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Rat Liver Subcellular Fractions Catalyze Aerobic Binding of 1-Nitro[^{14}C]pyrene to DNA

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The recently characterized environmental mutagen and potential carcinogen 1-nitropyrene (NP) is known to bind DNA in *Salmonella typhimurium*, and also in anaerobic incubations catalyzed by purified xanthine oxidase. In this study we show that rat liver S9 supernatant, microsomal and cytosolic subcellular fractions are also able to catalyze the binding of 1-nitropyrene labeled with ^{14}C to calf thymus DNA *in vitro*. In incubations conducted under air, S9 and microsomes from Charles River CD rats were the most active fractions, and NADPH was required for maximum activity (25–100 pmole NP bound/mg DNA/mg protein in 1 hr). S9 and microsomes had about one-fourth the activity under nitrogen, although less of this activity was NADPH-dependent. Binding in cytosolic incubations was generally low (1 to 5 pmole NP/mg DNA/mg protein in 1 hr), was somewhat enhanced under N_2, and was more extensive in the absence of NADPH. Treatment of rats (Harlan Sprague-Dawley) with the inducing agents phenobarbital (PB), Aroclor 1254 (A), or 3-methylcholanthrene (3-MC) enhanced NADPH-dependent binding in aerobic S9 (2- to 5-fold) and microsomal (10- to 20-fold) incubations. The effects of induction regimen on binding assays conducted under N_2 were more equivocal: 3-MC produced a 2-fold increase in binding in both S9 and microsomes, while the other two agents decreased binding from 50 to 75%. These results indicate that classic cytochrome P-450 inducers were able to stimulate activation of NP, but that this activation is not mediated solely by cytochrome P-450.

Introduction

1-Nitropyrene (NP), found in photocopier toners (*1,2*) and in particulate matter from diesel vehicle emissions (*3*) and ambient air (*4*), is a mutagen in bacterial and mammalian assay systems (*5–7*). NP has also been reported to cause mammary gland tumors in rats (*8*). Covalently bound adducts formed by interaction of NP with DNA have been detected (*9*) and characterized in *Salmonella typhimurium* (*10*) and in cell-free incubations catalyzed under anaerobic conditions by purified xanthine oxidase (*10*). The principal adduct formed under these conditions was identified as *N*-(deoxyguanosin-8-yl)-1-aminopyrene, thought to be generated from a hydroxylamine intermediate during the process of reductive metabolism of NP (*10*). On the other hand, Salmeen et al. (*11*) observed that although the mutagenicity of NP differed widely between nitroreductase-deficient and -proficient Salmonella strains, and V79 Chinese hamsters and primary rat hepatocytes, no corresponding variations were seen in the ability of all these cell types to reduce NP to 1-aminopyrene, and therefore nitroreduction might not be the only determinant of mutagenicity. NP was also shown to bind to endogenous DNA and protein when incubated with rabbit pulmonary tissues under aerobic conditions such that metabolism could occur by oxidative pathways (*12*).

The liver is the major site of oxidative metabolism in mammals and has been shown to convert NP to oxidized products which are themselves mutagenic (*13,14*). Although mutagenicity and DNA binding are not necessarily directly causally linked, both phenomena arise from the type of interactions with cellular genetic material which may ultimately lead to genotoxic damage to the organism. We have previously attempted to elucidate the role of oxidative metabolism in the activation of NP as measured by mutagenicity in the Salmonella plate incorporation assay (*14,15*).

The present study was undertaken to evaluate the ability of hepatic enzyme systems to catalyze the formation of NP metabolites capable of interacting with DNA by forming covalent adducts, and if so, whether these reactions could be linked to oxidative rather than to reductive metabolism. We also were interested to see whether the terminal enzyme of the mixed-function oxidase system, cytochrome P-450, known to be particularly active in the metabolism of foreign compounds, appears to be involved in this activation process.

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Materials and Methods

Chemicals

1-Nitro[4,5,9,10-^{14}C]pyrene (10 mCi/mmole, ≥99.9% radiochemical and chemical purity) was obtained from the Midwest Research Institute (Kansas City, MO) (*14,15*). NADPH and calf thymus DNA (Type I) were purchased from Sigma Chemical Co (St. Louis, MO). Laboratory reagents and solvents were obtained at the highest degree of purity from commercial suppliers and were used as supplied.

