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Multiphoton spectral analysis of benzo[*a*]pyrene uptake and metabolism in a rat liver cell line

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ABSTRACT

Dynamic analysis of the uptake and metabolism of polycyclic aromatic hydrocarbons (PAHs) and their metabolites within live cells in real time has the potential to provide novel insights into genotoxic and non-genotoxic mechanisms of cellular injury caused by PAHs. The present work, combining the use of metabolite spectra generated from metabolite standards using multiphoton spectral analysis and an "advanced unmixing process", identifies and quantifies the uptake, partitioning, and metabolite formation of one of the most important PAHs (benzo[*a*]pyrene, BaP) in viable cultured rat liver cells over a period of 24 h. The application of the advanced unmixing process resulted in the simultaneous identification of 8 metabolites in live cells at any single time. The accuracy of this unmixing process was verified using specific microsomal epoxide hydrolase inhibitors, glucuronidation and sulfation inhibitors as well as several mixtures of metabolite standards. Our findings prove that the two-photon microscopy imaging surpasses the conventional fluorescence imaging techniques and the unmixing process is a mathematical technique that seems applicable to the analysis of BaP metabolites in living cells especially for analysis of changes of the ultimate carcinogen benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide. Therefore, the combination of the two-photon acquisition with the unmixing process should provide important insights into the cellular and molecular mechanisms by which BaP and other PAHs alter cellular homeostasis.

Introduction

PAHs are persistent organic environmental contaminants formed as by-products of incomplete combustion of fossil fuels. These compounds have been identified in ground and rain water, tap water, waste water, sewage sludge and foodstuffs (Ramesh et al., 2004b; Samanta et al., 2002). Due to their ubiquitous presence and toxicity, PAHs are among the most important environmental pollutants; and several PAHs such as benzo[*a*]pyrene (BaP) and PAH mixtures are carcinogenic. BaP is an extensively studied prototype carcinogen; exposure to BaP by inhalation results in rapid uptake and distribution to several tissues with the highest levels found in liver, esophagus, small intestine, and blood within 30 min to 1 h after exposure (Ramesh et al., 2002; Weyand and Bevan, 1986). BaP exerts its toxic and mutagenic effects through bioactivation and generation of reactive metabolites (Phillips, 2005) and hepatobiliary excretion is a major route for elimination of hydroxylated metabolites and other conjugates.

The metabolism of BaP is complex and numerous metabolites are generated in the biotransformation process including hydroxylated intermediates, epoxides, quinones, dihydrodiols, dihydrodiol epoxides (see supplemental Fig. S1) and various metabolite-conjugates in cells (Bolton et al., 2000; Shimada et al., 2002). Some of these metabolites such as benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BPDE) can alkylate DNA to form BaP–DNA adducts which have been associated with BaP-induced carcinogenesis (Gammon et al., 2004). In addition, many of the major metabolites can be conjugated to glucuronic acid, sulfate and glutathione to become more water soluble facilitating excretion (Zhu et al., 2008).

An interesting and useful property of several PAHs and their metabolites is that they fluoresce efficiently in solution (Dabestani, 1999). The multiple fused aromatic ring systems are highly fluorescent due to their delocalized pi bonds which permit relatively low-energy photons to excite their electrons into excited states that return

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to the ground state with the emission of fluorescence. This property has been exploited to detect and evaluate environmental PAH contamination (e.g., Wild et al., 2007; Weston et al., 1993; Goryacheva et al., 2005), tissue accumulation of PAHs and their metabolites, and DNA and protein adducts by high performance liquid chromatography and fluorescence detection (Gmur and Varanasi, 1982; Xu and Jin, 1984; Boysen and Hecht, 2003). The fluorescence properties of BaP have also been used to investigate the processes of tissue penetration and metabolism in vivo in a mouse skin model (Lopp et al., 1986). At the cellular level, the characteristic fluorescence of BaP has previously been exploited to monitor mixed function oxygenase activity in cell populations by flow cytometry (Miller and Whitlock, 1982) and in individual anchored cells in culture (Plant et al., 1985; Moore et al., 1994).

One of the challenges in correlating exposure levels and adduct formation for the purpose of quantitative exposure assessments (Gammon et al., 2004) is the lack of analytical methods that can directly identify the presence and levels of BaP and its reactive metabolites in real time within living cells and tissues. Using laser cytometry, we previously evaluated the rapid uptake and partitioning of BaP into the plasma membrane and membranes of intracellular organelles within minutes after addition of the fluorescent genotoxicant (Barhoumi et al., 2000) and analyzed a number of non-genotoxic effects of BaP on cell signaling in cultured cells (Barhoumi et al., 2002; Barhoumi et al., 2006). Because BaP is photosensitive and breaks down when exposed to UV light, single cell assessment of BaP uptake and metabolism using conventional fluorescence microscopes or continuous wavelength laser confocal microscopes has been limited to BaP identification in cells without quantification. However, integration of pulsed femtosecond infrared laser systems in multiphoton microscopes provide high detection sensitivity and minimal fluorophore excitation volumes to reduce photobleaching, thereby providing new opportunities for investigating BaP uptake and metabolism in situ (Hornung et al., 2007; Barhoumi et al., 2009) as well as the consequences of BaP exposure within individual cells.

