Development of Methods to Monitor Exposure to 1-Nitropyrene

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On the basis of 32P-postlabeling analysis, treatment of rats with 1-nitropyrene (1-NP) resulted in the formation of multiple DNA adducts in the liver, mammary glands, and peripheral lymphocytes. The one adduct resulting from nitroreduction, N-deoxyguanosin-8-yl-1-aminopyrene, constitutes only a minor component among the adducts. In the present study, incubation of calf thymus DNA with mutagenic ring-oxidized metabolites of 1-NP in vitro in the presence and absence of xanthine oxidase also resulted in the formation of multiple adducts. On the basis of their chromatographic behavior, it appears that DNA adducts derived from such metabolites may have been formed in vivo; however, this needs to be confirmed. [3H]1-NP was given to male and female F344 rats and Sprague-Dawley rats by gavage at five dose levels in the range of 0.1 to 1000 μg/kg bw. This led to stable hemoglobin adducts accounting for 0.08 ± 0.05% of the dose (n = 3 rats). The radioactivity associated with hemoglobin following administration of [3H]1-NP was cleared with a half-life of about 14 days, which is faster than that of unmodified erythrocytes in the rat (t1/2 = 30 days). Treatment of the hemoglobin with 1% HCl in acetone, to precipitate the globin, released the radioactivity; it was all bound to the heme moiety. The structures of the heme adducts have not been elucidated; yet, because of their stability, they may be useful as dosimeters for human exposure to 1-NP. The results of this study demonstrate the potential of using hemoglobin adducts of 1-NP as dosimeters of uptake and metabolic activation of nitropolynuclear aromatic hydrocarbons (NO2-PAH). These indicators are a prerequisite for cancer risk assessment of NO2-PAH. — Environ Health Perspect 102(Suppl 6):31-37 (1994)

Key words: nitropyrene, 32P-postlabeling, DNA adducts, N-deoxyguanosin-8-yl-1-aminopyrene (NdG-AP), K-region oxide-DNA adducts, protein adducts, heme adducts

Introduction

Nitropolynuclear aromatic hydrocarbons (NO2-PAH) are widely distributed in the environment (1). Mutagenic activities in bacterial and mammalian systems and tumorigenic activity in laboratory animals of several members of the class of NO2-PAH have been clearly documented (2-5).

Risk assessment associated with human exposure to NO2-PAH has not been clearly defined, despite the widespread occurrence of such agents in the environment and their possible involvement in the etiology of some human cancers (6). Thus, our goal was to develop sensitive analytical methods for detection and quantitation of NO2-PAH and their metabolites in biologic fluids and assessment of their adducts with proteins and DNA in humans. Establishing the metabolic profiles of 1-nitropyrene (1-NP) in laboratory animals would be essential for developing sensitive methods for their detection and quantitation in humans. In the mammalian system, 1-NP is metabolized by nitroreduction, ring oxidation, and a combination of both pathways (Figure 1). Studies in our laboratory and elsewhere indicate that simple nitroreduction of 1-NP to yield N-deoxyguanosin-8-yl-1-aminopyrene (NdG-AP) cannot account for the observed DNA adducts in vivo and in vitro (7-12). To more clearly define the metabolic activation of 1-NP, structural elucidation of DNA adducts is required. Therefore, some of our previous efforts were directed towards the identification of additional DNA adduct markers derived from ring-oxidized metabolites of 1-NP (13). Seeking a highly sensitive method, we used the 32P-postlabeling technique (14) to establish fingerprints of the DNA adducts derived from ring-oxidized metabolites of 1-NP in vitro. The resulting 32P-postlabeled adducts were compared with those obtained from rat tissues such as liver, mammary tissues, and peripheral lymphocytes following the administration of 1-NP (12). In this study, we also present results on hemoglobin binding of 1-NP.

