Of course, it is also possible that saturated fatty acids inherently and directly promote mammary tumorigenesis by acting on the mammary epithelium. We know that saturated fatty acids inhibit the growth of both normal and neoplastic cells, but this inhibition is a little more dramatic in the case of normal cells (10). This difference may be significant, however, because the presence of normal mammary epithelial cells has been shown to suppress the conversion of preneoplastic to neoplastic mammary cells following transplantation into mammary fat pads (22). A differential inhibition of normal cells thus could favor tumor development. Clearly we are a long way from understanding how dietary lipids might influence breast cancer susceptibility but hopefully our observations and hypotheses will shed some light on the issue, or at least suggest new experimental approaches. Other exciting new ideas already are being brought forth on this subject. For example, Castenaga (23) recently has reported that tumor promoters bind to and activate a calcium and lipid dependent kinase, C kinase. The significance of this observation may be great because C kinase is markedly activated by unsaturated diacylglycerols (24) and thus these compounds may be natural promoters that act as transmembrane signals generated when growth factors interact with membrane receptors and activate phospholipase A_2.

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Lipid Peroxide Catalyzed Chemical Carcinogenesis

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ABSTRACT

Peroxides, including lipid peroxides, with heme catalysts cause the binding of C^{14}-acetylaminofluorene to DNA if microsomes are present. This binding was 96% inhibited by paraoxon, a deacetylase inhibitor. It is concluded that peroxide-peroxidase systems rapidly oxidize acetylated arylamines to proximate carcinogens following deacetylation by microsomal deacetylases. The DNA binding observed was greater than that observed with the liver microsomal mixed function oxidase catalyzed activation to N-OH-acetylaminofluorene, which binds to DNA following deacetylation by microsomal deacetylase. Lipid peroxidation or prostaglandin synthesis should therefore enhance carcinogenesis induced by arylamides.

INTRODUCTION

Multiple mechanisms for metabolic activation of chemical carcinogens exist. The activation by different target tissues also may reflect the different activating systems present. It is thought the initial activation usually involves a mixed function oxidase activity of the endoplasmic reticulum, and that this activation involves a two-electron oxidation of the polycyclic aromatic hydrocarbon to an epoxide or of an arylamine to an N-hydroxyarylamine. However, recently an initial one-electron oxidation to free radicals catalyzed by peroxidases or prostaglandin synthetase has been suggested as a first step for the activation of chemical carcinogens (1-4). Most tissues contain all three systems. However, the uterus, thyroid, salivary gland, Zymbal gland or Harderian glands are target tissues, with little cytochrome P450 and highly active peroxidases (5-8). The kidney medulla and bladder are target tissues with active prostaglandin synthetase (2). The liver hepatocyte, on the other hand, has very high levels of the mixed function oxidase activity of cytochrome P450 and little peroxidase or prostaglandin synthetase activity. The liver Kupffer cells contain a peroxidase (9). Even the apparent two-electron oxidation mechanism of mixed function oxidase function may still involve free radicals (10). It is well established that carcinogenesis induced by irradiation or ultraviolet light is free radical mediated.

Dietary fatty acid hydroperoxides can be toxic to the gastrointestinal tract and can be carcinogenic (11). Enhanced in vivo lipid peroxidation is associated with carcinogenesis induced by chlorinated hydrocarbons (12), hydrazines (13) and metals (14). Furthermore, enhanced in vivo lipid peroxidation following choline deficiency is associated with carcinogenesis (Farber, E., personal communication).

The induction of peroxisomes by hypolipidemic drugs also results in in vivo lipid peroxidation and carcinogenesis (15) although the drugs are not activated to mutagens or products which bind to DNA (16). Selenium and most antioxidants protect against chemical carcinogenesis, whereas selenium deficiency potentiates chemical carcinogenesis (17).

It has been known for some time that carcinogenic arylamines, hydrazines and polycyclic aromatic hydrocarbons are effective as antioxidants and free radical scavengers in lipid autoxidation (4). Indeed, some of them were used as such before their carcinogenic properties were discovered. This indicates that these carcinogens are readily oxidized during lipid peroxidation. Our research indicates that this oxidation can result in the carcinogen binding covalently to DNA and thus may be a carcinogenic activation mechanism (3). Lipid peroxides may therefore be able to act as a cocarcinogen or tumor promoter.

Bartsch and Hecker first demonstrated that nitroxyl free radicals were formed when N-OH-acetylaminofluorene (N-OH-AAF) was oxidized by peroxidases and H_2O_2 (18), and that they dismutate to N-acetoxy-AAF and 2-nitrosofluorene. The former readily binds covalently to DNA, tRNA and guanosine (19), whereas nitrosofluorene is one of the most mutagenic compounds known (20) and binds covalently to proteins. Floyd et al. also showed that nitroxyl free radicals were formed when N-OH-AAF was oxidized by linoleic acid hydroperoxide (LAHPO) with methemoglobin (21) or cytochrome P450 and particularly high spin cytochrome P420 (22).

