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Ibuprofen bioconcentration and prostaglandin E2 levels in the bluntnose minnow *Pimephales notatus*

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ABSTRACT

Prostanoids are oxygenated derivatives of arachidonic acid with a wide range of physiological effects in vertebrates including modulation of inflammation and innate immune responses. Nonsteroidal anti-inflammatory drugs (NSAIDs) act through inhibition of cyclooxygenase (COX) conversion of arachidonic acid to prostanoids. In order to better understand the potential of environmental NSAIDS for interruption of normal levels of COX products in fishes, we developed an LC/MS/MS-based approach for tissue analysis of 7 prostanoids. Initial studies examining muscle, gut and gill demonstrated that prostaglandin E2 (PGE2) was the most abundant of the measured prostanoids in all tissues and that gill tissue had the highest and most consistent concentrations of PGE2. After short-term 48-h laboratory exposures to nominal concentrations of 5, 25, 50 and 100 μg/L ibuprofen, 50 μg/L and 100 μg/L exposure concentrations resulted in significant reduction of gill tissue PGE2 concentration by approximately 30% and 80% respectively. The lower exposures did not result in significant reductions when compared to unexposed controls. Measured tissue concentrations of ibuprofen indicated that this NSAID had little potential for bioconcentration (BCF = 1.3) and the IC_{50} of ibuprofen for inhibition of PGE2 production in gill tissue was estimated to be 0.4 μM.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most abundant environmental pharmaceutical contaminants due to their high volume of consumption (about 50,000 tons a year) and incomplete removal during wastewater treatment (Nakada et al., 2006). Environmental concentrations, frequently dominated by ibuprofen, are reported in the low μg/L range in the final effluents of sewage treatment plants (STPs). NSAID STP effluent concentrations (with ibuprofen concentrations indicated parenthetically) have been reported from 3.3 (3.0) to 4.8 (4.2) with a maximum value of 27.6 (27.3) μg/L in the UK (Ashton et al., 2004; Roberts and Thomas, 2006), from 1.0 to 14.5 (6.7) μg/L in Canada (Gagne et al., 2006; Verenitch et al., 2006) and up to 7.1 μg/L in Sweden (Bendz et al., 2005). Some pharmaceutical drugs are capable of bioconcentration and ibuprofen has been reported to significantly bioconcentrate under some conditions of STP discharge (Brown et al., 2007).

NSAIDs are antipyretic, analgesic and anti-inflammatory drugs active through modulation of eicosanoid metabolism. Eicosanoids are cyclooxygenase, lipoxygenase and cytochrome P_{450} products of C20 polyunsaturated fatty acids (Rowley et al., 2005) and include the subclass of prostanoids (prostaglandins, prostacyclines and thromboxanes) (Masoodi and Nicolaou, 2006) which play important physiological roles in practically every animal organ, tissue and cell (Cha et al., 2006; Fortier et al., 2008; Rowley et al., 2005). In vertebrates, they have been shown to be critical in the central nervous (Rowley et al., 2005; Yeh and Wang, 2006), reproductive (Bradbury et al., 2005; Cha et al., 2006; Fortier et al., 2008; Lister and Van Der Kraak, 2008; Yeh and Wang, 2006), immune (Shibata et al., 2005; Villablanca et al., 2007) and hematopoetic systems (Yeh and Wang, 2006). In the euryhaline killifish (*Fundulus heteroclitus*), prostaglandins regulate ion secretion in chloride cells in the gill and may play a role in cell survival during acute osmotic shock (Choe et al., 2006). In invertebrates, a wide range of studies indicate that eicosanoids play critical signaling roles in defense response, reproduction and predator–prey interactions (Stanley, 2000).

