Bull Environ Contam Toxicol (2007) 79:319–322
DOI 10.1007/s00128-007-9259-7

Enhanced Bio-Mineralization by Riboflavin Photosensitization and its Significance to Detoxification of Benzo[a]pyrene

X. Zhao · Y. Zhang · X. Hu · H. M. Hwang

Received: 10 January 2007 / Accepted: 18 July 2007 / Published online: 23 August 2007

Abstract In this study, ^{14}C-benzo[a]pyrene (BaP) was chosen as a model compound to investigate if photosensitization by riboflavin enhances the subsequent microbial mineralization of polycyclic aromatic hydrocarbons (PAHs) in natural aquatic environments. After photolysis, BaP showed an increased toxicity to human epithelial cell and natural microbial assemblage. However, BaP mineralization rate in a river water sample containing riboflavin is roughly twice of that without riboflavin after the 2-day incubation. Thus, the results imply that microbial assemblage can mineralize BaP photoproducts to carbon dioxide and a combination of riboflavin photosensitization and microbial degradation could lead to complete detoxification of PAHs.

Keywords Benzo[a]pyrene · Mineralization · Photosensitizer · Riboflavin

Polycyclic aromatic hydrocarbons (PAHs) are a class of fused-ring aromatic compounds which are found in air, natural waters, soil, and in marine environments and usually exhibit high stability for high-molecular weight analogues with four or more fused rings (Manodori et al. 2006). PAHs mainly arise in incomplete combustion from both anthropogenic and natural activities such as power production, petroleum refining or by automobile emissions and forest fires. The industrial and chemical manufacturing processes such as coal gasification produce effluents containing PAHs which are released into the environment (Qiao et al. 2006). Many PAHs are of concern for both human and the environment health due to their acute toxicity, mutagenicity, or carcinogenicity (Arfsten et al. 1996). It is well-known that certain metabolites of benzo[a]pyrene (BaP), e.g., epoxides and diol epoxides, bind with DNA to form stable adducts and are responsible for the mutagenic activity (Baird et al. 2005). In addition, exposure to PAHs also induces immunosuppression in humans (Laupeze et al. 2002). Thus it is desirable to develop suitable techniques for remediation of the environment contaminated by these hazardous chemicals.

The use of biodegradation technologies has been generally considered having cost and technical advantages among the engineered processes for the treatment of PAHs (Shemer and Linden 2007). However, PAHs with five or more fused rings such as BaP are resistant to microbial degradation due to their extremely low water solubility and their complex chemical structure. Photolysis of PAHs is useful for enhancing their biodegradation because PAHs may readily absorb UV light energy and subject to photolytic breakdown. Some natural photosensitizers, for example riboflavin (vitamin B_2), have been demonstrated to facilitate the photochemical transformation of many persistent pollutants including PAHs (Zhao et al. 2006). Photochemical transformation served as the main pathway for degradation of selected higher molecular weight PAHs (Lehto et al. 2003). However, some intermediates of PAHs showed cytotoxic activity and caused damage of DNA (Hu et al. 2007; Lampi et al. 2005) thus bringing necessities to study environmental effects of photosensitization intermediates and their role in further biodegradation, i.e., mineralization of PAHs to CO_2 and H_2O. To our best knowledge, the role of photosensitizers in complete degradation (i.e., mineralization) or detoxification of these persistent pollutants, however, has been rarely studied.

X. Zhao · Y. Zhang · X. Hu · H. M. Hwang (✉)
Department of Biology, Jackson State University, Jackson, MS 39217, USA
e-mail: hwang@jsums.edu

In this report, ^{14}C labeled BaP was chosen as a model compound for PAHs in order to evaluate the effect of riboflavin photosensitization on the subsequent microbial mineralization of BaP. The objective of this study was to investigate the toxicity of photosensitized intermediates and the effect of the photosensitizer on mineralization of high molecular weight PAHs, i.e., BaP, in natural water, resulting in production of carbon dioxide as the terminal degradation products.

Materials and Methods

Radiolabled [7-^{14}C]BaP with specific activity of 26.6 mCi/mmol was obtained from Sigma Chemical Co. (Milwaukee, WI, USA). Radiochemcial purity was >98%. Riboflavin was also obtained from Sigma. 1,6-Benzo[a]pyrene-dione, 3,6-benzo[a]pyrene-dione, 6,12-benzo[a]pyrene-dione were purchased from Midwest Research Institute (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade purity.

