Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 134-140

TOPICALLY APPLIED NITROPYRENES ARE POTENT INDUCERS OF CUTANEOUS AND HEPATIC MONOOXYGENASES

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Received April 22, 1985

SUMMARY: The inducibility of skin and liver microsomal cytochrome P-450 dependent aryl hydrocarbon hydroxylase and other monooxygenases by a mixture of nitropyrenes was assessed and compared with the parent non-nitrated compound, pyrene. A single topical application of nitropyrenes to neonatal rats resulted in highly significant induction of aryl hydrocarbon hydroxylase, ethoxycoumarin O-de-ethylase, and ethoxyresorufin O-de-ethylase activities in skin and liver after 24 hours. Inducibility of the skin and liver enzymes was 3.9-5.7 fold and 1.8-10.3 fold respectively. On the other hand, aminopyrine N-demethylase, benzphetamine N-demethylase and epoxide hydrolase activities in the liver were unaffected by topically applied nitropyrenes. Furthermore, treatment with nitropyrenes produced a 1 nm shift to the blue region in the wavelength maximum of hepatic microsomal cytochrome P-450. Topically applied pyrene produced only marginal or no effects on cutaneous and hepatic enzyme activities. Our results suggest that nitration of pyrene, a relatively ineffective enzyme inducer, produces nitropyrenes which are potent inducers of hepatic and cutaneous monooxygenases and they resemble 3-methylcholanthrene in this inducing effect.

Nitropyrenes are a group of nitrated polycyclic aromatic hydrocarbons which are widely distributed in the environment chiefly as the result of incomplete combustion processes, e.g., automobile and diesel exhaust, coal power plants, kerosene heaters, grilled chicken yakitori and cigarette smoke (1-6). In the atmosphere, the nitropyrenes are also produced by the interaction of nitrogen oxides with pyrenes (7). There is growing concern about the potential toxicity and carcinogenicity of the nitropyrenes. For example, nitropyrenes are among the most potent mutagens yet identified (8) and they have been reported to be carcinogenic to rodents (1, 8-14).

Skin is the largest body organ and serves as a major portal of entry for many airborne environmental pollutants (15). Earlier reports from our laboratory have shown that topical application of several polycyclic aromatic hydrocarbons to neonatal rodents results in the induction of cutaneous and/or hepatic aryl hydrocarbon hydroxylase activity (16,17). AHH is a cytochrome P-450-dependent monooxygenase and it has been suggested that the

0006-291X/85 $1.50

inducibility of AHH in a target tissue may correlate with the tumor risk of chemical tumor induction in that tissue (18,19). Topical application of pyrene results in marginal or no induction of hepatic and cutaneous AHH and other monooxygenase activities (17). Since pyrene is ineffective as an inducer of monooxygenases and is noncarcinogenic and nonmutagenic (20) while nitropyrenes are carcinogenic and mutagenic, we studied the effect of topical application of a mixture of nitropyrenes, approximating their ambient distribution, on cutaneous and hepatic monooxygenase activities. Our data indicate that a topically applied mixture of nitropyrenes results in highly significant induction of cutaneous and hepatic aryl hydrocarbon hydroxylase and other monooxygenase activities in neonatal rats.

MATERIALS AND METHODS

Chemicals: (^{3}H)benzo(a)pyrene-4,5-oxide (specific activity 282.5 mCi/mmol) and unlabeled benzo(a)pyrene-4,5-oxide were provided by the Cancer Research Program of the National Cancer Institute, Division of Cancer Cause and Prevention (Bethesda, MD). Aminopyrine, 7-ethoxycoumarin, and pyrene were purchased from Aldrich Chemical Co (Milwauke, WI). Benzo(a)pyrene resorufin, bovine serum albumin, NADPH, and NADH were purchased from Sigma Chemical Co. (St. Louis, MO). 7-ethoxyresorufin was a product of Pierce Chemicals. The mixture of nitropyrenes was prepared as described earlier (1) with the ratio of dinitropyrenes and 1-nitropyrene of 7, which reflects their environmental distribution as well. All other chemicals were purchased in the purest form available.

Animals and Treatment: Pregnant rats were obtained from Holtzman Rat Farm, Madison, WI. Newborn rats were withdrawn from their mothers on day 4 after birth and topically treated with (10mg/kg) pyrene or mixture of nitropyrenes dissolved in 100 µl of THF:DMSO (1:1). Animals receiving solvent alone served as controls.

Preparation of Skin and Liver Microsomes: Twenty four hours after treatment, the animals were killed and skin and livers removed and their microsomal fractions prepared according to procedures established in this laboratory (21).