NSAIDs inhibit cyclooxygenase (COX) enzymes which are key enzymes in the conversion of arachidonic acid to prostanoids. COX-1 is a constitutively expressed enzyme present in most tissues (Cha et al., 2006; Fortier et al., 2008; Rowley et al., 2005) and involved in cellular housekeeping functions important in physiological homeostasis and mucosa protection (Cha et al., 2006; Fortier et al., 2008). COX-2, an inducible enzyme, is expressed in a more limited range of cell types in response to inflammatory signals (Fortier et al., 2008; Murphey et al., 2004; Schmidt et al., 2005). NSAIDs vary in their mechanisms for inhibition of COX enzymes (Garret and Grisham, 2005), but ibuprofen competes with arachidonic acid as a substrate for COX acting as a reversible non-selective competitive inhibitor. NSAIDs inhibit both COX-

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1 and COX-2, but the beneficial anti-analgesic and anti-inflammatory effects of NSAIDs are due to the inhibition of COX-2 (Dannhardt and Kiefer, 2001).

Although much is known about the pharmacological effects of NSAIDs in mammalian models, relatively little is known regarding their potential effects on the alteration of eicosanoid metabolism in aquatic lower vertebrates and invertebrates that may be exposed in wastewater receiving streams. The effects of diclofenac on fish have received the greatest attention (Hallare et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010; Schwaiger et al., 2004; Triebskorn et al., 2004) demonstrating reduced COX expression and liver and intestine pathology at exposures as low as 1 μg/L (Mehinto et al., 2010) and gill pathology with an exposure as low as 0.5 μg/L (Hoeger et al., 2005). Ibuprofen has been examined in Japanese medaka and reported to alter the spawning behavior (Flippin et al., 2007) and delay egg hatching when parental exposure was as low as 0.1 μg/L (Han et al., 2010) while in a freshwater mussel, ibuprofen exposure was shown to reduce COX activity (Gagne et al., 2006).

Our study was conducted to further understand the potential risk of environmental NSAID exposure to freshwater fish by establishing the relationships between exposure concentration and (1) bioconcentration and (2) inhibition of prostanoid production in the gill tissue of bluntnose minnows, *Pimephales notatus*. The bluntnose minnow is a small and slow growing species of temperate freshwater belonging to the cyprinid family. They have been used as physiological models (Weatherley and Gill, 2006) and in a variety of bioaccumulation and aquatic toxicity studies (Brix et al., 2001), are one of the most common freshwater fish in the Eastern U.S. and are frequently found in STP receiving streams. To our knowledge, this study represents the first direct laboratory examination of the immediate downstream molecular targets of NSAID exposure in a context relevant to environmental exposure of an aquatic vertebrate typical of wastewater receiving streams.

2. Materials and methods

2.1. Introduction

To achieve the goals described above, a single generalized extraction procedure that permits trace quantitative recovery of both the NSAID ibuprofen and a broad range of eicosanoids from fish tissue was developed and two different chromatography/mass spectrometry methods were applied to the resulting extract. Liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) was used for analysis of prostanoids, and gas chromatography-mass spectrometry (GC-MS) was used to quantify ibuprofen. Prostanoids can vary among individual animals with size, condition, stress and other factors, so 13–15 replicates were required in each group to adequately account for variability among individuals. Prostanoids are photo and temperature sensitive, so minimum light and cold conditions were maintained up to the time of analysis of prostanoids by LC/ESI-MS/MS. After completion of prostanoid analysis, ibuprofen was analyzed by GC/MS from the same sample extract.

2.2. Materials

Prostaglandin standards PGE2, PGD2, PGF2, PGE1, PGA2, PGB2, PGB1, PGA1, TXB2 and deuterated internal standard PGE2-D4 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Ibuprofen and deuterated internal standard, ibuprofen-D3, were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). All HPLC grade solvents, phosphate buffered saline (PBS), ethylene-diamine-tetraacetic acid (EDTA), butylated hydroxyl toluene (BHT) and citric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bluntnose minnows were purchased from Pond King Inc. (Gainesville, TX, USA).

