Formation of Genotoxic Nitro-PAH Compounds in Fish Exposed to Ambient Nitrite and PAH

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Mutagenic nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) have been known to arise in the environment through direct emissions from combustion sources and nitration of PAHs, primarily in the atmosphere. Here, we report the formation of nitro-PAH compounds in fish contaminated with PAH and exposed to nitrite (NO₂⁻) in the ambient water. Electrospray ionization mass spectrometric analysis of the bile of the euryhaline fish Oreochromis mossambicus exposed simultaneously to field-relevant, sublethal concentrations of phenanthrene (1 μg/g) and NO₂⁻ (1 μM) and collision-induced dissociation of selected ions revealed the presence of two strongly genotoxic nitro-PAH metabolites, namely phenanthrene-6-nitro-1,2-dihydrodiol-3,4-epoxide (mass/charge [m/z] 273) and dihydrodihydroxy acetylamino nitrophenanthrene (m/z 359). These two metabolite peaks present only in the bile of fish exposed simultaneously to phenanthrene and NO₂⁻ constituted, respectively, about 3.1 and 2.7% of the highest peak among the putative unconjugated phenanthrene metabolites in the mass spectrum. The presence of the oxidized phenanthrene metabolite dihydroxyphenanthrene (m/z 233) in fish exposed to phenanthrene alone as well as phenanthrene plus NO₂⁻ suggested that oxidation of phenanthrene precedes nitration in the sequence of reactions leading to the formation of the observed nitrophenanthrene metabolites. However, the route of PAH administration seems to determine the nature of metabolites formed. Nearly 92% of the hepatic cells of the fish exposed to phenanthrene in the presence of NO₂⁻ were found to have suffered extensive DNA fragmentation on comet assay.

Key Words: nitrite; coastal waters; nitro-PAH; fish; genotoxicity.

Potentially harmful chemical pollution and excess nutrient runoff remain top concerns in coastal areas. Coastal organisms are exposed to an assortment of environmental chemicals, natural as well as anthropogenic. Polycyclic aromatic hydrocarbons (PAHs) are one of the classic anthropogenic organic pollutants encountered in the marine environment, while the highly reactive nitrite (NO₂⁻) is produced naturally, mainly through biological processes such as denitrification (including assimilatory and dissimilatory nitrate reduction) and nitrification dominated by ammonia oxidation. NO₂⁻ accumulates in coastal waters under conditions that promote an imbalance in the above processes such as oxygen (O₂) deficiency or pollution with nitrogenous waste. In recent years, pelagic O₂ deficiency has intensified, essentially due to eutrophication, in many estuaries and coastal seas around the world (Nixon, 1995). An important biogeochemical consequence of this pelagic O₂ deficiency is the activation of alternate (anaerobic) pathways of respiration, mainly denitrification. The western continental shelf of India experiences a suboxic zone formation due to upwelling during southwest monsoon (June–September; Naqvi et al., 2000), which is presumably exacerbated by the burgeoning fertilizer use (consumption of fertilizers in the region has increased by a factor of 7 since the early 1970’s), contributing additional nitrogen loading from the land. As a result, large accumulations of the denitrification intermediates, notably NO₂⁻ (~ 1 μM near surface and > 6 μM near bottom) and nitrous oxide, occur in the water column (Naqvi et al., 2000, 2005).

Although more toxic to freshwater fish than to their marine counterpart (Eddy et al., 1983), NO₂⁻ can increase the toxicity of PAHs, leading to severe hepatic damage in estuarine fish (Shailaja and Rodrigues, 2003), impact the oxidative (Phase I) metabolism of PAHs, and enhance the formation of carcino- genic metabolites in fish exposed to refinery effluent (Shailaja et al., 2005). Mice injected with pyrene and subjected to inhalation exposure to nitrogen dioxide (NO₂) form highly mutagenic nitrogenated metabolites of pyrene (Kanoh et al., 1990). Whether marine organisms (e.g., fish) similarly produce endogenous nitrated PAHs (nitro-PAHs) when coexposed to PAHs and reactive NO₂⁻ in ambient water is not known. The main objective of the present study was to determine the manner in which NO₂⁻ present in seawater affects the toxicokinetics of PAH metabolism in fish by exposing the euryhaline fish Oreochromis mossambicus (tilapia) to a combination of phenanthrene, a three-ring PAH, and NO₂⁻ dissolved in water to yield field-relevant, sublethal concentrations of the two stressors and characterizing the biliary metabolites.