Enzyme assays: AHH, 7-ethoxycoumarin O-de-ethylase and epoxide hydrolase activities were determined as described earlier (21). Aminopyrine N-demethylase and benzphetamine N-demethylase activities were determined according to Cochine and Axelrod (22) by measuring the formaldehyde formed (23). Ethoxyresorufin O-de-ethylase activity was determined by a modification of the method of Pohl and Fouts (24). A typical reaction mixture in a final volume of 1.25 ml contained 0.1M phosphate buffer (pH 7.4), 0.96mM NADPH, 2mg BSA, and 0.3-0.7mg microsomal protein. The reaction was initiated by the addition of 1.5 µM ethoxyresorufin in 5µl of DMSO and was incubated for 10 or 30 min at 37°C, for liver and skin, respectively, in a Dubnoff metabolic shaker. The reaction was terminated by the addition of 2 ml methanol and the fluorescence of the deethylated product resorufin was measured at excitation and emission wavelenghts of 550nm and 585 nm, respectively. Quantification of the deethylated metabolites was based on the comparison of a standard solution of resorufin. Protein was estimated according to Lowry et al. (25) using bovine serum albumin as standard.

Cytochrome P-450: Cytochrome P-450 in liver microsomes was quantitated by the method of Omura and Sato (26). Carbon monoxide plus dithionite-reduced minus dithionite-reduced difference spectra were recorded using a Aminco DW 2a dual beam spectrophotometer equipped with a Midan microprocessor.

RESULTS AND DISCUSSION

The comparative effect of a single topical application of pyrene and the mixture of nitropyrenes on microsomal monooxygenase and epoxide hydrolase activities in the skin of neonatal rats is shown in Table 1. Pyrene treatment caused only a marginal increase in ethoxyresorufin O-de-ethylase and 7-ethoxycoumarin O-de-ethylase activities. However, the mixture of nitropyrenes resulted in 4.0, 3.9, and 5.7-fold increases in cutaneous AHH, ethoxycoumarin O-de-ethylase and ethoxyresorufin O-de-ethylase activities. Epoxide hydrolase activity was unaltered by nitropyrenes treatment. The comparative effect of a single topical application of pyrene and of the mixture of nitropyrenes on liver microsomal enzyme activities is shown in table 2. Following topical application of the nitropyrenes mixture there was a 4.3, 1.8 and 10.3-fold induction of AHH, ethoxycoumarin O-de-ethylase and ethoxyresorufin O-de-ethylase activities, respectively, in the liver. However this compound had no effect on hepatic aminopyrine N-demethylase, benzphetamine N-demethylase and epoxide hydrolase activities.

The effect of treatment with nitropyrenes on the concentration and wavelength absorption maximum of hepatic cytochrome P-450 is shown in table 3. A single topical

Table 1

Comparative effect of a single topical application of pyrene and nitropyrenes mixture on microsomal enzyme activities in the skin of neonatal rats

Parameters	Control (Vehicle)	Pyrene	Percent of control	Nitropyrenes Mixture	Percent of control
Aryl hydrocarbon hydroxylase	1.67 ± 0.03	1.67 ± 0.23	100	6.79 ± 0.16*	402
Ethoxyresorufin O-de-ethylase	0.58 ± 0.02	0.94 ± 0.02*	162	3.32 ± 0.08*	572
7-Ethoxycoumarin O-de-ethylase	2.10 ± 0.04	2.76 ± 0.04*	131	8.19 ± 0.17*	390
Epoxide hydrolase	30.2 ± 1.9	28.3 ± 1.6	94	27.5 ± 1.2	91

Data represent mean ± S.E. of 4 values. For each determination 4 neonatal rats (4-day old) were treated with a single topical application of pyrene or a nitropyrenes mixture (10 mg/kg) dissolved in the THF:DMSO (1:1). The vehicle had no detectable effect on enzyme activities. Animals were killed 24 hr after treatment and enzyme activities determined.

Enzyme specific activities are: AHH, pmol 3-hydroxy benzo(a)pyrene/min/mg protein; ethoxyresorufin O-de-ethylase, pmol resorufin/min/mg protein; 7-ethoxycoumarin O-de-ethylase, pmol 7-hydroxycoumarin/min/mg protein; epoxide hydrolase, pmol benzo(a)pyrene 4,5-diol/min/mg protein.

* $p < 0.001$ as compared to control by paired student's 't' test.

Table 2

Comparative effect of a single topical application of pyrene and nitropyrenes mixture on microsomal enzyme activities in the liver of neonatal rats

Parameters	Control (Vehicle)	Pyrene	Percent of Control	Nitropyrenes Mixture	Percent of Control
Aryl hydrocarbon hydroxlase	22.6 ± 0.5	24.2 ± 1.4	107	96.3 ± 2.3*	426
Ethoxyresorufin O-de-ethylase	10.3 ± 0.2	14.1 ± 0.1*	136	106.2 ± 1.2*	1029
Ethoxycoumarin O-de-ethylase	38.5 ± 1.3	50.6 ± 1.0*	131	70.9 ± 3.6*	184
Aminopyrine N-demethylase	0.82 ± 0.02	0.82 ± 0.04	100	0.86 ± 0.04	105
Benzphetamine N-demethylase	0.64 ± 0.05	0.71 ± 0.07	111	0.63 ± 0.07	98
Epoxide hydrolase	1135 ± 68	1208 ± 39	106	1242 ± 47	109

Data represent mean ± S.E. of 4 values.