Acetylaminofluorene is not oxidized by peroxidase-H_2O_2 or cytochrome P450-LAHPO systems so that the mixed function oxidase is still required for N-OH-AAF formation. Floyd (19) has suggested recently that mammary gland carcinogenesis induced by AAF may involve a peroxidase or prostaglandin synthetase catalyzed activation of N-OH-AAF.

No studies comparing different activating systems for DNA have been reported. In the following, the irreversible binding of C^{14}-AAF and C^{14}-NOH-AAF to DNA catalyzed by mixed function oxidase or peroxidase-H_2O_2 or methemoglobin-LAHPO systems are compared. It is concluded that a microsomal deacetylase is involved in AAF and NOH-AAF binding. The free radical mediated peroxidase catalyzed oxidation of N-OH-AAF results in much less binding than that with deacetylase/AAF catalyzed by the same oxidizing system. This indicates that a free radical mechanism for AAF activation may not involve an initial N-hydroxylation catalyzed by mixed function oxidase. Furthermore this mechanism, presented for the first time, shows that acetylarylamines can be activated by a free radical mechanism. It is more effective than mixed function oxidase which is normally considered the principal activation mechanism.

EXPERIMENTAL PROCEDURES

Chemicals. The following reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri); calf thymus DNA (type I), Horseradish peroxidase (type VI), porcine carboxy esterase (type I and II) and diethyl-p-nitrophenyl phosphate (paraoxon). Hydrogen peroxide was obtained from British Drug Houses Chemicals (Toronto, Canada). N-(9-^{14}C)-Acetyl-2-aminofluorene (AAF), (specific activity 50 mCi/mmol) was purchased from New England Nuclear (Boston, Massachusetts). (9-^{14}C)-N-hydroxy-acetylaminofluorene (NOH-AAF), (specific activity 25 mCi/mmol) was purchased from ICN Chemical and Radioisotopes (Irvine, California).

DNA binding. The reaction mixture (3.0 ml) for determining peroxidase-H_2O_2 catalyzed N-OH-AAF and AAF binding to DNA contained 5 μM N-(C^{14})-acetylaminofluorene or N-OH-acetylaminofluorene, 0.1 M Tris-HCl (pH 7.4), horseradish peroxidase (10 μg), hydrogen peroxide (0.5 mM) and calf thymus DNA (3 mg). Carboxyesterase II (0.1 mg) or 1 mM diethyl-p-nitrophenyl phosphate was added where indicated. The reaction was started by the addition of hydrogen peroxide and carried out for 30 min at 37 C with shaking. The reaction was stopped by extraction with 2.0 ml ethyl acetate-acetone (2:1), and the organic solvent was removed. The extraction was repeated 3 times. The residual organic solvent in the aqueous layer was then removed by bubbling with nitrogen. Following the removal of the residual organic solvent in the aqueous layer by bubbling with nitrogen gas, sodium dodecyl sulfate solution (10%, 200 μl) and protease (0.5 mg) were added and the mixture allowed to incubate at 37 C for 30 min. After digestion of any possible contaminating protein, the mixture was treated with water-saturated phenol (1 ml) and water-saturated $CHCl_3$ (1 ml) and the mixture was shaken vigorously. After centrifugation, the aqueous phase was transferred to a new test tube. The macromolecules were subsequently precipitated by the addition of NaCl (5 M, 100 μl) and ethanol (6 ml). After centrifugation, the supernatant was discarded. The macromolecules were dissolved in water (1 ml), reprecipitated with NaCl (5 M, 100 μl) and ethanol (2.5 ml), washed with ethanol (1 ml) and ether (1 ml) and dried under nitrogen. The isolated macromolecules were dissolved in water (1.0 ml). An aliquot was used for the determination of macromolecule concentration by UV absorption, and the rest was used for the measurement of the visible absorbance spectra and radioactivity of bound AAF and N-OH-AAF. The radioactivity was measured with a Beckman LS-330 scintillation counter.

Microsomal catalyzed binding. Rat liver microsomes were prepared from 200-250 g, overnight-fasted Sprague-Dawley derived albino rats. A microsomal protein concentration of approximately 1 mg/ml in Tris-HCl buffer pH 7.4 was used in the reaction mixture.