Materials and Methods

Chemicals

Commercial 1-NP (Aldrich Chemical Co., Milwaukee, WI) was purified by column chromatography on silica gel with elution by 10% benzene in hexane. Its purity, assessed by GC with electron capture detection (15) and GC-MS analysis, was >99.9%; no dinitropyrenes were detected. 1-Nitrosopyrene (1-NOP) was synthesized by oxidation of 1-aminopyrene (1-AP) using m-chloroperoxybenzoic acid (mCPBA) as described (16). 1-Nitroxyhydroxypyrene (1-NP-x-OH, x = 3, 6, or 8) was obtained by nitration of 1-acetoxy-pyrene followed by deacetylation (16). Refluxing of 1-NP with mCPBA in
CH$_2$Cl$_2$ yielded 4.5-epoxy-4.5-dihydro-1-nitropyrene (1-NP-4.5-oxide) and 9,10-epoxy-9,10-dihydro-1-nitropyrene (1-NP-9,10-oxide) (17). cis- and trans-4,5-Dihydro-4,5-dihydroxy-1-nitropyrene (cis and trans-1-NP-4,5-DHD) were also synthesized (18). [13C]1-NP was synthesized in our laboratory with >99% radiochemical purity, and a specific activity of 5 mCi/mmole (19). A sample of [3H]1-NP (13.7 Ci/mmole) was acquired from Chemsyn (Lenexa, KS), and pure samples were obtained by silica gel thin-layer chromatography with benzene as eluent; purity was >99.0% as ascertained by reverse-phase HPLC and monitoring radioactivity with a radioflow detector. [3H]1-NOP and [3H]1-NP-4,5-oxide were also prepared in our laboratory (11,13) as was N-dG-AP (12). The three major N$_2$-deoxyguanosine adducts derived from 1-NP-4,5-oxide were synthesized and structurally characterized (13).

Calf thymus DNA (type I), enzymes and other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Carrier-free [y$_{32}$]ATP (5000 Ci/m mole, Amersham) and T$_4$ polynucleotide kinase (30 units/ml, U.S. Biochemical Corp., Cleveland, OH) were purchased from commercial sources.

Instrumentation

HPLC was performed with a Waters Associates high-performance liquid chromatograph (Millipore, Waters Division, Milford, MA) equipped with a model 510 solvent delivery system, a model U6K septumless injector, a model 440 ultraviolet-visible detector and a model 680 automated gradient controller. HPLC conditions are described in the figure legends. A radioflow detector (Flo-One/Beta, Radiomatic Instruments and Chemicals Co., Tampa, FL) was employed in the radiochromatography. Quantitative measurements of radioactivity were made on a Beckman LS 9800 series liquid scintillation counter.

DNA Binding Studies in Vitro

Calf thymus DNA which had been modified with 1-NP-4,5-oxide (10,13), or with 1-NP in the presence of xanthine oxidase (12), was enzymatically hydrolyzed to 3'-monophosphates followed by $^{32}$P-postlabeling. trans-1-NP-4,5-DHD, 1-NP-x-OH (x = 3, 6, or 8), and 1-NP-9,10-oxide were each incubated with calf thymus DNA in the presence or absence of xanthine oxidase as reported (20,21). DNA which had been modified with ring-oxidized metabolites of 1-NP was hydrolyzed to 3'-monophosphates, followed by $^{32}$P-postlabeling.

Animal Protocols for 1-Nitropyrene-Hemoglobin Adducts

Dose-Response Study. Five groups of three male F344 rats weighing 272 ± 6 g (Charles River Breeding Laboratories, Kingston, NY) were given [3H]1-NP in pre-distilled triocanoin by gavage. They were housed under standard conditions (15) and given tap water and NIH-07 diet ad libitum. The compound was administered at dose levels of 0.1, 1.0, 10, 100, and 1000 µg/kg bw. Each rat was given 15 µCi except for the lowest dose, in which case 2.2 µCi was administered.Twenty-four hours after gavage, the rats were anesthetized with ether or halothane, and blood was obtained by cardiac puncture (average 8 ml/rat representing approximately 50% of the total blood volume) (22). The blood was drawn into syringes containing 0.1 ml EDTA solution, 0.25 M, pH 7.4, to prevent clotting. It was then transferred into polypropylene centrifuge tubes (35 ml) and placed on ice. All subsequent steps were carried out at 4°C. The blood was centrifuged at 900g to pack the red blood cells (RBC). The supernatant plasma was drawn off with a Pasteur pipette and processed as described below. The RBC were washed twice with 15-ml portions of 0.9% saline and centrifuged at 900g each time. The supernatant was kept frozen. Cell rupture and release of hemoglobin was minimal as evidenced by the slight coloration of the supernatant. The RBC were then lysed by vortexing with 15 ml ice-cold distilled H$_2$O for 5 min. Ten milliliters of 0.67 M KH$_2$PO$_4$ buffer, pH 6.5, were added, and the solution was centrifuged at 27,000g for 20 min. The supernatant containing the hemoglobin was placed into dialysis tubes (Spectra/Por 4, 14 x 10$^3$ molecular weight cutoff, Spectrum Medical Industries, Los Angeles, CA) and dialyzed against distilled H$_2$O, 2.5 liters × 4 changes over 4 days. The hemoglobin solutions were frozen at −20°C until analysis. The hemoglobin obtained from each rat was analyzed individually. Five-milliliter aliquots of each test solution were placed in scintillation vials containing 0.5 ml 10 N NaOH; these vials were brought up to 50°C for 2 hr to hydrolyze the hemoglobin. After cooling, 5 ml of 50% H$_2$O$_2$ was added to bleach the solution. The solutions were neutralized with 6 N HCl, and scintillation fluid (Monofluor, National Diagnostic, Manville, NJ) was added. The samples were stored at 4°C in the dark for several days to minimize chemiluminescence and then were counted. This set of experiments was also performed with female F344 rats and male and female Sprague-Dawley rats. The latter were selected because they are susceptible to tumor induction by nitrated pyrenes and can be used in experiments intended to compare levels of DNA adducts in target tissues to levels of hemoglobin adducts. Such data will be valuable as baseline studies for future investigations in humans.