The stock solution of [7-^{14}C]BaP was made in methanol. Natural river water sample was taken from Ross Barnett Reservoir (Ridgeland, MS, USA). For photolysis treatment, 0.016 μM labeled BaP was added to 150 mL quartz flasks (GM Associates Inc., Oakland, CA, USA) containing 25 mL autoclave sterilized river water with or without 5 μM riboflavin. This amount of riboflavin was confirmed to efficiently photosensitize BaP in our previous study (Zhao et al. 2006). The samples were irradiated under natural sunlight in a waterbath outdoors for 5 h (10:00 a.m. to 3:00 p.m.). To avoid the cell-damaging effect of the reactive chemical species formed in the irradiation process, samples were aged and cooled in darkness for 4 h. Then the irradiated solutions were added with another 25 mL sterilized or live river water and incubated for up to 2 days at 25°C in darkness. Abiotic controls were prepared by adding 1 mL formaldehyde solution (37%). At the end of the incubation, 0.5 mL of 2 N H_2SO_4 was added to the sample in order to terminate the biodegradation and the mineralization rate was determined by analysis of the triplicate samples.

The mineralization of radiolabeled BaP was quantified by trapping $^{14}CO_2$ produced from the incubated flasks. All samples were analyzed for ^{14}C-activity with 8 mL of Ultima Gold high flash point LSC cocktail from Parkard (Meriden, CT, USA). The $^{14}CO_2$ produced was trapped with 2-phenylethylamine soaked filter paper (Whatman #1) and the radioactivity of the filter paper was measured with liquid scintillation spectrometry (Packard Instrument, model TR 1600). Mineralization of benzo[a]pyrene was calculated based on the $^{14}CO_2$ evolved during the incubation period.

Cytotoxicity tests were conducted with human skin cell line. HaCaT cell line was chosen in this study because it was the permanent epithelial cell from adult human skin that exhibited normal differentiation capacity. HaCaT cells were grown and were harvested based on the method used in our previous research (Hu et al. 2007). One hundred micro liters (1×10^6 cells/mL) HaCaT cells were seeded in 96-well plates. Then, 100 μL of different concentrations of 1,6-benzo[a]pyrene-dione, 3,6-benzo[a]pyrene-dione, 6,12-benzo[a]pyrene-dione samples in DMSO (final concentration 4%) was added to each well to achieve the final concentrations of 0.05, 0.1, 0.5, 1 and 5 μM. The concentration range of samples was chosen according to their solubility in PBS buffer (containing 8% DMSO). A solvent control (containing 4% DMSO) and a positive control in PBS (100 μM H_2O_2) were included to assure all the experimental procedures were correctly followed. There were eight replicates in each concentration group. After 30 min of treatment, an aliquot of 100 μL of fluorescein diacetate uptake (FDA) (10 ng/mL) was added to the cell/chemical mixtures in 96-well plates. The plates were incubated at 37°C for 35 min in a CO_2 incubator and read by using a microplate reading spectrofluorimeter (Triad series, Dynex Technologies, VA, USA) with the filter set at emission wavelength of 538 nm and excitation of 485 nm, respectively. Microbial viability was determined by spread plate method (Zeng et al. 2004). River water (pH 6.8, 25°C) was collected from Ross Barnett Reservoir (Ridgeland, MS, USA) on the day of experiment. Different concentration of BaP and 1,6-benzo[a]pyrene-dione, 3,6-benzo[a]pyrene-dione, 6,12-benzo[a]pyrene-dione were tested by this method. Statistical analysis of the experimental results was conducted by factorial arrangement of treatments in a complete randomized design using SAS (Cary, NC, USA) with the general linear model.

Results and Discussion

BaP is one of the most prevalent PAHs with five condensed rings. The persistence is largely due to the resonance energy of its structure and extremely low solubility in water. Riboflavin, a well-known secretion product of phytoplanktons, is present in the waters of rivers, lakes, and seas (Momzikoff et al. 1983). The light absorption spectrum of BaP is complicated and consists of several bands, with major bands maxima at 265, 296, 364, and 385 nm. Our previous research showed that efficient sensitization of BaP can be achieved by using riboflavin under visible light irradiation to enhance the transformation of this recalcitrant pollutant (Zhao et al. 2006). The principal photoproduct of riboflavin, lumichrome, and a minor photoproduct, lumiflavin, were also shown to photosensitize BaP under natural