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MATERIALS AND METHODS

Exposure protocol. Juvenile O. mossambicus (9.3–11 g, n = 24) obtained from a local estuarine fish farm were acclimatized in the laboratory for 7 days in stored tap water at room temperature (~25°C) amended with reagent-grade NaCl (final salt concentration 15 g/l) to nullify the effect of nitrite in the experimental tanks. In one set of experiments, O. mossambicus (n = 6 per tank) were treated with phenanthrene (purity > 96%; Sigma, USA) dissolved in dimethylformamide and added to the tank with and without nitrite for 6 days. The final concentrations of phenanthrene and nitrite (added as analytical-grade sodium nitrite) in the exposure medium were 1 mg/l and 1μM, respectively. Control fish (n = 6) were exposed to equivalent concentrations of nitrite or phenanthrene only.

In another set of experiments, O. mossambicus (n = 6) were administered a single ip injection (1 μg/g) of phenanthrene dissolved in sunflower oil (100 μl) and exposed to dissolved nitrite (1μM) for 6 days as before. An equal number of control fish received equivalent amounts of phenanthrene without being exposed to nitrite.

The above experiments were conducted in several 30-l glass aquaria with continuous aeration and the water being changed every 24 h. Commercial feed was provided ad libitum during the period of experiment.

Comet assay for DNA strand break determination. Isolated hepatic cells of O. mossambicus (control and exposed) were subjected to modified single-cell gel electrophoresis or comet assay (Fairbairn et al., 1995; Singh et al., 1998). To obtain the cells, a small piece of the liver was washed with an excess of ice-cold Hank’s balanced salt solution (HBSS) and minced quickly into approximately 1-mm² pieces while immersed in HBSS, with a pair of stainless steel scissors. After several washings with cold phosphate-buffered saline to remove red blood cells, the minced liver was dispersed into single cells using a pipette (Lal and Singh, 1995). In brief, the protocol for electrophoresis involved embedding the isolated cells in agarose gel on microscopic slides, lysing them with detergent at high salt concentrations (overnight in the cold), treatment with alkali to denature the DNA (20 min), and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V. The slides after electrophoresis were stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX60 F-3) with a green filter at ×40 magnification. For each experimental condition, ~100 cells (about 25 cells per fish) were examined to determine the percentage of cells with DNA damage that appear like comets. Randomly selected nonoverlapping cells with DNA damage were scored on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail of length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the diameter of the nucleus), based on perceived comet tail length migration and relative proportion of DNA in the nucleus (Collins et al., 1997; Kobayashi et al., 1995). A total damage score for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing up the values. Slides were analyzed by one observer to minimize the scoring variability.

Electrospray ionization mass spectrometry. Electrospray ionization mass spectrometry (ESI-MS) analyses were performed on a quadrupole-time of flight (QTOF) mass spectrometer (Model QSTAR XL of Applied Biosystems, Rotkreuz-Switzerland) using an ion-spray source with the following settings: ion-spray voltage 5500 V, nebulizer gas (N2) 20 (arbitrary units), and curtain gas (N2) 20 (arbitrary units). The declustering potential and the collision energy were optimized around 15 and 3 V, respectively, during infusion experiment and 15 and 40 V, respectively, for MS/MS experiments. Composite untreated bile samples after dilution with methanol:water (1:1) were analyzed by direct ESI-MS. The sample was introduced at a constant flow rate of 10 ml/min into the electrospray source using an integrated syringe pump. Data acquisition and processing were carried out using mass Lynx NT version 3.5. The MS survey range was mass/charge (m/z) 0–950 (positive mode; or m/z 220–680 [negative mode] when used) with a scan duration of 1 s. Each mass spectrum was recorded over a period of 1 s and accumulated for 5 min for both single-analyzer profiles and collision-induced dissociation (CID) experiments. For each of the ion species examined, the lock mass in each product ion mass spectrum was the observed monoisotopic m/z ratio of the precursor ion. The samples were analyzed mainly in the positive ion mode. The discrepancies occasionally observed in the values for protonated molecular ions to the extent of 1 or 2 amu were due to the instrument not being calibrated each time.