Figure 1. 1-Nitropyrene metabolites (1–13) identified in vivo or in vitro. Acetylation of the amino functionality and conjugation (glucuronide and sulfates) of C-hydroxylated metabolites have also been demonstrated in vivo.
**Adduct Persistence in Vivo.** Three male F344 rats (162 ± 9 g body weight) were gavaged with 2 mCi 1-NP (0.71 μmole) in triocatoin. Approximately 0.5 ml blood was drawn from the orbital sinus of each rat at the following intervals: 1, 3, 6, 8, 14, 28, 35, 42, 49, 56, 63, and 68 days. The hemoglobin was isolated as described above, and the bound radioactivity was measured.

**Adduct Accumulation during Chronic Dosing.** Five male F344 rats (187 ± 5 g bw) were gavaged every Monday, Wednesday, and Friday for 5 weeks with NP (0.1 mCi, 0.006 mole; total dose: 1.8 mCi, 0.011 mole) in triocatoin. Blood was drawn weekly (0.5 ml/rat) via the orbital sinus on Tuesdays and Thursdays; the hemoglobin was isolated, and the bound radioactivity was quantified.

Isolation and chromatographic analysis of the hemoglobin adducts. The thawed hemoglobin samples were added dropwise to 200 ml of rapidly stirred 1% HCl in acetone on ice. The globin precipitate was filtered, dried, and kept frozen at −20°C for further analysis. The acidic acetone solution was evaporated to dryness under reduced pressure. The residue was resuspended in tetrahydrofuran, the radioactivity was measured, and an aliquot was analyzed by reverse-phase HPLC. The heme fraction that contained radioactivity was treated with H2SO4/CH3OH to derivatize the carboxylic acid groups on the heme moiety to methyl esters, followed by treatment with zinc acetate to form methylated zinc metalloporphyrins (23–26); it was then analyzed by HPLC. As an alternative approach, the whole hemoglobin was treated in a similar manner; this simultaneously precipitates the protein and derivatizes the heme moiety to the corresponding methyl ester.

Incubations of 1-nitrosopyrene and 1-nitropyrene-4,5-oxide with rat hemoglobin in vitro. Both 1-NOP and 1-NP-4,5-oxide are 1-NP metabolites that are known to bind to DNA. They also represent both metabolism pathways of 1-NP, nitroreduction and ring oxidation. Freshly isolated rat hemoglobin was incubated at room temperature with both compounds for 4.5 hr (27). [3H]1-NOP (0.7 μCi, 0.08 pmole) was incubated under aerobic and anaerobic conditions. The anaerobic conditions were employed to minimize further oxidation of 1-NOP to 1-NP. [3H]1-NP-4,5-oxide (0.94 μCi, 0.013 μM) was incubated under aerobic conditions only.

**Statistical Analysis**
Each point in Figures 2 and 3A represents the mean ± SD. Group means ± SD were plotted with linear regression lines using graphics software (Grapher, Version 1.75, Golden Software, Golden, CO and Statview II, Abacus Concepts, Berkeley, CA). Biologic half-lives were calculated by the method of Rumack and Lovejoy (28).

