The Role of Nongenotoxic Mechanisms in Arylamine Carcinogenesis

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The growth of preneoplastic nodules during the feeding of a carcinogenic 2-acetylaminofluorene (2-AAF) regimen is preceded by several alterations in the physiologic homeostasis. Many of these alterations can be considered adaptive responses to the drug exposure. One property of AAF could be identified that clearly distinguishes this complete rat liver carcinogen from at least two other, incomplete rat liver carcinogens. Highly specific redox cycling in mitochondria was demonstrated in vitro, and this observation could well contribute an explanation of the morphologic and histochmical observations in vivo. It is emphasized that nongenotoxic effects may play an important role in the generation of tumors by genotoxic carcinogens. — Environ Health Perspect 102(Suppl 6):173-176 (1994)

Key words: 2-acetylaminofluorene, 2-acetylamino phenanthrene, 2-acetylaminostilbene, chronic toxicity, initiation-promotion, oxidative stress, redox cycling

Introduction

Carcinogenic aromatic amines usually are mutagenic, and their genotoxic properties are made responsible for the biological effects. These effects are explained by the formation of reactive metabolites that react with DNA. Results from comparative studies, however, indicate that tissue-specific and species-specific tumor formation cannot be explained readily by the extent of DNA modifications. Within the initiation-promotion model of rat liver carcinogenesis, for instance, it seems that initiation — associated with DNA lesions — is necessary, but not sufficient to result in tumor formation. This raises the question of whether so-called complete rat liver carcinogens produce highly specific genotoxic effects, or whether they produce, in addition to the genotoxic effects, nongenotoxic effects because of properties that incomplete carcinogens do not have.

We have compared the properties of three model aromatic amines with regard to different end points. The amines are 2-acetylaminofluorene (2-AAF), 2-acetylamino phenanthrene (AAP), and trans-4-acetylaminostilbene (AAS). The end points in rat liver are a) tumor formation, b) tumor initiation, c) acute toxicity, d) chronic toxicity, and e) the generation of oxidative stress in mitochondria (1-3).

It is well known that feeding of 2-AAF produces liver tumors in the rat (4). Since this happens without any further treatment, 2-AAF is called a complete rat liver carcinogen. AAP and AAS do not produce liver tumors under comparable circumstances. The typical target tissue for AAP is the mammary gland (5), and for AAS the sebaceous glands, particularly Zymbal’s gland in the outer ear duct (6,7). Although they are complete carcinogens considering the whole animal, rat liver is not a target tissue. Since it was shown that in the liver both AAP and AAS yield reactive metabolites that bind to DNA, that the adducts are promutagenic lesions, and that liver tumors can be produced if an initiating treatment with these amines is followed by a promoting treatment in an initiation-promotion experiment, AAP and AAS may be called incomplete carcinogens and initiators for this tissue (2,8).

What are the properties of 2-AAF that make it a complete carcinogen? 2-AAF has been used as a promoter in initiation-promotion experiments, and the promoting properties have been ascribed to its cytotoxicity. The cytotoxicity, however, has not been really well defined. When analyzing some classical parameters of acute toxicity in the isolated perfused rat liver, neither of the three aromatic amines appears to be acutely cytotoxic, at least under circumstances relevant to liver tumor formation (2). 2-AAF, however, produces chronic toxicity, and we have detected one property by which it can be clearly distinguished from the other two aromatic amines: the generation of oxidative stress in mitochondria. This oxidative stress can be correlated with morphologic alterations seen in the livers of animals that were fed a carcinogenic dose of 2-AAF, and may well be instrumental in the generation of liver tumors.

Aromatic Amines as Initiators

In an initiation-promotion experiment, 2-AAF, AAP, and AAS in a total dose of 1.5, 1.5, and 0.15 mmoles/kg bw, respectively, were orally administered to newborn Wistar rats at 5, 7, 9, and 11 days postnataally. Promotion was started after weaning at day 35 by adding phenobarbital (500 ppm) to the drinking water. Each group of carcinogen-initiated animals exhibited a high incidence of liver tumors ranging from 50 to 100% in males, and 100% in females after 104 weeks. Considering the lower dose of AAS, the potency of initiators decreased in the order AAS, AAP, 2-AAF. All three of the chemicals clearly initiated liver tumors in this model system. 2-AAF, which was the only complete carcinogen in adult rats, was the least potent initiator in this tissue (9).

The purpose of this experiment was to obtain tumor material in order to study protooncogene mutation or activation, and to compare the lesions between the treatment groups in order possibly to demonstrate a specific lesion produced by 2-AAF. But so far we have not been successful. Of 38 tumors, 3 to 6 from animals in each group, including control livers, we found no mutations in the H-ras gene, neither in the critical codons 12, 13, and 61, nor in other sites. The latter statement is based on sequencing in 12 of the tumors, the first three exons and most of the introns, including intron D with the alternative

This paper was presented at the Fifth International Conference on Carcinogenic and Mutagenic N-Substituted Aromatic Compounds held 18–21 October 1992 in Würzburg, Germany.

