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Bile fluorescence, heme oxygenase induction, and increased biliverdin excretion by mixtures of environmental toxicants

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Abstract

The measurement of bile fluorescence has become a popular biomarker to demonstrate the exposure of fish to polycyclic aromatic hydrocarbons. Conflicting data has been published on how to normalize bile fluorescence. To investigate if normalization to biliverdin is a suitable method, experiments were performed to study the mechanisms related to biliverdin excretion in fish. In two separate experiments channel catfish (*Ictalurus punctatus*) were dosed with mixtures of benzo[a]pyrene with cadmium, chlorinated phenols or borneol. The results showed linear relationships between bile protein and biliverdin for each treatment group, but the slope of this relationship was significantly increased when fish received more chemical stress. Thus, under increasing toxicant stress, more biliverdin was excreted per amount of protein. To investigate if the increased biliverdin excretion was related to increased heme degradation, enzymatic activity of heme oxygenase (HO) was measured in liver homogenates of the dosed fish. The fish dosed with chemical mixtures had a significantly higher HO activity than the control fish, and in both experiments a significant correlation was observed between HO activity and biliverdin concentration in the bile. It is concluded that mixtures of environmental pollutants can induce HO activity and that this chemical stress leads to increased biliverdin excretion. The elucidation of this mechanistic pathway warrants that bile fluorescence should not be expressed per biliverdin absorption, and that expression per bile protein would be a more reliable method.

Keywords: Bile fluorescence; Biomarker; Biliverdin; Heme oxygenase; Channel catfish

1. Introduction

The release of polycyclic aromatic hydrocarbons (PAHs) into the environment has been recognized as a serious environmental problem for decades (Andelman and Suess, 1970). The main sources of PAHs are incomplete combustion of organic material and release from fossil fuel sources. This group of compounds is notorious for the potential carcinogenic effect of some of its members, while others exert a narcotic effect or, in combination with UV radiation, a phototoxic effect (Yu, 2002). While for some groups of environmental pollutants drastic regulatory measures have been taken to avoid further contamination of the environment, the continuing input of PAHs from anthropogenic and natural sources into the environment hamper comparable measures, leading to the assumption that these compounds will continue to pollute the environment for the decades to come.

To monitor the exposure and potential effects of PAHs in wildlife and aquatic organisms, a suite of biomarkers have been developed (Van der Oost et al., 2003). These biomarkers indicate a biochemical response to the PAH exposure which would result in a rapid metabolism and excretion of the compounds. Induction of cytochrome P450-1A, as measured by the EROD assay, has become the gold standard biomarker for PAH exposure. Biomarkers for actual damage by PAHs are the measurement of DNA adducts and lipid peroxidation leading to membrane damage. A relatively simple biomarker for PAH exposure is the measurement of PAH metabolites in bile (Krahn et al., 1984). Aromatic hydrocarbons are mostly insoluble in aqueous media, and thus, to enhance excretion through urine or bile, they need to be metabolized and changed into more water-soluble metabolites (Varanasi et al., 1989). Most organisms have a well-developed system of metabolizing enzymes, usually described as Phase 1 and Phase 2 enzymes, which facilitate the excretion of poorly water-soluble xenobiotic compounds. This explains why PAHs do not accumulate in most organisms, and why tissue concentrations of PAHs are poor indicators for environmental concentrations and exposure.

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Aromatic hydrocarbons, including their metabolites, are highly fluorescent, and can easily be measured in bile samples (Lin et al., 1996; Aas et al., 2000). Thus, if animals have been exposed to PAHs, and PAH metabolites are being formed and excreted, fluorescence of bile samples will increase. A problem with measuring fluorescence of bile samples is the normalization of the fluorescence data (Aas et al., 2000). Three methods for normalization have been proposed and used: fluorescence units per bile volume or weight, units per mg protein in bile, and units per amount of biliverdin in bile. The first method appears to be the most straightforward, but bile volume is highly variable, depending on feeding rates, and under reduced feeding regimes, bile appears to undergo concentration (Richardson et al., 2004). This can make expression of fluorescence per bile volume highly variable. Expression of fluorescence in relation to the amount of biliverdin in bile has been investigated, but led to contradictory results (Aas et al., 2000; Ruddock et al., 2003). In these studies, recommendations for normalization were based on reducing the variability in the data, not on underlying mechanistic pathways.