**Results**

**Analysis of 1-NP-DNA Adducts Using 32P-Postlabeling**

Because multiple adducts were observed upon treatment of rats with 1-NP, including only one minor adduct spot (N-dG-AP) that was derived from nitroreduction (12), our current efforts were directed toward examining the nature and the origin of the other adducts. We prepared markers derived from ring-oxidized metabolites of 1-NP. The structures of 1-NP-4,5-oxide-DNA adducts were described previously (13). The 32P-fingerprints of DNA that had been modified with 1-NP-4,5-oxide are shown in Figure 4. By comparing chromatographic behaviors, we deduced that these oxide adducts may be responsible for the formation of some of the putative 1-NP-DNA adducts in vivo (12). The 32P-maps of DNA modified with 1-NP-4,5-oxide in the presence of xanthine oxidase showed additional adduct spots, including those observed in the absence of xanthine oxidase (Figure 4). The maps of DNA modified with 1-NP-9,10-oxide in the presence and absence of xanthine oxidase were more complex (Figure 4). Multiple adducts also were observed by the 32P-postlabeling assays of DNA that had been modified with 1-NP-x-OH (x = 3, 6, or 8, Figure 5) and trans-1-NP-4,5-DHD in the presence of xanthine oxidase (Figure 6).

**Analysis of Hemoglobin Adducts Following the Oral Administration of [3H]1-Nitropyrene to Rats**

After dialysis, the hemoglobin solution was extracted with EtOAc. A putative 1-NP metabolite was covalently bound to the hemoglobin as evidenced by the absence of radioactivity in the EtOAc layer. [3H]1-NP forms hemoglobin adducts at 0.08 ± 0.05% (mean ± SD, n = 3 rats) of the dose given orally in triocatoin. The dose-response data were obtained by measuring the radioactivity in the whole hemoglobin compartment (i.e., after dialysis but prior to acidic acetone treatment). Using linear regression analysis, the dose-response curve is linear over five orders of magnitude (p<0.01, r² = 0.963; Figure 2).

Following the disappearance of radioactivity after administering a single dose of [3H]1-NP, it appears that these adducts are cleared (by first-order kinetics with a half-life of about 14 days) faster than unmodified rat erythrocytes (28,29); compare Figure 3A. Thus, these adducts would be expected to accumulate as a result of chronic exposure; this result was observed (Figure 3B). After cessation of dosing (after 35 days), these adducts decreased rapidly, as indi-
Figure 4. $^{32}$P-PEI cellulose TLC maps of DNA modified with 1-NP-4,5-oxide (A,D) and 1-NP-9,10-oxide (B,E) in the absence and presence of xanthine oxidase; solvent systems used for 4-D TLC (10 x 10 cm PEI cellulose plates, Brinkmann Instruments, Westbury, NY) were: D1, 1 M sodium phosphate, pH 6.8; D2, 2.5 M ammonium formate, pH 3.5; D3, 3 M lithium formate: 7 M urea, pH 3.5; and D4, 0.8 M lithium chloride: 7 M urea: 0.5 M Tris-buffer, pH 8.0.

Figure 5. $^{32}$P-PEI cellulose TLC maps of DNA modified with 1-NP-x-OH (x = 3, 6, or 8) in the presence of xanthine oxidase; see legend to Figure 4 for details.

Figure 6. $^{32}$P-PEI cellulose TLC maps of DNA modified with trans-1-NP-4,5-DHD in the presence of xanthine oxidase; see legend to Figure 4 for details.

The radioactivity from the dark brown heme by silica gel column chromatography. Upon analysis of the radioactive material, using a reverse-phase C$_4$ column and a H$_2$O/CH$_3$CN gradient containing 0.1% trifluoroacetic acid, two radioactive peaks...
eluted after the unmodified heme (Figure 7B). Treatment of the radioactive fraction with 6 and 12 N HCl, or with concentrated HBr, concentrated HI, or 5 and 10 N NaOH did not release a recognizable derivative of 1-NP. In fact, such treatment did not alter the retention time of the original radioactive peak in the C<sub>18</sub>-system (Figure 7A). This indicated that a radioactive metabolite of 1-NP was covalently bound to the heme. Following a general procedure described by Ortiz de Montellano (see "Materials and Methods"), the heme fraction was derivatized to its methyl ester by treating with 10% H<sub>2</sub>S<sub>2</sub>O<sub>3</sub> in CH<sub>3</sub>OH. This simultaneously precipitated the proteins, removed the iron ligand, and methylated the free carboxylic acid groups on the porphyrin. The methylated porphyrins were then isolated by extraction and complexed with zinc using Zn(CH<sub>3</sub>COO)<sub>2</sub>. The Zn-complex was purified by means of silica gel chromatography. HPLC analysis of the methylated Zn-complex on a C<sub>4</sub> column indicated the presence of two major radioactive peaks that eluted slightly later than those observed before derivatization (data not shown). This is consistent with ester formation. When the in vivo experiments were repeated using <sup>14</sup>C]1-NP instead of <sup>3</sup>H]1-NP, similar results were obtained. More of the Zn complex is needed to enable field desorption mass spectrometry analysis for structural information.