RESULTS

Bartsch and Hecker (18) showed that adducts of tRNA, guanosine and N-acetylmethionine were formed in a peroxidase-H_2O_2-N-OH-acetylaminofluorene system. The adducts were similar to that formed with N-acetoxy-2-acetylaminofluorene, a product of this system. In Table I, it can be seen that such a system also results in DNA binding. About 14% of the radioactive N-OH-acetylaminofluorene was trapped by denatured DNA, and a binding level of 158 p moles N-OH-acetylaminofluorene per mg DNA was found. Double stranded DNA had a binding level of only about one-third of that of the single stranded DNA (thermally denatured DNA). At pH 7.4 the product responsible for the binding was stable as similar binding was observed when DNA was added 2 minutes after starting the reaction. N-acetoxy-2-acetylaminofluorene is believed to be formed by the dismutation of the nitroxy radicals observed following the oxidation of N-OH-acetylaminofluorene. A yield of 17-19% N-acetoxy-2-acetylaminofluorene was observed (18). About 3% of the N-OH-AAF was trapped by tRNA (18) and 8% was trapped by N-acetyl-DL-methionine (19).

In Table I, it can be seen that methemoglobin could catalyze similar adduct formation with H_2O_2, cumene hydroperoxide or linoleic acid hydroperoxide. Floyd et al. (21) have shown that such systems form nitroxy radicals and nitrosofluorene, the other dismutation product (20).

TABLE I

NOH-Acetylaminofluorene Binding to DNA

Peroxide system	DNA binding (p mol/mg DNA) None	+ Paraoxon
None	0.6	0.6
H_2O_2 + HRP	145	140
H_2O_2 + mHb	52	48
CHP + mHb	108	99
LAHPO + mHb	6	6
Microsomes	122	5
NADPH + Microsomes	153	5
CHP + Microsomes	22	4
Esterase	247	6

The reaction mixture (3 ml) contained 3 mg single stranded DNA, 5 μM C^{14}-acetylaminofluorene, 0.1 M Tris-HCl buffer (pH 7.4), 0.5 mM H_2O_2 or cumene hydroperoxide (CHP) or 0.12 mM linoleic acid hydroperoxide (LAHPO) or 0.5 mM NADPH; 10 μg horseradish peroxidase (type VI) (HRP) or 5 μM methemoglobin (mHb) or 1 mg rat liver microsomes. The mixture was incubated for 1 hour at 37 C. Carboxyesterase (0.1 mg) or 1 mM diethyl-p-nitrophenol phosphate (paraoxon) was added where indicated. DNA was isolated as described in "methods."

They were unable to detect N-acetoxy-AAF with cumene hydroperoxide. The major product found, nitrofluorene, may be formed by the further oxidation of nitrosofluorene (34). N-acetoxy-AAF was found with a hematin-linoleic acid hydroperoxide system (22). However, as shown in Table I, cumene hydroperoxide was found to be more effective than linoleic acid hydroperoxide in DNA adduct formation. A comparison with microsomal mixed function oxidase system showed a similar level of DNA adduct formation but, surprisingly, microsomes were effective in the absence of NADPH. The latter binding was inhibited 96% by paraoxon, a microsomal deacetylase inhibitor. No inhibition of the peroxidase systems by paraoxon was found. It is concluded that DNA adduct formation catalyzed by microsomes involves NOH-aminofluorene formed by deacetylation.

C^{14}-Acetylaminofluorene was not bound by peroxidase systems to DNA, and the acetylaminofluorene (as measured by HPLC) was unmetabolized. The acetyl group presumably prevents the one electron oxidation of the amine group. However, the addition of liver microsomes or carboxyesterase (type II) to the peroxidase systems resulted in extensive binding to DNA. Adduct formation with H_2O_2, cumene hydroperoxide or linoleic acid hydroperoxide catalyzed by methemoglobin was also observed in the presence of carboxyesterase or liver microsomes. Up to 9% of the C^{14}-acetylaminofluorene was trapped by DNA to a level of 154 pmol AAF/mg DNA in a peroxidase system. This corresponds to a level of one AAF bound/20,000 nucleotides. The mixed function oxidase system (microsomes/NADPH) was much less effective and was prevented by paraoxon. Liver microsomes from 3-methylcholanthrene injected rats were much more effective. Other investigators have shown a large induction of N hydroxylation of AAF in such microsomes (24). The cytochrome P450 inhibitors, SKF-525A (0.2 mM) or 2-(2,4 dichloro-6-phenyl)-phenoxy-ethylamine (0.5 mM), inhibited the binding. Added carboxyesterase increased the microsomal activity by only a small degree, so it is clear that the microsomal mixed function oxidase mechanism involves an N-hydroxylation followed by deacetylation.

TABLE II

Acetylaminofluorene Binding to DNA

Peroxide system	DNA binding (p mol/mg DNA) None	+ Paraoxon	+ Esterase
H_2O_2 + HRP	0.2	0.2	154.1
H_2O_2 + mHb	0.2	0.2	6.2
CHP + mHb	0.2	0.2	57.1
LAHPO + mHb	0.2	0.2	7.5
H_2O_2 + HRP + Microsomes	107.5	0.9	143.2
H_2O_2 + Microsomes	0.2	0.2	0.2
CHP + Microsomes	3.7	1.2	7.5
NADPH + Microsomes	6.5	1.4	8.9
NADPH + 3MC-Microsomes	5.1	0.4	6.4

Reaction conditions as described in the legend to Table I.