Selected ions with m/z values corresponding to putative metabolites were further subjected to MS/MS or CID with N2 gas and the subsequent product ion mass spectra analyzed to produce a product ion mass spectrum.

RESULTS AND DISCUSSION

Genotoxic Effects of Coexposure to Phenanthrene and NO2⁻ in Oreoichromis mossambicus

Phenanthrene is found in the highest concentrations in aquatic samples, but unlike other PAHs such as benzo(a)pyrene and chrysene, it is not a procarcinogen (IPCS, 1998). Yet, the hepatic cells of O. mossambicus exposed simultaneously to phenanthrene and NO2⁻ in three different experiments showed considerable DNA damage when evaluated by comet assay for DNA strand break in nuclei from individual cells. In a representative experiment (Table 1), DNA fragmentation was observed in nearly 92% of the hepatic cells as compared to 9% in the control fish exposed to NO2⁻ alone and 23% in fish

<table>
<thead>
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<th>TABLE 1</th>
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<tr>
<td>No. of cells Classa Total score DNA damaged cells (% mean)</td>
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<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Nitrite (control N)</td>
</tr>
<tr>
<td>Phenanthrene (control P)</td>
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<tr>
<td>Phenanthrene + nitrite (test group)</td>
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Note. The total score was obtained by multiplying the number of cells in each class by the numeric value assigned to the class and ranged from 0 (all undamaged) to 300 (all maximally damaged).

aClass 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1× and 2× the diameter of nucleus; and 3 = tail length > 2× the diameter of nucleus.

bFrom four slides; one slide per fish scored.
exposed to only phenanthrene. The liver was chosen for study because it is the major organ for organic xenobiotic metabolism and biotransformation enzymes.

While genomic injury is the initial step in the triggering of mutagenesis and carcinogenesis, the carcinogenic potential of PAHs is linked to the formation of ultimate carcinogens generated by metabolic biotransformations. In order to characterize the compounds formed by the interaction between NO and PAH in *O. mossambicus*, the bile of the exposed fish was subjected to ESI-MS, and some selected ions therein were further analyzed by CID. Untreated bile was analyzed for the presence of free metabolites as they are potentially genotoxic. The positive ion mass spectra of the bile from fish exposed to waterborne phenanthrene alone and phenanthrene (via ip injection/water) plus NO are shown in Figures 1a, 1b, and 1c, respectively.

**FIG. 1.** Positive ESI-mass spectra showing biliary metabolites of *Oreochromis mossambicus* (a) administered phenanthrene through ip injection, (b) injected with phenanthrene and exposed to nitrite in water, and (c) coexposed to phenanthrene and nitrite through water.
Identification of Biliary Nitrophenanthrene Metabolites in Oreochromis mossambicus

Diols are one of the major metabolites of phenanthrene formed in fish (Livingstone, 1998; Pangrekar et al., 1995, 2003; Varanasi et al., 1986), about 20% of them being present as free metabolites in the bile. Putative unconjugated metabolites of phenanthrene could, therefore, be expected to have m/z values in the range of 200–400. We identified two biliary metabolites, namely phenanthrene-6-nitro-1,2-dihydriodiol-3,4-epoxide and a dihydriodihydroxy acetylamino nitrophenanthrene, based on the mass of the MH + ions and the product ion spectrum generated by CID. These metabolite peaks (m/z 273 and m/z 359) present in the bile of fish exposed to both phenanthrene and NO 2 (Fig. 1b) constituted, respectively, about 3.1 and 2.7% of the highest peak in the considered m/z range. With a collision energy of 45 eV, the CID spectrum of dihydriodihydroxy acetylamino nitrophenanthrene (disodium) ion of m/z 359 yielded product ion signals of significant intensity at m/z 313, 255, 176, 59, 46, 38.9, and 22.9 (Fig. 2a). Of these, the last three fragments could be attributed to NO 2, potassium, and sodium ions, respectively, and the signal at m/z 313 resulted from the elimination of the NO 2 radical from the molecular ion (i.e., M + 46). Removal of the acetylamino (-NHCOCH 3 ) group from the fragment of m/z 313 produced the peak corresponding to m/z 255, while the signal noted at m/z 59 is characteristic of a protonated acetylamino group. The product ion signal observed at m/z 176 represented the structure resulting from the loss of two -ONa moieties from the fragment corresponding to m/z 255. Based on the logical fragmentation pattern, the metabolite was identified as the disodium salt of dihydriodihydroxy acetylamino nitrophenanthrene. However, the regiodistribution of the -NO 2, -NHCOCH 3, and -OH groups on the molecule is unclear.