Animals

Livers of adult male Charles River CD rats (150–180 g) were excised and homogenized in sterile 1.15% KCl–0.02 M HEPES, pH 7.4 (4 vol). S9 supernatant, cytosol and microsomes were prepared by differential centrifugation. The microsomal pellet was resuspended in 0.1 M HEPES buffer, pH 7.4. Groups of five male Sprague-Dawley rats (body weight 100–200 g; Harlan Sprague-Dawley, In., Madison, WI) were treated by intraperitoneal injection with the following cytochrome P-450 inducers: phenobarbital (Mallinkrodt Chemical Co., St. Louis, MO; 20 mg in 0.5 mL of 0.85% NaCl daily for 4 days), Aroclor 1254 (Monsanto, St. Louis, MO) 120 mg in 0.5 mL corn oil 4 days before sacrifice), or 3-methylcholanthrene (Eastman Organic Chemicals, Rochester, NY; 1 mg in 0.2 mL corn oil four days before sacrifice (*16*). A further group of five rats served as untreated controls. Rats were starved for 20 hr prior to sacrifice; hepatic microsomes and S9 fraction were prepared as described (*17*). Protein concentration was assayed (*18*) with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the protein standard. Cytochrome P-450 content was determined by the dithionite difference method (*19*). All incubations were carried out on freshly prepared tissues.

Incubations

S9, cytosol or microsomes (1–1.4 mg protein) were suspended in 0.95 mL of 0.1 M HEPES buffer, pH 7.4, containing 2 mg calf thymus DNA. The incubation mixture was warmed to 37°C for 3 min, then 100 μM ^{14}C-NP was added in 10 μL of DMSO. The reaction was started by addition of NADPH (1 mg in 50 μL of 0.1 M HEPES buffer, pH 7.4), and terminated after 60 min by addition of an equal volume of $CHCl_3$/isoamyl alcohol/phenol (CIP; 24:1:25 by volume, saturated with HEPES buffer) and vortexed. Where incubations were conducted under nitrogen, the incubation mix was purged with N_2 as it was equilibrating to 37°C, purged again after addition of ^{14}C-NP and NADPH, then sealed for the duration of the incubation.

Determination of Radioactivity Bound to DNA

The protein was removed by CIP extraction (3 × 1 volume), and the DNA precipitated from the aqueous phase by addition of NaCl and ice-cold EtOH. The DNA was washed twice with EtOH, then harvested by winding onto glass pipettes, dried, and redissolved in 0.1 × standard saline/citrate. The ^{14}C content of the DNA solution was determined by liquid scintillation counting (Model 2660, Packard Instrument Co., Downers Grove, IL). The UV spectrum of the DNA solution was scanned (240–320 nm; DW-2, American Instrument Co., Silver Spring, MD); the DNA content was assayed by measurement of the OD_{260} and the purity of the DNA was verified by examination of the OD_{260}/OD_{280} ratio.

Results

After incubation of ^{14}C-NP with rat liver subcellular fractions and calf thymus DNA, radioactivity was detected in the isolated DNA fraction. DNA isolated from similar incubations conducted in the absence of tissue protein contained little or no ^{14}C detectable above background, which corresponded to limits of detectability of 0.1 to 0.2 pmole NP bound/mg DNA (Table 1). In incubations where tissues were derived from untreated Charles River CD rats, the highest rates of binding were seen in microsomes in the presence of air and NADPH (Table 1). This reaction was verified to be linear with respect to time up to 60 min and with respect to protein up to 2 mg of protein (results not shown). Although NADPH was required for maximum activity in S9 and microsomes, measurable rates of binding were also found in the absence of this co-factor. Both S9 and microsomes were about one-fourth as active under nitrogen as under air, although the activity under nitro-

Table 1. Activity of rat hepatic subcellular fractions in catalyzing the binding of ^{14}C from 1-nitro[^{14}C]pyrene to calf thymus DNA *in vitro*.[a]