In vitro studies were performed to gain insight into the nature of the binding and to provide ample material for spectral analysis. We incubated <sup>3</sup>H]1-NOP and <sup>3</sup>H]1-NP-4,5-oxide with rat hemoglobin. Both compounds bound to globin, the former more so (37% of the initial radioactivity) than the latter (25%). In addition, radioactivity from both compounds was also found to be associated with the heme fraction (20%). The in vitro results do not exclude the possible contribution of these two metabolites to adduct formation in vivo. However, we did not investigate further whether the radioactivity that was covalently bound to the heme moiety was similar to that observed in vivo.

**Discussion**

**32P-Postlabeling Studies**

It was our aim to compare qualitatively the <sup>32</sup>P-maps from in vivo experiments with those obtained from DNA samples following in vitro incubations of metabolites derived from ring oxidation and ring oxidation combined with nitroreduction of 1-NP. The results suggest that such metabolites may contribute to the formation of putative 1-NP-DNA adducts found in vivo (12); however, confirmation is required. Comparing our results with those obtained by Jeffrey et al. (32) and Bond et al. (33), who exposed rats to a source containing 1-NP (eg., Diesel engine emissions), should provide insights regarding the utility of the adduct markers. It was demonstrated that animals exposed to Diesel engine emissions had higher adduct levels in the lung than did unexposed rats (32,33). Although the chemical identity of these adducts has not been determined, they were chromatographically different from the major adduct spots derived from (2)-anti-benzol[a]-pyrene-7,8-dihydrosulfone-9,10-oxide. It is possible that adducts derived from 1-NP are present in the lungs of rats after exposure to Diesel engine emissions; however, the analysis of DNA from such exposed animals and of DNA modified with metabolites of 1-NP in vitro should be conducted under identical conditions.

**Hemoglobin Studies**

We have ascertained that 1-NP binds to hemoglobin of both male and female F344 rats in a dose-response related manner over five orders of magnitude at a level of 0.08% of the dose; levels of binding were the same in male and female Sprague-Dawley rats. With the exception of 4-aminothiophenyl binding to hemoglobin (approximately 5%) (27), most other carcinogens or xenobiotics studied have been found to bind to hemoglobin at levels comparable to those measured in the present study (34). Clearance of hemoglobin-associated radioactivity (half-life = about 14 days) upon administration of a single dose of 1-NP occurred at a faster rate than that of unmodified rat erythrocytes (half-life = 30 days). The clearance rate of hemoglobin adducts varies, depending on their structure. For example, binding of hemoglobin to ethylene oxide, dimethylnitrosamine, vinyl chloride, methylmethane sulfonate, benzophenone, fluoranthene, and benzene yields adducts that have lifetimes equivalent to that of the erythrocyte life-span (35-40). In contrast, Neumann reported that several aromatic amines form hemoglobin adducts that have shorter lifetimes than that of the erythrocyte in the rat (41). Carmella and Hecht have also reported a half-life of 9.1 days for adducts formed with metabolites of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (42).

We attempted to elucidate the structure of 1-NP-hemoglobin adducts. Knowledge gained during the past decade on the metabolism of 1-NP led us to reason initially that nitroreduction could be responsible for hemoglobin binding via formation of 1-NOP. The latter compound would be expected to react with cysteine residues on hemoglobin. The adduct containing the sulfonamide bond would be labile under the acidic conditions used in this study for the hydrolysis of 1-NP-hemoglobin adducts. The acid hydrolysis of hemoglobin adducts revealed that none of the radioactive activity remained bound to the globin and more than 80% of the initial radioactivity was recovered in the heme fraction. Concentrated acid and base treatments of the heme fraction did not release any detectable derivative of 1-NP, suggesting that the radioactivity was strongly bound to the heme moiety. Other compounds (for example phenylhydrazine, 3-alkylindones, diethylaminothiazole, aminobenzotriazole, and norethindrone) have also been shown to form adducts with the heme moiety of hemoglobin as well as with heme-containing enzymes (23-26,43-45).
a lesser extent, to the heme moiety. These findings contrasted with those obtained in vivo, thus leading us to believe that these particular metabolites are not required in the binding of 1-NP to the heme moiety in vivo. However, one cannot exclude the possibility that such metabolites contribute to the in vivo binding solely on the basis of in vitro findings. Even though the structures of the 1-nitropyrene-heme adducts have not been elucidated, the fact that they are stable may render them useful as dosimetry for human exposure to 1-nitropyrene.

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