The objective of the current study was to extend the previous single cell multiphoton microscopic analysis of cellular BaP uptake and partitioning in order to better evaluate BaP metabolism in situ using: 1) multiphoton microscopy spectral acquisition to minimize the effect of UV exposure on BaP autofluorescence and metabolite generation, and 2) a spectral unmixing process that uses the unique spectral properties of metabolites generated from standards in order to detect their presence and levels in cells treated with BaP. The normal rat liver cell line (Clone 9) used in this study exhibits an increase in ethoxyresorufin-*O*-deethylase (EROD) activity in response to PAH treatment and has been utilized as a model system for investigating the nongenotoxic effects of BaP (Barhoumi et al., 2002). These liver cells were used to identify BaP parent compound and reactive metabolites generated within cells in real time, to quantify these metabolites using database reference spectra generated with reference standards, and to use specific metabolic inhibitors to modify metabolite profiles. The development of this real time approach for investigating BaP metabolism in living cells will provide insights on the rates of metabolite formation and persistence and also facilitate development of agents that will block critical mutagenic/carcinogenic metabolic pathways (Hecht, 2002).

Materials and methods

Materials. Ham's F-12 culture media, Dulbecco's phosphate buffered saline (PBS), Janus green, β-glucuronidase (keyhole limpet, #G8132), triclosan (Irgasan), pyrene (Pyr), resorufin ethyl ether and 3,3′-methylene-bis(4-hydroxycoumarin) (dicumarol) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from Equitech-Bio (Kerrville, TX). Benzo[*a*]pyrene, benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide(±), (*anti*) (BPDE), benzo[*a*]pyrene-3,6-dione (3,6BPQ), 3-hydroxybenzo[*a*]pyrene (3OH), 9-hydroxybenzo[*a*]pyrene (9OH), benzo[a]pyrene-*trans*-7, 8-dihydrodiol(±) (*t*7,8), benzo[*a*]pyrene-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol(±) (*c*-tetrol), benzo[*a*]pyrene-*r*-7,*t*-8,*t*-9,*t*-10-tetrahydrotetrol(±) (*t*-tetrol), benzo[a]pyrene-3-sulfate, potassium salt (3-S), benzo[a]pyrene-9-sulfate (9-S), 9-benzo[a]pyrene-β-D-glucopyranosiduronic acid (9-G) were purchased from Midwest Research Institute (Kansas City, MO) which operates the Chemical Carcinogen Reference Standard Repository. Analytical data provided with each standard was reported as >99% pure by HPLC and UV/visible spectra. These UV/visible spectra were also confirmed prior to use in cells. Soluble epoxide hydrolase (sEH) inhibitors used included 1-(1-acetyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS, 1709; Rose et al., 2010) and *trans*-4-[4-(3-trifluoromethoxyphenyl-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-TUCB, 1728; Hwang et al., 2007). Microsomal epoxide hydrolase (mEH) inhibitors used included 2-nonylsulfanyl-propionamide (mEH #16) and 10-hydroxy-octadecanamide (mEH #29) (Morisseau et al., 2008). Tissue culture flasks, 2-well Lab-Tek chambered coverglass slides and 96-well Greiner glass bottom multi-well plates were purchased from Thermo Fisher Scientific (Waltham, MA). BaP, Pyr, 3,6BPQ, 3OH, 9OH, 3-S, 9-S, 9-G, *t*7,8, and BPDE were each prepared as 10 mM stocks in DMSO. Resorufin ethyl ether was prepared as a 7 mM stock in methanol and diluted to 7 μM for EROD activity measurement. Janus green was prepared in PBS at 1 mg/ml.

Cell culture. The normal rat liver cell line, Clone 9 (CRL 1439) was obtained at passage 17 from American Type Culture Collection (Manassas, VA) and used between passages 25 and 35. Cultures were approximately 80% confluent at the time of analysis.

Ethoxyresorufin-O-deethylase (EROD) activity. EROD activity is a biomarker of exposure to planar halogenated and polycyclic aromatic hydrocarbons (PHHs and PAHs, respectively) and provides evidence of aryl hydrocarbon receptor-mediated induction of cytochrome P450-dependent monooxygenases (Donato et al., 1993). To identify the contribution of BaP and each of its metabolites to the induction of EROD activity, cells were plated on 96-well plates at 25,000/well for 48 h prior to treatment with different concentrations of BaP, 3OH, 9OH, *t*7,8, BPDE, 3-S, 9-S, 9-G, or 3,6BPQ. Following treatments for 3 h, cells were then washed twice with PBS and loaded with 7 μM resorufin ethyl ether and 10 μM dicumarol for 30 min. EROD activity was measured using a BioTek Synergy 4 plate reader (Biotek Instruments Inc., Winooski, VT, USA) with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. For comparison of EROD activity between different concentrations within the same treatment, cell number per well was determined using the Janus green assay (as described below) and EROD fluorescence intensities measured were corrected accordingly. Eight samples per concentration were collected and at least 3 experiments were preformed on different days.

Cell count/Janus green assay. Cells were incubated in 96-well plates, and after treatments were washed with PBS (2×) and fixed with 100% methanol for 30 min at room temperature. Methanol was then completely removed and 1 mg/ml Janus green was added to the cultures for 3 min. Following removal of Janus green, cultures were washed twice with PBS and 100 μl of 50% methanol was added to each well. Cell counts were then determined with a BioTek Synergy 4 plate reader set to an absorbance of 630 nm (Raspotnig et al., 1999).

Single cell multiphoton spectral analysis of BaP and BaP metabolite standards. Clone 9 cells were cultured for 24 h in Ham's Nutrient Mixture F-12 with 10% fetal bovine serum on 2-well Lab-Tek slides following plating at a density of 10^5 cells per well. Cells were then washed and incubated for 24 h with 2 μM of one of the following