Short Communication

Brief exposure to copper induces apoptosis and alters mediators of olfactory signal transduction in coho salmon

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HIGHLIGHTS

- Short-term Cu exposure increased cell death in salmon olfactory epithelium.
- Decreased *omp* expression was associated with increased cell death.
- ACIII expression decreased in apical olfactory epithelium at all Cu levels.
- Intracellular cGMP was significantly reduced at all Cu doses.

ABSTRACT

Pacific salmon are particularly susceptible to copper (Cu)-induced olfactory injuries that can ultimately inhibit neurobehaviors critical to survival. However, the molecular mechanisms underlying Cu-mediated olfactory impairment remain poorly understood. In the present study, we conducted a short-term Cu exposure at levels relevant to urban runoff (5, 25 and 50 ppb), and investigated the roles of impaired olfactory signal transduction and induced apoptosis as underlying mechanisms of olfactory injury. Increased cell death in the olfactory epithelium was evident in coho receiving 4 h exposures to 25 and 50 ppb Cu. Expression of olfactory marker protein (*omp*), a marker of mature olfactory sensory neurons, also decreased at 50 ppb Cu. Immunohistochemical analysis of coho olfactory epithelium demonstrated a loss of type 3 adenylate cyclase (ACIII) in the apical olfactory epithelium cilia at all levels of Cu exposure, suggesting an inhibitory effect of Cu in olfactory signaling. Accompanying the loss of ACIII in Cu-exposed coho were reduced intracellular cyclic guanosine monophosphate (cGMP) levels in the olfactory rosettes. Collectively, these results support a linkage among the initial steps of olfactory signaling in Cu-induced salmon olfactory injury, and suggesting that monitoring olfactory cGMP levels may aid in the assessment of salmon olfactory injury.

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1. Introduction

The decline of salmon populations in the Western United States has been linked to the deterioration of coastal habitats and the contamination of surface waters and riverine sediments (Lackey, 2003; Scholz et al., 2011). Key components of the ecological risk of chemical exposures to salmon are sublethal neurological injury and, in particular, injury to the peripheral nervous system. Peripheral neurotoxicity is often associated with impaired olfactory

function, leading to the loss of critical behaviors such as predator avoidance, prey capture, mate selection, and migration (Scott et al., 2003; McIntyre et al., 2012). Accordingly, understanding the mechanisms of olfactory injury can lead to the development of sensitive biomarkers to better evaluate the effects of pollution on sublethal injury in salmonids.

The peripheral component of the fish olfactory system includes a pair of olfactory rosettes lodged in the olfactory pits. These organs are covered by a sensory epithelium containing olfactory receptor neurons (ORNs) that are highly vulnerable to the toxic effects of dissolved contaminants (Tierney et al., 2010). The initiation of an olfactory response occurs in the ORNs and involves the sequential activation of: G-protein coupled receptors via binding with odorant molecules and stimulation of an enzymatic cascade, leading to the generation of second messengers, the opening of cyclic nucleotide-gated (CNG) channels, and the elicitation of a generator current which depolarizes the cell (Hara, 1994; Schild

and Restrepo, 1998). Olfactory neuron CNG channels also respond to both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), underlying the key role of second messengers in olfactory signaling (Nakamura and Gold, 1987). Chemicals that interfere with any component of the signaling process can thus impair key behavioral responses by blocking proper olfactory function.

Copper (Cu) is a ubiquitously distributed olfactory toxicant and a pervasive contaminant in urban runoff at concentrations that vary from 3 to 64 ppb (Soller et al., 2005). Short-term exposure to Cu concentrations within this range of urban watersheds can inhibit the physiological responsiveness of olfactory receptor neurons in a concentration-dependent manner (Sandahl et al., 2004, 2007). Using zebrafish as a laboratory model, we previously demonstrated that Cu alters the expression of olfactory signaling genes including odorant receptors, G-proteins, and ion transport proteins (Tilton et al., 2008). The aforementioned study identified multiple olfactory signal transduction (OST) targets and complex biochemical responses to toxicants possibly occurring within the olfactory cilia. Despite these advances, the underlying mechanisms of Cu-mediated olfactory injury are still poorly understood. The objective of the current study was to assess whether Cu exposure affects the initial steps of olfactory signaling in juvenile salmon. Our goal was to define the linkages among cellular injury and secondary messenger cascades in order to potentially generate novel biomarkers of metal-induced olfactory injury in salmon.

