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Gene transcription and biomarker responses in the clam *Ruditapes philippinarum* after exposure to ibuprofen

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ABSTRACT

Pharmaceuticals are a class of emerging environmental contaminants that continuously enter aquatic environments. Presently, little information is available about the effects of these substances on non-target organisms, such as bivalves. We investigated the effects of ibuprofen (IBU) on the clam *Ruditapes philippinarum*. Clams were exposed for 1, 3, 5 and 7 days to 0, 100 and 1000 μg IBU/L, and established biomarker responses (haemolymph lysozyme, gill acetylcholinesterase and digestive gland superoxide dismutase activities) as well as digestive gland transcriptome were evaluated. A two-way ANOVA revealed significant effects of both "IBU concentration" and "exposure duration" on biomarker responses. Overall, the enzyme activities were generally lower in IBU-exposed clams than in controls. Although limited knowledge of the mollusc transcriptome makes it difficult to interpret the effects of IBU on clams, the gene transcription analysis using DNA microarrays enabled the identification of the putative molecular mode of action of the IBU. The functional analysis of differentially transcribed genes suggests that IBU can interfere with various signalling pathways in clams, such as arachidonic acid metabolism, apoptosis, peroxisomal proliferator-activated receptors, and nuclear factor-kappa B. In addition, several genes involved in the metabolism of xenobiotics (e.g., glutathione S-transferase, sulfotransferase, cytochrome P450) were also found to be significantly affected by IBU exposure. In summary, the integrated approach of gene transcription analysis and biomarker responses facilitated the elucidation of the putative mechanisms of action of IBU in non-target species.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a class of emerging environmental contaminants that include various substances that are either used by humans for personal health and cosmetic reasons or by agribusiness to enhance the growth or health of livestock. Thousands of tonnes of PPCPs are produced annually and include prescription drugs, veterinary drugs, diagnostic agents, fragrances, lotions, and cosmetics (Fent et al., 2006).

Among the non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen (IBU) is one of the most widely used drugs worldwide as an analgesic, antirheumatic and antipyretic (Fent et al., 2006; Praveen Rao and Knaus, 2008). IBU decreases the formation of prostaglandins and thromboxane by inhibiting cyclo-oxygenase COX-1 and COX-2 enzymes (Gierse et al., 1999). In the European Union, the consumption of IBU is several tonnes per year (see Fent et al., 2006 for a review). In Germany, the annual consumption of IBU in 2001 was approximately 345 tonnes (Huschek et al., 2004), whereas in Italy it was approximately 1.9 tonnes (Calamari et al., 2003).

IBU is not fully metabolised by humans, and it is mostly excreted in its native form (70–80% of the therapeutic dose) or as metabolites (hydroxy- and carboxy-ibuprofen and carboxy-hydratropic acid) (Heberer, 2002; Pounds et al., 2008). Consequently, IBU can be detected at concentrations ranging between ng/L and μg/L in wastewater and surface water. Relatively high concentrations of IBU have been found in effluents (up to 22 μg/L) and influents (up to 84 μg/L) from sewage treatment plants (STPs) (Brun et al., 2006; Gómez et al., 2007). In the U.K., IBU (median 3086 ng/L) has been detected in 84% of the STP effluent samples that have been analysed (Ashton et al., 2004). In Spain, Farré et al. (2001) recorded IBU concentrations of up to 85 μg/L in sewage effluent samples and up to 2.7 μg/L in surface waters. In Italy, Zuccato et al. (2005) reported IBU levels of approximately 120 ng/L in the effluents from nine STPs, while Calamari et al. (2003) recorded levels of approximately 17 ng/L in the Po River.

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In aquatic ecosystems, PPCPs can affect non-target species (Fent et al., 2006). To our knowledge, information concerning the toxic effects of IBU on molluscs is limited to just a few species, such as *Planorbis carinatus*, *Dreissena polymorpha*, *Mytilus galloprovincialis*, *Mytilus edulis trossulus* and *Ruditapes philippinarum*. In adult snails (*P. carinatus*), both 48 and 72 h LC_{50} values were 17.1 mg/L, whereas 21-day reproduction (hatching success) LOEC and NOEC values were 45.36 and 5.36 mg/L, respectively (Pounds et al., 2008). In *D. polymorpha*, in vitro exposure of haemocytes to IBU (45, 450, and 909 μg/L) caused lysosomal membrane destabilisation and increased the percentage of apoptotic cells (Parolini et al., 2009), whereas in vivo exposure (0.2, 2 and 8 μg/L) altered antioxidant and detoxifying enzyme activities, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) (Parolini et al., 2011). In *D. polymorpha*, increased oxidative stress was noted in the digestive gland of IBU-exposed mussels (0.206, 2.06, 20.6 and 206.3 μg/L) (Contardo-Jara et al., 2011). IBU was also found to be an endocrine disruptor in *M. galloprovincialis* (Gonzalez-Rey and Bebianno, 2012), whereas in *M. edulis trossulus*, it caused significant reductions in both byssus strength and scope for growth (Ericson et al., 2010). In *R. philippinarum*, exposure to sublethal IBU concentrations (100, 500 and 1000 μg/L) for 7 days caused significant alterations in immunomarker responses (Matozzo et al., 2012).