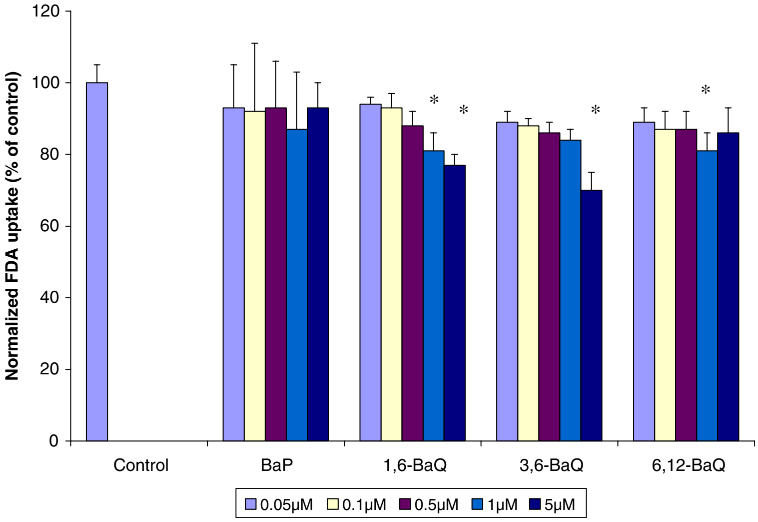

Fig. 1 Normalized FDA uptake by HaCaT cells of BaP, 1,6-benzo[a]pyrene-dione (1,6-BaQ), 3,6-benzo[a]pyrene-dione (3,6-BaQ), 6,12-benzo[a]pyrene-dione (6, 12-BaQ) at the concentrations of 0.05, 0.1, 0.5, 1, and 5 μM. *Asterisks* significantly different from the corresponding control ($p < 0.05$)

sunlight after riboflavin diminished. The major photoproducts of BaP were identified as 1,6-benzo[a]pyrene-dione, 3,6-benzo[a]pyrene-dione, 6,12-benzo[a]pyrene-dione.

Quinones are toxic intermediates which could create a variety of hazardous effects in vivo, including acute cytotoxicity, immunotoxicity and carcinogenesis (Hu et al. 2007). In the FDA uptake experiments with HaCaT cells, treatments with quinone intermediates exhibited significant cytotoxicity compared to the control group ($p < 0.05$). Mostly, quinone intermediates caused a dose-dependent decrease in cell viability at the concentration as low as of 1 μM (Fig. 1). Results from spread plate measurement showed that two of three BaP quinones, i.e., 1,6-benzo[a]pyrene-dione, 3,6-benzo[a]pyrene-dione, were far more toxic to microbial assemblage than the parent compound (data not shown). BaP showed no significant toxicity in the tests, probably due to the fact that BaP has not undergone a metabolic activation to form reactive intermediates for causing toxic effects in the skin cells. The increased toxicity of the quinone intermediates could raise human and environmental health concerns. Thus, further degradation of these toxic intermediates into inorganic and non-toxic chemicals through other means (e.g., microbial degradation) is needed.

The microbial degradation of ^{14}C-BaP was conducted in river water after photolysis under natural sunlight. The BaP mineralization patterns observed in the different treatment groups are shown in Fig. 2. The mineralization rate of BaP is increased for the samples with riboflavin. The mineralization rate of BaP with riboflavin is roughly twice of that the samples without 5 μM riboflavin after the 2-day incubation. In sterilized control groups, abiotic mineralization rate was negligible. Apparently the presence of riboflavin greatly enhanced the rate of BaP photolysis by natural sunlight. The major photoproducts of BaP, including 1,6-benzo[a]pyrene-dione, 3,6-benzo[a]pyrene-dione, 6,12-benzo[a]pyrene-dione, were subjected to microbial degradation; consequently caused the increased evolution of $^{14}CO_2$ in the photosensitized samples. In addition, the increased aqueous solubility of quinone intermediates and their further degradation intermediates could increase their bioavailability for the natural microorganisms, become the growth substrates for them, and are mineralized to carbon dioxide.

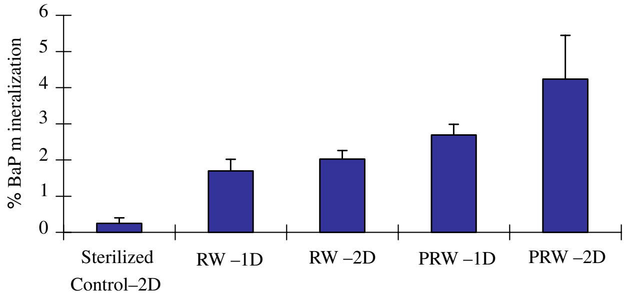

Fig. 2 Trapped $^{14}CO_2$ from biodegradation of labeled benzo[a]pyrene after photolysis in the presence and absence of riboflavin. *Sterilized Control-2D* Sterilized control groups incubated with river water treated with formaldehyde solution after 2 days; *RW* incubated with live river water without riboflavin after 1 or 2 days; *PRW* incubated with live river water with riboflavin photosensitization after 1 or 2 days