We determined a second biliary metabolite peak (m/z 273, Figs. 1b and 1c) as corresponding to phenanthrene-6-nitro-1,2-dihydriodiol-3,4-epoxide from its CID-MS/MS product ion spectrum with signals at m/z 215, 129, 114, 99, and 46 (Fig. 2b). The fragment at m/z 215 resulted from the combined loss of (NO 2 + 2H 2 O + Na) from the molecule, while the peak at m/z 129 comprised a portion of the phenanthrene ring system (C 4 H 3 NO 2 ) carrying the hydroxy and epoxy groups. Loss of a molecule of water from this ion resulted further in the signal at m/z 114. Another portion of the phenanthrene ring system containing the -NO 2 group (C 4 H 3 NO 2+2H + ) yielded the signal at m/z 99 even as the signal observed at m/z 46 represented the NO 2 fragment.

Nitro-PAHs are mostly produced either during incomplete combustion processes (pyrosynthesis) or as a result of reactions taking place in the atmosphere involving PAHs adsorbed on air particulates and gaseous nitrogenous species (Atkinson and Arey, 1994; Ciccioli et al., 1996; Sasaki et al., 1995). First detected in atmospheric particulate matter in the 1970's (Pitts et al., 1978), they are studied mainly as potential genotoxic risk factors present in the atmosphere, based on their strongly mutagenic and carcinogenic properties (Möller et al., 1993). In the water phase, nitro-PAHs can result from a photochemical reaction of PAH with nitrite (NO 2− ) donating the nitro group (Ohe, 1984; Suzuki et al., 1987). Endogenous formation of nitro-PAH compounds, however, has not been reported so far in fish although the ability of 1-nitropyrene to form DNA adducts in fish has been demonstrated both in vitro and in vivo in brown trout (Salmo trutta) and turbot (Scophthalmus maximus; Mitchelmore et al., 1998). From the mass spectra in the present study, it appears that a number of phenanthrene metabolites (not fully identified) are present in the bile, many of them as conjugates (m/z value > 400). The formation of the various nitro compounds, very likely depends on the route of PAH administration as suggested by the fact that dihydriodihydroxy acetylamino nitrophenanthrene (m/z 359) was not observed in the mass spectrum of the bile of fish coadministered phenanthrene and NO 2 through water (Fig. 1c).

The basis for the observed genotoxic effects in the hepatic cells of O. mossambicus exposed to phenanthrene in presence of NO 2 is not certain, but, presumably, the nitrophenanthrene metabolites were responsible given that very little damage occurred in the case of control fish exposed to either phenanthrene or NO 2 alone. Mitchelmore and Chipman (1998) have reported concentration-dependent increases in DNA strand breaks (using the “comet” assay) in isolated brown trout (S. trutta) hepatocytes incubated in vitro with 1-nitropyrene. Both the nitrophenanthrene metabolites identified by us are capable of inducing DNA strand breaks in O. mossambicus since hydroxylated and diol epoxide metabolites of nitro-PAHs are potent genotoxic compounds (Chae et al., 1993; Fu et al., 1982; Li et al., 1994; Möller, 1994). Conjugation reactions involving acetylation also activate chemical carcinogens (Grant et al., 1992), and according to McCann et al. (1975), hydroxylated acetylamino-PAHs (e.g., OH-acetylamino fluorene) do exhibit a low mutagenic potency. Also, interestingly, the major part of the urinary mutagenicity in the case of 2-nitrofluorene–treated rats has been found associated with the unconjugated fraction of metabolites consisting of a range of hydroxylated acetylamino- fluorenes and hydroxyfluorenes (Kiellhorn et al., 2003; Möller et al., 1987).