2.3. Methods

2.3.1. Control fish and treatment exposures

Control and treatment (100, 50, 25 and 5 μg/L ibuprofen) groups of 45–60 bluntnose minnows (adult, mixed sexes) each were subjected to a static 48 h exposure in tanks without feeding. De-chlorinated tap water from the City of Denton, Texas (USA) was used to dilute high concentration ibuprofen stock (5000 ppm in methanol) to the different exposure level concentrations in each tank. Ibuprofen has been demonstrated to reach equilibrium with fish blood plasma within 48 h (Brown et al., 2007). An equivalent methanol concentration (0.002% v/v) was also added to control tanks. The concentration of methanol used as a carrier solvent is well below that shown to be toxic to fish (Kaviraj et al., 2004). Immediately following exposure, euthanized fish (tricaine methane sulfonate) were dissected for removal of the entire gill arch which was used for subsequent extraction. All experiments were conducted in accordance with protocols approved by the University of North Texas Institutional Animal Care and Use Committee.

2.3.2. Tissue extraction for eicosanoids and ibuprofen

Approximately 100 mg gill fresh tissue weight/replicate (each replicate consisted of combined entire gill arches from 2 to 3 fish) was homogenized with 3.0 mL of 1:1 hexane:ethyl acetate extraction solvent, 0.5 mL PBS buffer with 1 mM EDTA, 150 μl of 1% BHT (to prevent peroxidation of prostaglandins) and 150 μl 1 N citric acid using a Mini Beadbeater-8™ (Biospec Products, Bartlesville, OK, USA) to extract eicosanoids (Yang et al., 2002, 2006) and ibuprofen. Both internal standards, D4-PGE2 (250 μg/L in final volume) and D3-IBU (500 μg/L in final volume) were added to the mixture at the time of homogenization. Homogenate mixtures were transferred to glass test tubes that were kept on ice.

Prostaglandins and ibuprofen were extracted by vortexing each sample for 2 min followed by storage for 4 to 8 h at 4 °C (Yang et al., 2002, 2006). Samples were centrifuged at 1800 *g* (Yang et al., 2002, 2006) for 10 min at minimum light levels to reduce the potential for photo and thermal degradation (Yang et al., 2006) and then the upper organic layer was transferred to a glass vial. The lower aqueous layer was extracted one more time with 3.0 mL of extraction solvent and the resulting organic layer was combined with the initial organic extract. After drying under nitrogen, final extracts were reconstituted in 100 μL of 30% acetonitrile (ACN) and 70% water with 5 mM ammonium acetate. Insoluble precipitate was filtered using a 0.22 μM syringe driven filter (13 mm Millex-GV) before LC/MS injection.

2.3.3. LC-ESI/MS/MS analysis for prostanoids

Eight μL of the sample was injected into the LC-ESI/MS/MS in negative ion mode for prostanoid analyses. For the liquid chromatography, an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA) was used with a reverse phase C18 column (2.1 mm × 150 mm with 5 μM Zorbax Extend; Agilent Technologies). The LC-ESI/MS/MS method was setup using nitrogen dry gas at 350 °C and 8 L/min and a nebulizer pressure of 30 psi. Gradient elution (300 μL/min) of water with 5 mM ammonium acetate (pump-A) and 95% acetonitrile with 5 mM ammonium acetate (pump-B) was set up to separate the different prostanoids on the C-18 column. Gradient elution started from 30% B for the first minute then increased linearly to 50% at 10 min, 55% at 15 min, 100% at 15.1 min and held until 20 min when it was returned the starting conditions of 30% B.

The LC-ESI/MS/MS was operated in the multi reaction mode (MRM) with five different windows in which the first and last windows diverted LC effluent to waste and the other three windows were used for analysis of different prostanoids. The second window was used for TXB2 with a collision induced dissociated (CID) energy 0.5, the third window was used for PGF2, PGE2, PGE2-D4, PGD2 and PGE1 with CID energy 0.8, and finally the forth window was used for