In this study, solar irradiation of BaP in the presence of riboflavin readily degrades BaP and the photoproducts, e.g., BaP-quinones, are further mineralized by microorganisms. The results imply that a combination of natural photosensitizer such as riboflavin and microbial degradation is a promising approach to the removal of PAHs (Fig. 3). The mineralization data are based on [7-^{14}C]BaP used in this study; therefore, the degradation of other aromatic rings in the BaP structure was overlooked. An underestimation of mineralization might happen. On the other hand, detection of $^{14}CO_2$ did not represent destruction of all the rings in the BaP molecule either. Nevertheless, the evidence of ring opening and transformation of BaP to

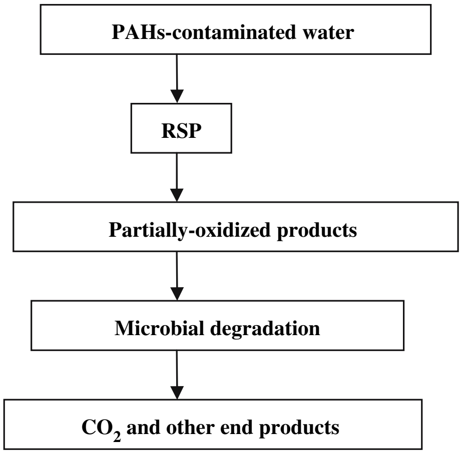

Fig. 3 Proposed treatment of PAHs-contaminated water with riboflavin-sensitized photolysis (RSP) and microbial degradation

CO_2 was unequivocally provided. Our finding indicates that photolysis of BaP and similar PAHs using riboflavin represents a useful treatment option that could be applied to facilitate the detoxification and environmentally safe disposal of these pollutants.

Acknowledgments This research was supported by the following grants: (1) NIH-SCORE S06GM08047 (to JSU) and (2) U.S. Department of the Army # DAAD 19-01-1-0733 and # W911NF-04-1-0327 to JSU. This project was performed, in part using compounds provided by the National Cancer Institute's Chemical Carcinogen Reference Standards Repository operated under contract by Midwest Research Institute, No. N02-CB-07008. Authors also appreciate Sean Cook of Department of Biology at JSU for his help in the experiment.

References

Arfsten DP, Schaeffer DJ, Mulveny DC (1996) The effects of near ultraviolet radiation on the toxic effects of polycyclic aromatic hydrocarbons in animals and plants: a review. Ecotoxicol Environ Saf 33:1–24

Baird W, Hooven L, Brinda M (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ Mol Mutagen 45:106–114

Hu X, Zhang Y, Zhao X, Hwang HM (2007) Cytotoxicity and genotoxicity of benzo[a]pyrene and its enzymatic degradation intermediates to HaCaT and A3 cells. Environ Mol Mutagen 48:106–113

Lampi M, Gurska J, McDonald K, Xie F, Huang XD, Dixon DG, Greenberg BM (2005) Photoinduced toxicity of polycyclic aromatic hydrocarbons to *Daphnia magna*: ultraviolet-mediate effects and the toxicity of polycyclic aromatic hydrocarbon photoproducts. Environ Toxicol Chem 25:1079–1087

Laupeze B, Amiot L, Sparfel L, Ferrec EL, Fauchet R, Fardel O (2002) Polycyclic aromatic hydrocarbons affect functional differentiation and maturation of human monocyte-derived dendritic cells. J Immunol 168:2652–2658

Lehto K, Puhakka J, Lemmetyinen H (2003) Photodegradation products of polycyclic aromatic hydrocarbons in water and their amenability to biodegradation. Polycycl Aromat Comp 23:401–416

Manodori L, Gambaro A, Piazza R, Ferrari S, Stortini AM, Moret I, Capodaglio G (2006) PCBs and PAHs in sea-surface microlayer and sub-surface water samples of the Venice Lagoon (Italy). Mar Pollut Bull 52:184–192

Momzikoff A, Santus R, Giraud M (1983) A study of the photosensitizing properties of seawater. Mar Chem 12:1–14

Qiao M, Wang C, Huang S, Wang D, Wang Z (2006) Composition, sources, and potential toxicological significance of PAHs in the surface sediments of the Meiliang Bay, Taihu Lake. China Environ Int 32:28–33

Shemer H, Linden KG (2007) Photolysis, oxidation and subsequent toxicity of a mixture of polycyclic aromatic hydrocarbons in natural waters. J Photochem Photobiol A Chem 187:186–195

Zeng K, Hwang HM, Zhang Y, Cook S (2004) Assessing cytotoxicity of photosensitized transformation products of 2,4,6-trinitrotoluene (TNT) and atrazine with freshwater microbial assemblages. Environ Toxicol 19:490–496

Zhao X, Hu X, Hwang HM (2006) Effects of riboflavin on the phototransformation of benzo[a]pyrene. Chemosphere 63:1116–1123