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Inhibitory effects of pain relief drugs on neurological enzymes: Implications on their potential neurotoxicity to aquatic animals

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ABSTRACT

Pain relief medications commonly occur in the aquatic environment at measurable levels. While the neurotoxicity of pain relievers to higher vertebrates is currently known, little is known about their effects on aquatic animals. This study investigated the neurotoxicity of pain relievers to aquatic animals. We used three neurological enzymes, cholinesterase (ChE), adenosine triphosphatase (ATPase), and monoamine oxidase (MAO), from a freshwater planarian (*Dugesia japonica*) and green neon shrimp (*Neocaridina denticulata*) as biomarkers to examine the effects of pain relievers on *in vitro* activity. The activity of MAO and ChE, but not ATPase, was significantly inhibited by acetaminophen, but not by other pain relievers examined. It was likely that the inhibitory effects of acetaminophen on shrimp neurological enzymes were more severe than on the planarian. These findings suggest that acetaminophen is potentially neurotoxic to aquatic animals, at least in terms of neurotransmission disturbance.

1. Introduction

Pharmaceuticals are developed to bestow specific biological benefits on human and animal health. After usage, these medical compounds can enter the environment through excretion in feces and/or urine in forms that are only slightly transformed or even unchanged, plus the direct disposal of surplus drugs in sewage (Halling-Sørensen et al., 1998). The ubiquitous occurrence of medical compounds in the environment was first observed at least two decades ago (Halling-Sørensen et al., 1998). Pain relief medications are frequently prescribed pharmaceuticals and are commonly detected in the environment. In Taiwan, the average concentrations of pain relievers in streams range from non-detected (ND) to 36.9 μg/L (Lin et al., 2008). Due to the presence of pain relievers in the environment at high levels, their potential toxicity to non-target species are of concern to environmental toxicologists. For example, declining populations of vultures in Pakistan is considered to be associated with diclofenac (Oaks et al., 2004). Additionally, after aquatic animals are exposed to pain relievers, survival rate, mobility, growth, and reproduction are

Abbreviations: ANOVA, analysis of variance; Aspirin, acetylsalicylic acid; ATPase, adenosine triphosphatase; ChE, cholinesterase; COX, cyclooxygenase; DOPAC, 3,4-dihydroxyphenylacetic acid; LC_{50}, median lethal concentration; MAO, monoamine oxidase; Naproxen, (S)-(+)-2-(6-methoxy-2-naphthyl)propionic acid; ND, non-detected; NSAID, non-steroidal anti-inflammatory drugs; PGHS, prostaglandin H_2 synthase; $PhSe_2$, diphenyl diselenide; POX, PGHS peroxidase; ROS, reactive oxygen species.

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decreased; morphology and oxidative status are altered; and stress biomarkers are induced (Haap et al., 2008; Heckmann et al., 2007; Parolini and Binelli, 2012; Pounds et al., 2008; Quinn et al., 2011).

The adverse effects of acetaminophen on neural systems and behavior of rodents have recently been demonstrated through *in vivo* and *in vitro* studies (Da Silva et al., 2012; Posadas et al., 2010; Viberg et al., 2014). The potential for neurotoxicity from other pain relievers is also suspected (Sheppard et al., 2014), although some of them were once considered to have neuro-protective effects (Grilli et al., 1996). Courade et al. (2001) found that mice treated with acetaminophen at high doses exhibited altered brain levels of an enzymatic product of dopamine, which is one of the major neurotransmitters in animals. In addition, acetaminophen-treated rotifers suffered inhibited gene expression and activity of cholinesterase (ChE), which is the major enzyme involved in regulating cholinergic neurotransmission (Rhee et al., 2013). These findings suggest that enzymes involved in normal neural functions might be the targets of neurotoxicity by pain relievers. Hence, it leads to our hypothesis that acetaminophen and other pain relievers exert their neurotoxic effects on aquatic animals possibly by influencing neurological enzymes.

Multi-biomarker approaches are used to comprehensively evaluate the toxicity of environmental contaminants to aquatic animals exposed to these chemicals in the field (Fonseca et al., 2011) or laboratory (Gravato et al., 2014). A successful application of multi-biomarker approach to evaluate the toxicity of environmental contaminants relies on the choice of biomarkers (Beliaeff and Burgeot, 2002). In other words, the biomarkers chosen should be relevant and closely related to the specific objectives of research. ChE, adenosine triphosphatase (ATPase), and monoamine oxidase (MAO) are important neurological enzymes critical for maintaining normal neural functioning in animals (Wu et al., 2014). Any fluctuation in their activities can cause a change in neural functions. Thus, activities of these neurological enzymes could be served as practical biomarkers for dysfunction and abnormality occurred in neural system.