Aminopyrine N-demethylase and benzphetamine N-demethylase activities are expressed as pmol formaldehyde/min/mg protein. For other enzyme units, treatment schedule and other details see Table 1.

* $p < 0.001$ as compared to control by paired student's 't' test.

application of nitropyrenes caused no detectable increase in the concentration of cytochrome P-450. However, a shift of 1 nm to the blue in the wavelength maximum of the hepatic hemeprotein was observed in the animals treated with the nitropyrenes mixture.

Nitrated polycyclic aromatic hydrocarbons have recently been recognized as potent toxic and carcinogenic chemicals. Long-term exposure of nitropyrenes may have adverse

Table 3

Comparative effect of a single topical application of pyrene and nitropyrenes mixture on cytochrome P-450 concentration and absorption maxima of the heme protein in liver microsomes from neonatal rats

Treatment	Cytochrome P-450 (pmole/mg protein)	λ_{max} (nm)
Control	350 ± 14	452
Pyrene	353 ± 18	452
Nitropyrenes Mixture	359 ± 22	451

For treatment details see Table 1.

effects on human health (2,27). In an effort to define the mode of toxicity of the nitropyrenes, we studied the effects of topical application of a mixture of nitropyrenes, in the proportion of their occurrence in the environment, on hepatic and cutaneous monooxygenase activities. The effects were compared with the non-nitrated parent compound, pyrene, which is nonmutagenic and noncarcinogenic (20). Our data clearly indicate that a single topical application of the nitropyrenes mixture to rats resulted in a several-fold induction of hepatic and cutaneous AHH, ethoxyresorufin O-de-ethylase and ethoxycoumarin O-de-ethylase activities.

The deethylation of ethoxyresorufin (or ethoxyresorufin O-de-ethylase) is highly specific for cytochrome P-448 (28) whereas benzo(a)pyrene hydroxylation (or AHH) is predominantly catalyzed by cytochrome P-448 and deethylation of 7-ethoxycoumarin (or 7-ethoxycoumarin O-de-ethylase) is metabolized by both cytochrome P-448 and cytochrome P-450 (29,30). Cytochrome P-448 is selectively induced by 3-methylcholanthrene type of chemical inducers (30). The N-demethylation of aminopyrine or benzphetamine is catalyzed by cytochrome P-450 which is induced by phenobarbital type of chemical inducers (31). Our results demonstrating the high inducibility of hepatic and cutaneous AHH and ethoxyresorufin O-de-ethylase activities following treatment with mixture the nitropyrenes suggest that nitropyrenes behave like the 3-methylcholanthrene type inducers of monooxygenase activities. This is further supported by the lack of effect on hepatic epoxide hydrolase activity. Prior studies have shown that 3-methylcholanthrene has no effect whereas phenobarbital results in the induction of hepatic epoxide hydrolase activity in rodents (32).

The absorption maximum of the CO-difference spectra from control neonatal rat liver was at 452 nm which is consistent with that reported previously (16). In the present study a shift of 1 nm to the blue region was observed in the absorption maximum of the hepatic hemeprotein following treatment with the nitropyrenes mixture. Since this mixture was applied to animals topically for only 24 hours, it is possible that prolonged application or parenteral administration may result in greater shifts in the wavelength absorption maximum and increased levels of the hemeprotein as is observed after prolonged administration of 3-methylcholanthrene.

Another interesting finding of our studies is the observation that a topically applied mixture of nitropyrenes can produce effects on hepatic monooxygenase activities. This suggests that nitropyrenes present in the ambient environment can penetrate the cutaneous barrier and gain entry into the circulation to produce effects on hepatic drug metabolizing enzyme activities and thereby may produce toxicity including carcinogenicity in cutaneous as well as extracutaneous tissues.

To our knowledge, the present study is the first to demonstrate that the nitropyrenes are inducers of skin and liver monooxygenase activities. Furthermore, our results indicate that nitropyrenes are inducers of the 3-methylcholanthrene type. Our data do not imply that the responses we observed are due to a single nitropyrene or to a group of nitropyrenes. It may be that the induction of monooxygenase activities observed in this study is due to a combined effect of several nitropyrenes whose individual effects might be too low to measure. Further studies are needed to define the relative inducibility of individual anthropogenic nitropyrenes known to be present in mixtures of nitropyrenes. Our findings further emphasize that airborne nitrated polycyclic aromatic hydrocarbons may be potent environmental toxins and that the skin is a potential target tissue for their effects.

ACKNOWLEDGMENTS

Supported NIH Grants ES-1900 and CA 38028 and funds from the Veterans Administration. Thanks are due to Dr. P.C. Howard for helpful discussions and to Mrs. Cherice Edmonds for preparing the manuscript.

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