	Incubation under air		Incubation under nitrogen	
Tissue	+ NADPH	− NADPH	+ NADPH	− NADPH
None[b]	<0.2[c]	—	<0.1	—
S9	24	2	6	3
Cytosol	0.7	0.3	0.9	1.7
Microsomes	44	0.3	11	1

[a] S9, cytosol and microsomes were prepared from the livers of untreated Charles River CD rats, and incubated (1–3.4 mg protein) for 60 min at 37°C with 1-nitro[^{14}C]pyrene (100 μM) and calf thymus DNA (2 mg) in HEPES buffer, pH 7.4, final volume 1 mL, under air or under nitrogen, with or without NADPH (1 mg). DNA was isolated and the ^{14}C bound to it quantitated as described in the text.

[b] Substrate, DNA, and NADPH were incubated without protein in order to assess limits of detectability and the occurrence of spontaneous reaction.

[c] The values given are pmole NP bound/mg DNA/mg protein in 60 min.

Table 2. Influence of induction on S9- and microsome-mediated binding of 1-nitro[^{14}C]pyrene to calf thymus DNA.[a]

	S9			Microsomes		
		Incubation conducted under[b]			Incubation conducted under	
Treatment of animals	P-450 content[c]	Air	Nitrogen	P-450 content	Air	Nitrogen
None	0.21 (100)	103.0 ± 10.7[d] (100)	27.6 ± 0.6 (100)	0.79 (100)	2.8 ± 1.3 (100)	20.0 ± 5.5 (100)
Phenobarbital	1.11 (530)	181.6 ± 3.3 (170)	67.4 ± 16.2 (240)	3.24 (410)	89.1 ± 28.8 (2400)	49.8 ± 0.4 (250)
Aroclor 1254	1.48 (710)	579.6 ± 40.2 (560)	40.4 ± 6.2 (150)	4.49 (570)	46.3 ± 15.0 (1300)	24.1 ± 1.5 (120)
3-Methylcholanthrene	0.33 (160)	209.5 ± 31.4 (200)	75.4 (270)	0.95 (120)	89.6 ± 6.7 (2500)	74.2 ± 0.9 (380)

[a] Tissues were obtained from Harlan Sprague-Dawley rats treated with various cytochrome P-450-inducing agents as described in "Materials and Methods."

[b] Tissues (1.0–1.4 mg) were incubated with 1-nitro[^{14}C]pyrene (100 μM), NADPH (1 mg) and DNA (2 mg) at 37°C for 60 min under air or nitrogen as specified.

[c] Values are expressed as nmole of cytochrome P-450/mg protein, with the percent of the untreated value in parentheses.

[d] Values given are the means (± SEM) of duplicate determinations (pmole of 1-nitro[^{14}C]pyrene-derived material bound/mg DNA/mg protein) from the pooled tissues of five animals for each treatment. Percentages of the corresponding untreated value are shown in parentheses.

gen was somewhat less dependent on NADPH. The activity of the cytosol was low under air, and slightly higher under nitrogen. When the cytosol was incubated with ^{14}C-NP and NADPH, but without DNA, under the conditions which had resulted in uniformly low DNA binding, and the incubates analyzed by HPLC for NP metabolites as described previously (*14*), this tissue was readily able to reduce NP to 1-aminopyrene (results not shown).

Treatment of the animals used as sources of hepatic tissues with inducing agents duly increased the P-450 content in the S9 and microsomal fractions (Table 2), and also the extent of DNA binding catalyzed by these fractions, although the increase in binding was not proportional to the enhancement in P-450 content. Aroclor 1254 produced the highest increase in cytochrome P-450 content in both S9 and microsomes and the highest specific binding in S9 under air, whereas phenobarbital and 2-methylcholanthrene were more effective than Aroclor in enhancing binding under nitrogen in S9 and in microsomes under both air and nitrogen. Although in this study 3-methylcholanthrene was by far the least potent inducer of cytochrome P-450, our phenobarbital and 3-methylcholanthrene treatments were almost equivalent in their ability to induce binding of ^{14}C-NP to DNA.

