a flow rate of 0.2 mL/min at 4°C with 20 mM sodium molybdate (8S ER) or 400 mM NaCl (4S ER) in the buffer.

For the *in vivo* studies, female Wistar rats (180–220 g) were injected IP once daily for 5 days with 2-AAF (100 μmole/kg), AAS (20 μmole/kg), or AAP (100 μmole/kg).

Results and Discussion

Affinity of Aromatic Amines to the Ah Receptor

Competition experiments with aromatic amines with the Ah receptor have demonstrated that binding of the ligand ^{3}H-TCDD is decreased by the aromatic amines 2-AAF and AAP in a concentration-dependent manner (*12*). In contrast, the 2-AAF isomer, 4-AAF, and AAS do not bind to the Ah receptor. The 50% inhibition concentrations (IC_{50}) and the apparent inhibitor constants (K_i) of the aromatic amines are given in Table 1. For comparison, the IC_{50} value and the K_i value of TCDF was determined with the same experimental protocol; K_i values were calculated as previously described (*13*). The binding constants of the aromatic amines are nearly three orders of magnitude higher than that of TCDF. This raises the question of the specificity of the assay in the presence of high competitor concentrations. The solubility of TCDD in aqueous solution is a matter of controversy (*14*). We have routinely checked the concentration of TCDD after the incubation period by measurement of radioactivity. The solubility of the radioligand remained unchanged in the presence of the high competitor concentrations. The purity of the compounds was greater than 99%, and a contamination with a potent Ah receptor ligand is unlikely.

Table 1. Binding of aromatic amines and TCDF to the rat hepatic cytosolic Ah receptor *in vitro*.

Compound	IC_{50}, M	K_i, M
2-AAF	5.0×10^{-6}	2.3×10^{-6}
4-AAF	$> 100.0 \times 10^{-6}$	NA[a]
2-AAP	6.0×10^{-6}	2.7×10^{-6}
AAS	$> 100.0 \times 10^{-6}$	NA
TCDF	1.8×10^{-8}	7.1×10^{-9}

[a] NA, no affinity.

The significance of the binding studies *in vitro* was examined *in vivo*. Rats were treated with the aromatic amines and monooxygenase activities were measured. 2-AAF and AAP increased ethoxyresorufin-*O*-deethylase (EROD) and aromatic hydrocarbon hydroxylase (AHH). Induction of EROD activity is highly correlated with the induction of P450IA1 (*15,16*). In addition, microsomal proteins were separated on SDS polyacrylamide gel electrophoresis, and a Western blot with isoenzyme-specific antibodies was performed. Treating rats with 2-AAF and AAP led to the induction of P450IA1 as well as of P450IA2 (unpublished data). Microsomes of rats treated with 3-methylcholanthrene (MC) (20 mg/kg) were used as a control. MC induces both P450IA1 and P450IA2, but preferentially P450IA1. The amines 2-AAF and AAP are much less effective than MC as inducers. The relative proportion of the P-450 isoenzymes induced is reversed by 2-AAF and AAP compared to MC, the amount of P450IA2 protein being more enhanced. Comparing the two amines, AAP is a more potent inducer than 2-AAF in female rats at the same dose. This was shown on the basis of enzyme activities as well as qualitatively by immunoblotting. Microsomes of untreated rats did not contain any detectable amount of P450IA1 or P450IA2.

Induction of Ornithine Decarboxylase Activity by Aromatic Amines in Rat Liver

The induction of ornithine decarboxylase (ODC)

Table 2. Induction of ODC activity by aromatic amines in rat liver.