In these studies (except for that by Contardo-Jara et al., 2011), a biomarker approach was generally adopted to assess the IBU toxicity in bivalves. However, established biomarkers alone do not provide exhaustive information about the possible mechanisms of action (MOA) of contaminants. Consequently, we decided to combine biomarker responses with a transcriptomic analysis to obtain a more complete evaluation of IBU toxicity and the putative MOA in bivalves. The hypotheses we tested were that IBU affects clams (*R. philippinarum*) at different biological organisation levels and that IBU effects vary depending on both the exposure concentrations and duration. To test these hypotheses, clams were exposed for 1, 3, 5 and 7 days to 0, 100 and 1000 μg IBU/L, and haemolymph lysozyme, gill acetylcholinesterase (AChE) and digestive gland SOD activities were measured. In addition, a transcriptomic analysis of the digestive gland was performed using an oligo-DNA microarray representing 11,906 transcripts. We are aware that the IBU concentrations tested in this study were higher than those generally recorded in aquatic environments. Clams were exposed to IBU concentrations similar to those tested in previous studies (Pounds et al., 2008; Parolini et al., 2009; Ericson et al., 2010; Matozzo et al., 2012) to provide further information about the MOA of the contaminant in bivalves.

2. Materials and methods

2.1. Clams and IBU solutions

Specimens of *R. philippinarum* (3.5–3.8 cm shell length) were collected from a reference site that was located inside a licensed area for clam culture in the southern basin of the Lagoon of Venice (Italy) and acclimated in the laboratory for 5 days before exposure to IBU. Clams were maintained in large aquaria with sandy bottoms and aerated seawater (35 ± 1 psu salinity, 17 ± 0.5 °C) and were fed with microalgae (*Isochrysis galbana*). IBU was purchased from Sigma–Aldrich (Milano, Italy). Due to its low solubility in water, a stock solution of IBU was prepared in ethanol and stored at room temperature (18–20 °C) for the duration of the experiments. Working solutions were prepared daily by diluting the stock solution in seawater.

2.2. IBU exposure and tissue collection

The experiments were performed during the winter to avoid periods of sexual maturity for clams. This non-reproductive condition avoided spawning and reduced possible additional stress during the experiments. Clams (30 per concentration) were exposed to 100 and 1000 μg IBU/L. The nominal concentrations were chosen based on the reported data on IBU toxicity in molluscs (Pounds et al., 2008; Parolini et al., 2009; Ericson et al., 2010; Contardo-Jara et al., 2011; Matozzo et al., 2012). In the control, ethanol was added at the highest concentration (10 μL/L) used in the IBU treatments (1000 μg IBU/L). Only one control (seawater + solvent) was prepared because no significant differences in immunomarker responses between seawater controls and solvent controls were recorded in our recent study (Matozzo et al., 2012). Clams were maintained in glass aquaria (without sediment) containing aerated seawater (1 L per animal) in the same thermohaline conditions that were used during the acclimation period. Seawater was changed daily, and the IBU and microalgae (*I. galbana*, at an initial concentration of approximately 150,000 cells/L) were added.

Haemolymph, gills, and digestive gland from 7 clams per concentration were individually collected after 1, 3, 5 and 7 days. Haemolymph was collected from the anterior adductor muscle in a 1-mL plastic syringe, placed in Eppendorf tubes, and stored on ice. Haemolymph samples were then centrifuged at 780 × *g* for 10 min, and the supernatants, corresponding to cell-free haemolymph (CFH), were collected. After haemolymph sampling, the shell was opened, and the gills and digestive gland were excised and placed in tubes on ice. A sub-sample of each digestive gland was stored in an RNA later solution (Qiagen) for gene transcription analysis. The CFH, gills and digestive gland that were used for biomarker measurement were immediately frozen in liquid nitrogen and stored at −80 °C until the analysis.

2.3. Haemolymph lysozyme activity assay

Fifty μL of CFH were added to 950 μL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in a 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance (Δ*A*/min) was continuously recorded at 450 nm for 3 min at room temperature (18–20 °C). The results were expressed as μg lysozyme/mg protein. Protein concentrations in CFH were quantified according to Bradford (1976).

2.4. Gill AChE activity assay

Gills were thawed on ice and homogenised (1:4, w:v) in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT, Sigma) and 40 μg/mL aprotinin (a trypsin inhibitor, Sigma). The gills were then sonicated for 2 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles and centrifuged at 12,000 × *g* for 45 min at 4 °C. The supernatant (SN) was collected for the enzyme assay. AChE activity was determined according to Ellman et al. (1961), as adapted for microplate readers by Bocquené and Galgani (1998). Changes in the absorbance at 405 nm were recorded for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg protein. Tissue protein concentrations were quantified according to Bradford (1976).

2.5. Digestive gland SOD activity assay

The total SOD activity was measured in the gills and digestive glands in triplicate using the xanthine oxidase/cytochrome *c* method (Crapo et al., 1978). Tissues were homogenised as described above, and the cytochrome *c* reduction by the superoxide anion