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 172.

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splicing sites. This result is remarkable since 2-AAF has been shown to produce C to A transversions in codon 61 of the H-ras gene in the liver of B6C3F1 mice (10). Mutations in this codon have been also demonstrated with other bulky carcinogens like 9,10-dimethylbenzanthracene (DMBA) (11). Moreover, the H-ras gene was not overexpressed. Our results indicate that the H-ras gene is not involved in a number of rat liver tumors produced by three different initiators. At present, no 2-AAF-specific DNA lesion has been identified that could explain the tissue-specific effects.

**Acute Toxicity of 2-Acetylaminofluorene**

When 2-AAF is added to the feed of male Wistar rats at a concentration of 0.02%, a commonly used regimen to produce tumors with this chemical, the increase in body weight is markedly retarded in comparison to controls, which indicates acute toxicity (Figure 1). This is underlined by the observation that body weight increase returns to normal when the chemical is removed from the feed.

If acute toxicity is caused by immediate effects on the biochemistry of cells resulting, for instance, in cell death, one should be able to demonstrate this under controlled exposure conditions in isolated perfused livers or in rats with a bile fistula. The latter situation was studied particularly in order to demonstrate increased excretion of oxidized glutathione (GSSG) as an indication for oxidative stress. Although glutathione (GSH) levels in liver decreased with doses up to 1 mmole/kg, which is by far more than the 0.045 mmole/kg corresponding to 0.02% in the feed, GSSG was not increased in bile. There were also no changes in the ratio of lactate/pyruvate, an indicator of oxidative stress, or in thiobarbiturate reactive sites, indicator of lipid peroxidation, or in leakage of enzymes like glutamate-pyruvate-transaminase (GPT) into serum, indicator of cell necroses (12,13).

Thus, 2-AAF toxicity cannot be detected readily within a matter of hours with single administrations, and must develop more slowly or in a more subtle way. In experiments recently completed, Muster and Cikryt (14) administered 0.02% 2-AAF in the diet to male Wistar rats for up to 3 weeks and looked for non-genotoxic alterations. They observed significant changes in enzyme activities. Among the enzymes of the endogenous metabolism, glucose-6-phosphatase (G-6-P) was decreased, glucose-6-phosphate dehydrogenase (G-6-PDH) was increased, and phosphoenolpyruvate carboxykinase was decreased, changes which indicate an inhibition of gluconeogenesis. Therefore, enzyme activities controlling glucose homeostasis are apparently not only altered in preneoplastic foci, but seem to be subject to an adaptive response of the whole liver. Enzymes involved in drug metabolism were also affected. In this study (14), the increase of cytochromes P450, CYP1A1, CYP1A2, CYP1B1, and CYP1B2 was measured, confirming that 2-AAF is a mixed-type inducer, increasing both the methylcholanthrene and the phenobarbital-inducible cytochromes. In line with this response is the observed affinity of 2-AAF to the Ah receptor (15). Moreover, a number of other effects became evident that indicate that 2-AAF interacts with receptor-mediated processes. The concentration of insulin and glucocorticoid receptors decreased, the binding of epidermal growth factor (EGF) to its receptor decreased, and the binding of phorbol esters to phosphokinase C decreased. The effect of 2-AAF on EGF binding to its receptor is clearly different from that of the isomeric, non- or at least far less carcinogenic, 4-AAF. This may indicate a role for the EGF receptor in the carcinogenic process (14).

The activity of phase II enzymes was measured in more detail in a study in which 0.02% 2-AAF was fed for up to 84 days to male Wistar rats. UDP-glucuronyltransferase (UDP-GT, 1) and glutathione S-transferase (GST, tested with CDNB = p-chlorodinitrobenzene as a substrate) increased, whereas aryl sulfotransferase IV (AST-IV, tested with N-OH-AAF as the substrate) decreased significantly over the entire feeding period (Figure 2). These changes would be consistent with an improved detoxification and enhanced excretion of 2-AAF metabolites in the induced state, and they seem not to be restricted to enzyme-altered foci as it has been described above.

**Oxidative Stress Produced by 2-AAF Metabolites**

During the experiments with isolated perfused livers, it was observed that livers from 2-AAF treated animals consumed more oxygen than those from untreated animals (3,13). This led us to examine mitochondria as a possible target for 2-AAF toxicity.

![Figure 1](image1.png)

**Figure 1.** Body weight increase of male Wistar rats that were fed 0.02% AAF in the diet either continuously or for 42 days (n = 4) (13). △, controls; ●, continuous AAF; *, 42 days AAF.

![Figure 2](image2.png)

**Figure 2.** Relative activities of phase II enzymes in livers from male Wistar rats that were fed 0.02% AAF in the diet for the time indicated (13).