Compound	Mean of ODC activity, mU/mg protein[a]	Peak time of ODC activity, hr	Confidence level by Wilcoxon test
Control	36	—	—
MC	128	12	0.025
2-AAF	142	2.5	0.025
4-AAF	135	4	0.025
AAP	186	2.5	0.025
AAS	205	9	0.01

[a] $n = 4$, control; $n = 8$, 2-AAF; $n = 5$.

seems to be associated with the *Ah* gene locus in mouse liver (*17*). In rat liver, however, a comparable association has so far not been established (*18*). In the mouse skin tumor model, the induction of ODC was proposed as a marker of tumor-promoting activity. In order to correlate the Ah receptor affinity with the ODC inducing capacity, we have measured the induction of ODC activity in rats by treatment with various aromatic amines (*19*). MC was used as a reference compound. The results are given in Table 2. All aromatic amines tested induce ODC activity *in vivo* irrespective of their Ah receptor affinity. It is concluded that the induction of ODC in the Wistar rat is not (strictly) under the control of the Ah receptor.

Binding Studies of Aromatic Amines to the 8S and 4S Estrogen Receptor of Rat Uterus and Rat Liver

The analysis of the ER of rat uterus and rat liver was performed by means of HPLC gel filtration and SDG centrifugation. In the presence of molybdate, the chromatography of ^{3}H-E_2 binding proteins of uterus cytosol yielded, with both techniques, only one peak in the separation range of proteins: the 8S ER. In the presence of high ionic strength, the chromatograms of uterus cytosol showed two peaks. The first peak in the void volume of the column or at the bottom of the sucrose gradient contained protein aggregates of high molecular weight with E_2 binding capacity. The second peak eluted after serum albumin, and we assume that this peak contains the 4S estrogen receptor. The chromatograms of the 8S and 4S ER of rat uterus obtained by HPLC gel filtration are shown in Figure 1. With rat hepatic cytosol, the elution pattern of the 4S ER is unchanged. However, the analysis of the 8S ER showed an additional peak which coeluted with the 4S ER. In contrast to the 4S ER, the binding of ^{3}H-E_2 to the latter peak is not entirely suppressed by an excess of unlabeled DES.

The competition experiments with aromatic amines and ^{3}H-E_2 binding to the 8S and 4S ER of rat uterus and rat liver were analyzed using SDG. The concentration of the amines in the assay ranged from 0.1 μM to 0.5 mM. Typical binding curves of ^{3}H-E_2 to the 4S ER of rat uterus as a function of increasing concentrations of aromatic amines are shown in Figure 2. The binding

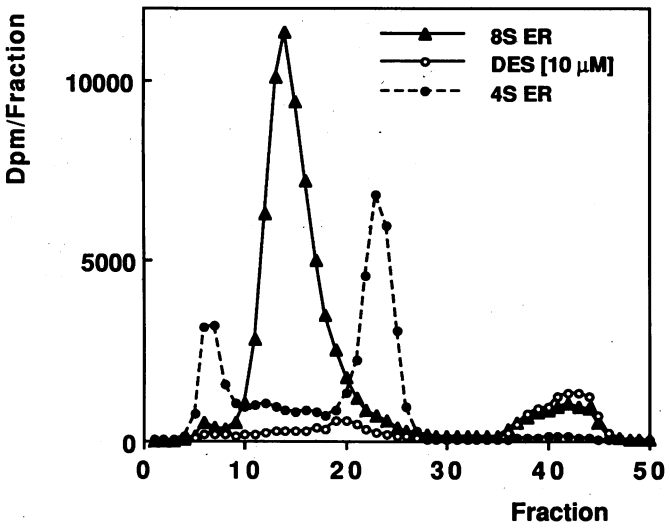

FIGURE 1. Analysis of the 8S and 4S estrogen receptor of rat uterus using HPLC gel filtration. Binding of ^{3}H-17-β-estradiol to rat uterine cytosolic proteins in the presence of 20 mM sodium molybdate (▲) (8S ER), and in the presence of a 10,000-fold excess of diethylstilbestrol (○) or in the presence of 400 mM NaCl (●) (4S ER).