Possible Mode of Formation of Nitrophenanthrene Metabolites in Oreochromis mossambicus

A tentative metabolic pathway for the formation of nitrophenanthrene and its metabolites identified by ESI-MS/MS is presented in Figure 3. We speculate that, in the sequence of reactions leading to the formation of the nitrophenanthrene metabolites, ring oxidation of phenanthrene precedes nitration since the presence of the oxidized metabolite dihydroxyphe- nanthrene (m/z 233) was noted in fish exposed to phenanthrene alone as well as phenanthrene plus NO 2 (Figs. 1a–1c) and confirmed by CID-generated product ion spectrum (Fig. 2c).
FIG. 2. CID spectrum of (a) (1,2,3,4,9,10)-dihydroxyphenanthrene (m/z 233, Fig. 1a–1c), (b) dihydrodihydroxy acetylamino nitrophenanthrene, disodium salt (m/z 359, Fig. 1b), and (c) phenanthrene-6-nitro-1,2-dihydrodiol-3,4-epoxide (m/z 273, Figs. 1b and 1c).
FIG. 3. Suggested metabolic pathway of formation of nitrophen anthrenes and some identified metabolites (1,2/3,4/9,10)-dihydroxyphenanthrene (m/z 233), phenanthrene-6-nitro-1,2-dihydrodiol-3,4-epoxide (m/z 273), and dihydrodihydroxy acetylamino nitrophenanthrene, disodium salt (m/z 359) in *Oreochromis mossambicus* exposed to phenanthrene in the presence of nitrite. (1) Oxygenation reaction catalyzed by CYP1A (Buhler and Williams, 1989). (2) Nitration, possibly catalyzed by a peroxidase (Budde *et al.*, 2001). (3) Ring oxidation of nitrophenanthrene(s) by CYP1A (Fent, 2001). (4) Hydrolysis by epoxide hydrolase (Kielhorn *et al.*, 2003). (5) Catalyzed consecutively by a nitroreductase and an N-acetyltransferase (Kitamura and Tatsumi, 1996).
Results of studies conducted in several animals (hamsters, mice, etc.) to elucidate the mechanism of in vivo nitration of pyrene have also suggested that PAH undergoes hydroxylation in the liver before being nitrated (Miyanishi et al., 1996). Nitration of the hydroxyl groups by NO\textsubscript{2} possibly involves the action of a peroxidase (Budde et al., 2001). The further sequence of reactions likely includes ring oxidation of the nitro-PAH (resulting in the formation of phenanthrene-6-nitro-1,2-dihydrodiol-3,4-epoxide; \textit{m/z} 273) and nitroreduction and acetylation (leading to the formation of dihydrodihydroxy acetylamino nitrophenanthrene; \textit{m/z} 359). The ring oxidation of the nitro-PAH involves the biotransformation enzyme CYP1A whose induction by a mixture of PAH and nitro-PAH is additive (Fent, 2001). This is also supported by our previous observations of nearly 100 and ~270% increase in hepatic 7-ethoxyresorufin O-deethylase activity compared to normal in \textit{O. mossambicus} coexposed to phenanthrene and NO\textsubscript{2} and a PAH-containing effluent and NO\textsubscript{2}, respectively (Shailaja and Rodrigues, 2003; Shailaja et al., 2005).

It is known that interactions with other pollutants can play important modulatory roles in the expression of PAH toxicity (Chaloupka et al., 1993). But this study presents the only observation so far of potentiation of mutagenicity of a noncarcinogenic PAH (e.g., phenanthrene) in fish by ambient NO\textsubscript{2}. Elevated NO\textsubscript{2} levels are generally limited to certain hypoxic habitats and habitats receiving nitrogenous pollutants; however, the ubiquitous nature and strong bioconcentration capacities of PAHs in the marine environment increase the likelihood that the type of interactions discussed will occur. Also, given the importance of coastal systems and the increasing pressure on them, it would be important to investigate the immediate as well as long-term effects of such interactions on ecosystem health for, in the final reckoning, human health depends on thriving ocean ecosystems.

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