Acetaminophen, aspirin, diclofenac, ibuprofen, mefenamic acid, and naproxen are pain relievers that commonly used by humans and found in aquatic environments. The major aim of this study was to evaluate the potential neurotoxicity of these pain relievers on aquatic animals, including freshwater planarian (*Dugesia japonica*, hereinafter referred to as planarians) and green neon shrimp (*Neocaridina denticulata*, hereinafter referred to as shrimp). These two animals are widely distributed in the freshwater environments of Taiwan. For this purpose, the *in vitro* activities of planarian and shrimp ChE, ATPase, and MAO were quantified after these enzymes were directly exposed to pain relievers. Also, the species-specific difference in neurotoxicity of these pain relievers was discussed.

2. Materials and methods

2.1. Chemicals

Amplex®Red reagent was obtained from Life Technologies Corporation (Carlsbad, CA, USA). Naproxen ((S)-(+)-2-(6-methoxy-2-naphthyl)propionic acid) was obtained from Alfa Aesar (Ward Hill, MA, USA). Other chemicals used in this study – acetaminophen, aspirin (acetylsalicylic acid), diclofenac sodium salt, ibuprofen sodium salt, and mefenamic acid – were purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals used were at A.C.S. or analytical grade. Acetaminophen was dissolved in methanol to prepare a stock solution at a concentration of 2000 mg/L. Stock solutions of other pain relievers at 200 mg/L were prepared by completely dissolving powders/crystals of the drugs in buffers as done with enzyme assays. These buffers were 25 mM Tris–HCl (pH 7.0) for the ChE assay, 50 mM Tris–HCl containing 250 mM sucrose and 1 mM EDTA (pH 7.4) for the ATPase assay, or 50 mM KPO_4 buffer (pH 7.4) for the MAO assay. All stock solutions of pain relievers were freshly prepared and diluted to desired concentrations with the same buffers mentioned above.

2.2. Animals

The planarian (*D. japonica*) stock used in this study was obtained from Taikong Corporation (Taipei, Taiwan) and acclimated in our laboratory for more than 2 weeks (Wu et al., 2012). Size-selected intact planarians (~1 cm TL) were used for this study. Green neon shrimp (*N. denticulata*) were purchased from local aquarium shops. According to shop owners, shrimp were collected from freshwater environments in New Taipei City, Taiwan. Shrimp identification to species was based on Shy and Yu (1998). In the laboratory, shrimp were maintained in dechlorinated tap water (pH 7.4–7.8) with continuous water circulation and filtration at room temperature (25 ± 3 °C) for acclimation for more than 2 weeks. Sub-adult shrimp with body lengths of 1–1.5 cm measured from the tip of the rostrum to the end of the telson were used for this study. Because most major organs/systems are distributed in the cephalothorax of shrimp, including the neural organs/systems, only their cephalothoraxes were used in this study.

2.3. Effects of pain relievers on in vitro neurological enzyme activity

Intact planarians and shrimp cephalothoraxes were homogenized in homogenizing buffers, followed by centrifugation at 12,000 × *g* for 20 min at 4 °C. The homogenizing buffers were 25 mM Tris–HCl (pH 7.0) for ChE assays and 50 mM Tris–HCl containing 250 mM sucrose and 1 mM EDTA (pH 7.4) for ATPase assays. After centrifugation, pellets were discarded and supernatants used for assays. For MAO assays, intact planarians or shrimp cephalothoraxes were homogenized with 250 mM sucrose (pH 7.6, adjusted by 1 M K_2HPO_4) and centrifuged at 1500 × *g* for 20 min at 4 °C, followed by mitochondria extraction (Tipton and Dawson, 1968). Mitochondria were re-suspended in 50 mM potassium phosphate buffer (pH 7.4) and kept on ice until they were used for assays. Supernatants from tissue homogenates or mitochondria extracted from planarians and shrimp were incubated with pain relievers at different concentrations for 30 min at 25 °C prior to enzyme assays. Concentrations of acetaminophen used in this study were 0, 0.001, 0.01, 0.1, 1, 10, and 100 mg/L. Concentrations of aspirin, diclofenac, ibuprofen, mefenamic acid, and naproxen, used