![Figure 3](image3.png)

**Figure 3.** Schematic presentation of the electron flow in the respiratory chain in mitochondria in the presence of AAF metabolites (see text for details).
In isolated rat liver mitochondria, the oxygen consumption can be blocked by cyanide (Figure 3). If 2-nitrosofluorene (NO-F) or N-hydroxy-2-aminofluorene (N-OH-AF) is added to such a preparation, the consumption of oxygen starts immediately, and this is a dose-dependent process. A comparable cyanide-insensitive, state 4 oxygen consumption can be produced also with N-hydroxy-2-aminophenanthrene (N-OH-AP), but not with any one of the other N-hydroxy-metabolites tested. All of them are able to affect liver mitochondria as can be seen from calcium (Ca^{2+}) release, a typical indicator of mitochondrial toxicity. However, only 2-N-OH-AF (or NO-F) is able to increase the level of oxidized glutathione in mitochondria, which can be taken as a measure of oxidative stress. (For comparison with the treatment of mitochondria with tertiary butylhydroperoxide see Figure 7).

This observation can be related to the in vivo situation. Although cytosolic glutathione equivalents are higher than in controls—and as a consequence GSSG release in response to challenging menadione administrations is significantly lower than in controls—glutathione equivalents decrease constantly in liver mitochondria during 2-AAF feeding (Figure 4).

From the above observations, together with a number of supporting findings, the following hypothesis was developed: The nitroso group of NO-F accepts electrons from the respiratory chain via cytochrome c (Figure 3). They are used to establish a redox cycle in the inner mitochondrial membrane between either NO-F and N-OH-AF, or between NO-F and the nitroxy radical, which results in the formation of superoxide anion radicals (Figure 5). Their inactivation produces oxidized glutathione and consequently a decrease of the NADPH/NADP ratio, which then leads to Ca^{2+} release and a breakdown of the mitochondrial membrane potential. A number of other reactions may contribute to the stress situation.

The quantitative difference between 2-AAF metabolites to induce Ca^{2+} release from liver mitochondria is impressive. One nmole N-OH-AF/mg mitochondrial protein is sufficient to produce a significant effect. Fifty times higher concentrations are required of N-OH-AAF or N-acetoxy-AAF for comparable activity (Figure 6). If such a comparison is made between N-OH-AAF and hydroxylamines of other aromatic amines, the high potency of N-OH-AAF becomes also apparent (Figure 7).

Role of Chronic Toxicity in the Development of Tumors
How can oxidative stress in mitochondria be brought together with the in vivo observations? When 2-AAF is fed to male Wistar rats, enzyme-altered foci and preneoplastic

Figure 4. Alteration of glutathione equivalents in cytosol and mitochondria in livers from male Wistar rats that were fed 0.02% AAF in the diet, expressed in percent of controls (n = 4)(13).

Figure 5. Schematic presentation of redox cycling of AAF metabolites in mitochondria (see text for details).

Figure 6. The potency of various AAF metabolites to induce calcium release from isolated rat liver mitochondria (13).

Figure 7. Potency of metabolites from various arylamines to induce calcium release from isolated rat liver mitochondria (13). (Bi, biphenyl; Be, benzene, 4-CA, 4-chloroaniline, fBuOOH, tertiary butylhydroperoxide).
nODULES are usually viewed as the first indication of tumor development. Individual GST-P positive cells (GST-P = placental form of glutathione S-transferase) can be seen rather soon after the onset of treatment, yet foci start to grow only after 7 to 8 weeks of feeding (Figure 8). Necroses and, as a consequence, increased cell division can be seen only at that time, with a maximum at 8 to 12 weeks. But the alterations begin much earlier. After a few days of feeding 2-AAF, oval cells develop to proliferate, starting from the perportal area, which is the oxygen-rich area. The hepatocytes in this area are particularly rich in mitochondria, and have the highest oxygen turnover. These are the first cells thought to encounter oxidative stress in mitochondria and presumably to suffer first from it. Within the first 2 or 3 weeks, the number of bile ductlike cells that stain positive for γ-glutamyl transpeptidase, and presumably replace stressed hepatocytes, increases slowly, thereafter more rapidly. Interestingly, the appearance of these cells is still restricted to the oxygen-rich areas. In the neighborhood of these cells, the production of reticulin fibers can be demonstrated, which begin to encapsulate the liver lobule. Only when these netlike structures have reached advanced stages, the enveloped hepatocytes seem to be suppressed and begin to die, presumably as a result of a disturbance of the microcirculation. At this time (i.e., after 8 to 12 weeks of feeding) necroses can be seen, starting from the area around the central vein. Concurrently, regeneration of hepatocytes sets in, leading to a highly nodular cirrhoselike liver. This can be demonstrated best by counting dividing cell nuclei by means of the proliferation markers S3 or Ki 67 (Figure 9) (16). At early time points, cells thus marked are evenly distributed, whereas at the time of exponential proliferation, marked cells are concentrated in the centrilobular area.

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