Differences in activity between strains were also apparent, since the microsomal fraction from the Harlan Sprague-Dawley rats bound ^{14}C-NP under air at very low rates, in contrast to the more extensive binding seen in the microsomes from Charles River CD rats. These low figures for aerobic untreated microsomal binding resulted in consequently high induction ratios for this parameter (Table 2).

Discussion

DNA binding of ^{14}C-NP in hepatic tissues was largely (though not entirely) dependent on air and NADPH. This binding was enhanced by cytochrome P-450 inducers: 3-methylcholanthrene and phenobarbital proved more effective than Aroclor 1254 in inducing binding in microsomes, whereas Aroclor was more effective in S9. This pattern was seen under both air and nitrogen. In contrast, much more enhancement in cytochrome P-450 content was seen here following Aroclor 1254 and phenobarbital treatment, indicating that though 3-methylcholanthrene was the least effective inducer of total P-450 here, it was the agent most effective at inducing binding of NP to DNA. The correlation between percentage increase in binding and in cytochrome P-450 content was also closer in the incubations catalyzed by tissues from 3-methylcholanthrene-treated animals than in those from the other two agents.

Binding in the previously studied systems (*9,10*) is thought to be due to the reductive intermediate *N*-hydroxy-1-aminopyrene. In this study we have shown that incubations conducted under conditions most conducive to NP reduction resulted in only low levels of binding, thus confirming the report that in mammalian cells the mutagenicity of NP does not necessarily correlate with the reduction of NP (*11*). In rabbit liver microsomes NP can be reduced by both cytochrome P-450 and aldehyde oxidase, and NADPH was reported to be the most effective electron donor (*20*). Mammalian microsomal nitroreductase activity appears to occur via the oxygen-sensitive one-electron pathway, which generates a nitro anion free-radical intermediate, whereas some strains of bacteria can metabolize nitro compounds by both the one-electron pathway and the two-electron pathway, which does not form the free radical (*21*). The latter pathway may therefore be more efficient in generating hydroxylamine intermediates capable of interacting with macromolecules, whereas the former can produce cytotoxicity by means of the superoxide arising from reoxidation of the nitro free radical.

Our findings that maximal levels of NADPH-dependent binding of NP to DNA occur under air suggest that the active intermediates responsible for binding are being generated either by oxidative routes, or by some

previously unsuspected oxygen-insensitive mammalian nitroreductase. The enzyme(s) involved would also appear to respond to inducing agents (especially 3-methylcholanthrene) in a manner similar to cytochrome P-450. The effects of mixed-function oxidase inducers are not limited to cytochrome P-450 (*22,23*), so that this response is not diagnostic for P-450; moreover, the specific binding activity does not increase consistently on going from S9 to microsomes as would be the case for reactions due entirely to cytochrome P-450. The overall pattern of activity indicates that both soluble and microsomal enzymes contribute to the overall process, but that the microsomal components are capable of functioning independently of the cytosolic portions, while the converse does not hold. In contrast, activation of NP in the Ames assay was clearly shown to reside in the microsomal fraction (*24*). Further studies are in progress with specific inhibitors and co-factors in order to clarify the pathways responsible, singly or in conjunction, for catalysis of the *in vitro* binding of NP to DNA.