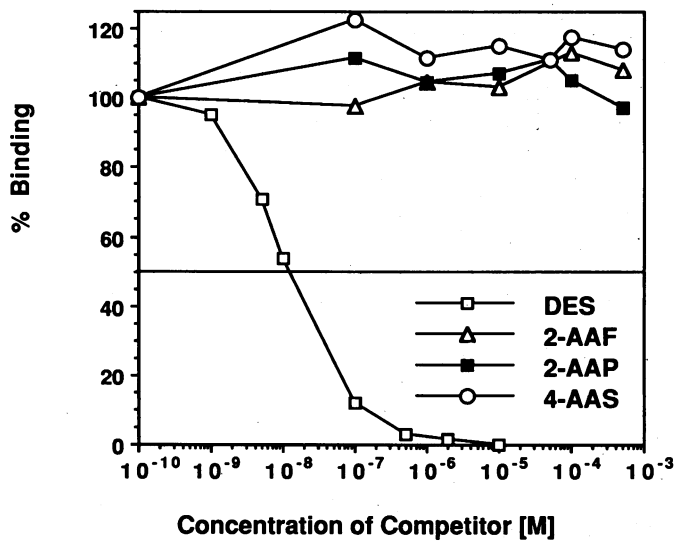

FIGURE 2. Competition experiments with aromatic amines and the rat uterine 4S estrogen receptor. Binding of ^{3}H-17-β-estradiol to the rat uterine 4S estrogen receptor in the presence of increasing concentrations of 2-acetylaminofluorene (Δ), 2-acetylaminophenanthrene (■), and *trans*-4-acetylaminostilbene (○). Diethylstilbestrol (□) was used as a control. The same results were obtained with the nonacetylated amines.

curves reveal that aromatic amines do not bind to the 4S ER of rat uterus. This also holds true for the 8S ER of rat uterus and rat liver. The competition experiments of aromatic amines with the 4S ER of rat liver demonstrate a displacement of the ligand in a concentration-dependent manner (data not shown). However, comparing the 4S ER analysis of rat uterus and rat liver, it is obvious that DES is less effective as a competitor

in the case of the hepatic receptor. We assume that another estrogen-binding protein of rat liver coelutes with the 4S ER and that DES has no or only a low affinity to this protein. The binding studies with the 4S ER of rat uterus clearly show no displacement of E_2 by aromatic amines. Therefore, we conclude that the aromatic amines bind to the coeluting estrogen-binding protein.

The competition experiments *in vitro* did not show a direct interaction of the aromatic amines with the steroid binding domain of the estrogen receptor. However, we cannot exclude the possibility that aromatic amines modulate the estrogen receptor concentration *in vivo* or that aromatic amines affect the binding of the estrogen receptor to DNA. In summary, we have shown that structurally related aromatic amines do interact differentially with a specific cellular target protein: the cytosolic Ah receptor.

Specific binding to key cellular receptors determines at least in part the biological activity of numerous xenobiotics. On the other hand, the development of cancer as a multistep process involves a series of cellular events that are not yet fully understood even in the case of tumor initiation. Because of the multiple factors, a direct correlation between the carcinogenic activity of a compound and a single cellular event is not to be expected. In the case of the Ah receptor, it has been suggested that the receptor is involved in the growth control of the cell and participates in the process of tumor promotion (*8*). However, the experimental basis for this hypothesis is frail. The role of the Ah receptor in the induction of drug-metabolizing enzymes is well known, especially in the case of P450IA1 (*20*). It has been noted that the induction of drug-metabolizing enzymes by many enzyme inducers leads to an increase in their own metabolism. 2-AAF is an example of this type of inducer. From this point of view, binding to the Ah receptor *in vivo* should be related to the process of tumor initiation rather than promotion if more or less reactive intermediates were produced as a result of the induction process. On the other hand, enzyme induction is a common property of many tumor promoters. The correlation between Ah receptor affinity and the promoting activity of the model compounds in rat liver, however, is also not perfect. 2-AAF is a complete carcinogen for rat liver, and promoting properties have been deduced from many experiments. AAP has been less thoroughly studied, and promoting activity in liver has not yet been demonstrated but cannot be excluded because high doses have not been tested.

We are now focusing our interests on receptors that are involved in the cellular signal transduction and in the growth control of the cell: the growth factor receptors of the plasma membrane. Even if the aromatic amines do not bind to these receptors, they could change receptor levels or their binding constants, thereby interfering with signal transduction.

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