DISCUSSION

N-hydroxylation of AAF by a cytochrome P448 dependent monoxygenase followed by activation by cytosolic sulfotransferases or seryltransferase is believed to form the AAF-DNA adducts that are formed in vivo (25). However, 80% of the adducts formed in vivo are deacetylated and activation by a microsomal-N,O-acetyltransferase has been implicated (25). The experiments reported above suggest that a microsomal deacetylase catalyzes the activation.

As the deacetylase inhibitors paraoxon and toluenesulfonyl-fluoride completely inhibited microsomal mixed function oxidase catalyzed DNA binding by acetylaminofluorene or N-OH-acetylaminofluorene suggests that microsomal deacetylase activates N-OH-AAF and that N-OH-aminofluorene binds to DNA. A nonenzymatic reaction of N-OH-aminofluorene with nuclear DNA was also the explanation given for the DNA binding following the incubation of aminofluorene with rat liver nuclei in the presence of a NADPH generating system (28). The N-OH-aminofluorene formed probably reacts via the nitrenium ion with nucleophilic sites on nucleic acids and proteins at an acidic pH (28). Nuclei also have a paraoxon sensitive deacetylase (29). N-OH-AAF can be converted readily to N-OH-AF by microsomal deacetylases (26,27). Deacetylase inhibitors also decrease the covalent binding of N-OH-AAF to microsomal protein (29,30), its mutagenic activation (32) and its binding to nuclear DNA (31). The O-glucuronide detoxification product of N-OH-AAF is also activated by a microsomal deacetylase to form tRNA adducts (33).

In the absence of a microsomal deacetylase, N-OH-AAF could be activated to DNA reacting species by a free radical mechanism involving H_2O_2-peroxidase or lipid peroxide-methemoglobin systems (Table II). However, in the presence of microsomes the deacetylase mechanism predominated as shown by the inhibition by paraoxon. The activation by a prostaglandin synthetase-arachidonate system using sheep vesicular gland microsomes also was found to be inhibited by paraoxon.

The liver microsomal catalyzed activation of N-OH-AAF was unaffected by NADPH. Cumene hydroperoxide can catalyze cytochrome P450 function when substituted for NADPH (33). However, the liver microsomal catalyzed activation of N-OH-AAF was decreased 90% by cumene hydroperoxide, which may indicate that a 1e oxidation to nitroxy radicals does not occur but rather a competing 2e oxidation to nitrosofluorene occurs followed by further oxidation to nitrofluorene (34). Electron spin resonance studies also indicate no nitroxy radical formation (Nagata, C., personal communication).

By contrast, AAF was poorly oxidized by the above free

radical systems but was readily activated to DNA reacting species in the presence of microsomes or carboxyesterase. The activation was prevented by paraoxon. Interestingly, the free radical systems were much more effective than the mixed function oxidase-cytochrome P450 activity. Although microsomal deacetylases deacetylate AAF more slowly than NOH-AAF (27), they are clearly active enough to participate in the activation of AAF. It is not known which of the aminofluorene oxidation products bind to DNA. Recently, evidence has been presented showing that aminofluorene is oxidized by peroxidase-H_2O_2 or prostaglandin synthetase-arachidonate systems to azofluorene and 2-nitrofluorene (39). The above findings have important implications for arylamine carcinogenesis. The action of deacetylases in vivo on acetylated arylamines will result in the formation of excellent substrates for free radical systems. The latter systems include peroxidases and prostaglandin synthetase and are particularly active in nonhepatic tissues, e.g., bladder, mammary gland, Zymbal gland and Harderian gland where mixed function oxidase is very low.

An alternative mechanism for the association of lipid peroxidation and carcinogenesis could be the result of dialdehyde and aldehyde products formed following the decomposition of lipid peroxides. Our previous research demonstrates extensive irreversible binding of C^{14}-arachidonate to DNA following peroxidation catalyzed by lipoxygenase, prostaglandin synthetase or microsomal fractions from various tissues (35). Others have also demonstrated fluorescent Schiff base formation with the nucleic acid bases when malondialdehyde binds to DNA (35). However, malondialdehyde is much less mutagenic than formaldehyde or glutaraldehyde or glyoxal (36). Recently formaldehyde has been shown to be a carcinogen (37). However, the mutagenicity and carcinogenicity of the various aldehydes and dialdehydes formed during lipid peroxide decomposition is unknown. The lipid peroxides may also prove to be highly mutagenic, as cumene hydroperoxide and t-butyl hydroperoxide are more mutagenic than the above aldehydes (37).

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