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REFERENCES

1. Löfroth, G., Hefner, E., Alfheim, I., and Møller, M. Mutagenic activity of photocopies. Science 209: 1037–1039 (1980).
2. Rosenkranz, H. S., McCoy, E. C., Sanders, D. R., Butler, M., Kiriazides, D. K., and Mermelstein, R. Nitropyrenes: isolation, identification and reduction of mutagenic impurities in carbon black and toners. Science *209: 1039–1043 (1981).*
3. Schuetzle, D., Riley, T. L., Prater, T. J., Harvey, T. M., and Hunt, D. F. Analysis of nitrated polycyclic hydrocarbons in diesel particulates. Anal. Chem. 54: 265–271 (1982).
4. Gibson, T. L. Nitroderivatives of polynuclear aromatic hydrocarbons in airborne and source particulate matter. Atmos. Environ. 16: 2073–2076 (1982).
5. Mermelstein, R., Kiriazides, D. K., Butler, M., McCoy, E., and Rosenkranz, H. S. The extraordinary mutagenicity of nitropyrenes in bacteria. Mutat. Res. 89: 187–196 (1982).
6. Nachtman, J. P., and Wolff, S. Activity of nitro-polynuclear aromatic hydrocarbons in the sister chromatid exchange assay with and without metabolic activation. Environ. Mutag. 4: 1–7 (1982).
7. Li, A. P., and Dutcher, J. S. Mutagenicity of mono-, di- and trinitropyrenes in Chinese hamster ovary cells. Mutat. Res. Lett. 119: 387–392 (1983).
8. Hirose, M., Lee, M. S., Wang, C. Y., and King, C. M. Induction of rat mammary gland tumors by 1-nitropyrene, a recently-recognized environmental mutagen. Cancer Res. 44: 1158–1162 (1984).
9. Messier, F., Lu, C., Andrews, P., McCarry, B. E., Quilliam, M. A., and McCalla, D. R. Metabolism of 1-nitropyrene and formation of DNA adducts in *Salmonella typhimurium*. Carcinogenesis 2: 1007–1011 (1981).
10. Howard, P. C., Heflich, R. H., Evans, F. E., and Beland, F. A. Formation of DNA adducts *in vitro* and in Salmonella typhimurium upon metabolic reduction of the environmental mutagen 1-nitropyrene. Cancer Res. 43: 2052–2058 (1983).
11. Salmeen, I., Zacmanidis, P., and Ball, J. 1-Nitropyrene reduction by *Salmonella typhimurium*, V79 chinese hamster and primary rat liver cells. Mutat. Res. 122: 23–28 (1983).
12. King, L. C., Jackson, M., Ball, L. M., and Lewtas, J. Binding of 1-nitro-[^{14}C]pyrene to DNA and protein in cultured lung macrophages and respiratory tissues. Cancer Letters 19: 241–246 (1983).
13. El-Bayoumy, K., and Hecht, S. S. Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. Cancer Res. 43: 3132–3137 (1983).
14. Ball, L. M., Kohan, M. J., Claxton, L. D., and Lewtas, J. Mutagenicity of derivatives and metabolites of 1-nitropyrene: activation by rat liver S9 and bacterial enzymes. Mutat. Res. 138: 113–125 (1984).
15. Ball, L. M., Kohan, M. J., Inmon, J., Claxton, L. D., and Lewtas, J. Metabolism of 1-nitro[^{14}C]pyrene *in vivo* in the rat and mutagenicity of urinary metabolites. Carcinogenesis 5: 1557–1564 (1984).
16. Coombs, M. M. The influence of various enzyme inducers on the microsomal metabolism of 7,8-benzoflavone. Carcinogenesis 3: 229–230 (1982).
17. van der Hoeven, T. A., Haugen, D. A., and Coon, M. J. Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. J. Biol. Chem. 249: 6302–6310 (1974).
18. Lowry, O. H., Rosebrough, N. G., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275 (1951).
19. Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem 239: 2370–2378 (1964).
20. Kitamura, S., Narai, N., Hayashi, M., and Tatsumi, K. Rabbit liver enzymes responsible for reduction of nitropolycyclic aromatic hydrocarbons. Chem. Pharm. Bull. 31: 776–779 (1983).
21. Peterson, F. J., Mason, R. P., Hovsepian, J., and Holzman, J. L. Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes. J. Biol. Chem. 254: 4009–4014 (1979).
22. Marselos, M., and Hänninen, O. Enhancement of D-glucuronolactone and acetaldehyde dehydrogenase activities in the rat liver by inducers of drug metabolism. Biochem. Pharmacol. 23: 1457–1466 (1974).
23. Lind, C., and Ernster, L. A possible relationship between D-T-diaphorase and the aryl hydrocarbon hydroxylase system. Biochem. Biophys. Res. Commun. 56: 392–400 (1974).
24. Kohan, M., and Claxton, L. Mutagenicity of diesel-exhaust particle extract, 1-nitropyrene, and 2,7-dinitrofluorenone in *Salmonella typhimurium* under various metabolic activation conditions. Mutat. Res. 124: 191–200 (1983).