Biliverdin is the breakdown product of heme, the iron containing moiety in hemoglobin and cytochrome enzymes. Heme is in a continuous physiological cycle because of the limited life time of red blood cells and the natural fluctuations of heme containing enzymes. Excess heme is metabolized in the liver, where heme oxygenase catalyzes the conversion step of heme into biliverdin (Dennery, 2004). Thus, biliverdin excretion into the bile is modulated by heme oxygenase activity. From previous studies it is known that HO activity can be modified by environmental pollutants, most specifically by heavy metals and other oxidative stressors (Alam and Cook, 2003). This was not only observed in mammalian species, but also in several fish species (Jorgensen et al., 1998). The induction of heme oxygenase activity by exposure to environmental pollutants led to the hypothesis that also biliverdin excretion is related to chemical stress, and that therefore relating bile fluorescence to biliverdin in animals that are exposed to a mix of PAHs and other environmental toxicants, is not a reliable method. The objectives for the studies presented here were: (1) to evaluate if bile protein and biliverdin concentrations are equally suitable as normalization parameters for bile fluorescence caused by benzo[a]pyrene metabolism, (2) to investigate if heme oxygenase activity is increased by exposure to mixtures of environmental pollutants, and (3) to investigate if increased heme oxygenase activity in the liver is related to increased biliverdin excretion into the bile.

2. Methods

2.1. Experiments

Results from two separate experiments were used for this study. Both experiments were performed with channel catfish (*Ictalurus punctatus*), which were cultured and raised in the Clemson University Fish Health Laboratory. The fish cultures and the experiments described here were performed according to guidelines of the Clemson University Animal Research Committee. During the experiments, the fish were kept in flow-through tanks with filtered lake water, with a turn-over of three volumes per hour. The temperature was kept at 20 °C and the fish were fed daily. The fish had an average length of 241 mm (±18 S.D.) and an average weight of 151 g (±43 S.D.).

2.1.1. Experiment 1

Channel catfish were dosed with 100 μl intraperitoneal injections of benzo[a]pyrene (5 and 50 mg/kg, in canola oil), cadmium (0.5 and 5 mg/kg in deionized water) or a combination of both (0.5 mg/kg Cd + 50 mg/kg BaP). Animals were maintained in flow-through tanks and harvested after 3 days. Livers and gall bladders were frozen in liquid nitrogen and stored at −80 °C until further analysis. Sample size was five fish per treatment.

2.1.2. Experiment 2

Channel catfish were dosed (i.p.) with benzo[a]pyrene (10 mg/kg) alone or in combination with pentachlorophenol (PCP), trichlorophenol (TCP) and borneol (each 25 mg/kg). These compounds were selected because of their known effects on biotransformation enzymes, and because animals can be exposed to combinations of these compounds in contaminated environments. Animals were maintained in flow-through tanks and harvested at days 2, 3 and 6. Livers and gall bladders were frozen in liquid nitrogen and stored at −80 °C. Sample size was three fish per treatment per sampling day.

2.2. Bile analysis

After thawing, gall bladders were punctured, and bile was dissolved in 1 ml acetate buffer (0.1 M, pH 5.0). Samples were centrifuged at 14,000 rpm for 10 min. Two subsamples (25 μl) of the supernatant were analyzed for protein content with the Pierce^{TM} bicinchoninic acid (BCA) protein assay, using bovine serum albumin to prepare a standard curve. Biliverdin absorption in the supernatant was measured at 365 nm in a 1:5 dilution in methanol/water (50:50, v:v), using a Molecular Devices Spectramax 190 UV microplate reader. Biliverdin absorption is often measured at 660 nm, but the molar absorption is higher at 365 nm (Gray et al., 1961). Benzo[a]pyrene fluorescence was measured in diluted samples in a Molecular Devices Gemini fluorescence microplate reader at 380 nm excitation, 430 nm emission. To check for inner-filter effects, a dilution series was prepared of a control and a highly fluorescent sample, and it was determined that a 1:1000 dilution was optimal to avoid quenching but maintain accuracy.

2.3. Subcellular liver fractions

Frozen liver samples were thawed and homogenized in ice-cold 0.25 M Sucrose/0.05 M Tris buffer (pH 7.4), containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 0.2 mM α-toluene sulfonyl fluoride (PMSF). Cell and nuclear debris were removed by centrifuging at 10,000 × *g* for 20 min at 4 °C. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4 °C to separate microsomes from the cytosol. Microsomes were resuspended in ice-cold 0.25 M Sucrose/0.01 M HEPES buffer, containing 0.1 mM EDTA, 0.1 mM DTT and 5% glycerol. Samples were frozen and