2. Materials and methods

2.1. Cu exposure and tissue processing

All animal welfare and experimental procedures were carried out in strict accordance with the University of Washington's Institutional Animal Care and Use Committee (IACUC) guidelines. Juvenile coho salmon were provided by the National Oceanic and Atmospheric Administration (NOAA), Seattle, Washington and raised under natural photoperiod in cylindrical tanks containing recirculated, tempered (10–12 °C) freshwater from Lake Washington. The fish were fed Bio Vita Fry Feed (Bio-Oregon Inc., OR) and water quality conditions were typically 80–120 mg/L total hardness as calcium carbonate, pH 7.4 ± 0.2, and 8.1 mg/L dissolved oxygen content. Exposures were preceded by a 24 h clean water acclimation period during which the fish received no food. For the 4 h exposures, 10–12 coho (body length: 11.86 ± 1.95 cm and body mass: 17.95 ± 7.97 g) were exposed to the intended concentrations of 0, 5, 25, and 50 ppb Cu (as $CuCl_2$) in 70 L, individually-aerated aquaria contained within a large, chilled (11 °C), re-circulating water bath. The targeted nominal Cu concentrations closely tracked the measured waterborne Cu concentrations (Table 1). The background concentration of total dissolved copper in the source water was 1 ppb, which is extremely low relative to other studies (Baldwin et al., 2003). Following the Cu exposures, fish were euthanized and the olfactory rosettes were isolated and

prepared for molecular and biochemical endpoints as described below.

2.2. Analysis of cell death (TUNEL assay)

Cu-induced cell death was measured in olfactory rosette cryosections by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to the manufacturer's protocol (*In situ* Cell Death Detection Kit, TMR red; Roche Diagnostics). Slides were counterstained with Hochest 33 342 prior to microscopic analysis, and images were collected using a Nikon Labophot 2 microscope equipped with a Nuance Multispectral Imaging System (Caliper Life Science, Hopkinton, MA). TUNEL signals were detected with a red filter set (Nikon G-1B, EX540/10, DM580, BA590), and images were captured with Nuance 3.0.1 software. Quantitative measurement of apoptosis was conducted by counting the number of TUNEL-positive cells per 10^4 μm^2 of olfactory tissue.

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated from coho olfactory rosettes using the Trizol (Invitrogen, Carlsbad, CA) method. First strand cDNA synthesis and qPCR analysis of olfactory gene expression was conducted as previously described (Wang et al., 2012). Expression of olfactory marker protein (*omp*) was quantified using real-time PCR (forward primer: 5′-GACCCCTGACCTCACACACT-3′ and reverse primer: 5′-GTACATGACCTTGCGGACCT-3′). *Omp* gene expression was normalized against the housekeeping gene β-actin, which was unaffected by the exposures.

2.4. Immunohistochemical analysis of type 3 adenylate cyclase (ACIII) expression

Cryosections of olfactory tissue were incubated overnight with anti-ACIII (1:200, sc-588 Santa Cruz Biotechnology) in blocking solution (10% normal donkey serum, 0.1% Triton X-100, and 3% BSA). A Cy3-conjugated donkey anti-rabbit secondary antibody (1:500, Jackson Laboratories) was used in the immunohistochemical analysis. As a negative control, the primary antibody was replaced with blocking solution. Images were captured at 610 nm with Nuance 3.0.1 software (Caliper Life Science, Hopkinton, MA). The threshold values obtained from the negative controls were subsequently used when analyzing captured images with Image J software (NIH).

2.5. Analysis of cAMP and cGMP

Pooled coho olfactory rosettes were homogenized on ice with 5 volumes of 5% trichloroacetic acid (TCA) (1 mL of solution/gram of tissue) and centrifuged at 1500 × g for 10 min at 4 °C. The supernatants were extracted (3×) with water-saturated ether to remove the TCA, and the residual ether was removed from the aqueous layer by heating samples to 70 °C for 5 min. To prepare the standard curve matrix solution, 20 mL of the 5% TCA preparation was treated similarly by extraction of water-saturated ether and heating. Subsequently, all standards and sample dilutions were prepared according to manufacturer guidelines (Cyclic AMP and Cyclic GMP EIA Kit, Cayman Chemical Company). Samples were acetylated with potassium hydroxide and acetic anhydride, and cAMP and cGMP levels were assayed in triplicate in a 96-well plate format at 405 nm.

2.6. Statistical analysis

The Mann–Whitney *U* test was used to assess differences in the number of TUNEL-positive cells among Cu treatments and controls.

Table 1
Nominal and measured copper concentrations.[a]

Sample	Nominal (ppb)	Measured (ppb)
Control	0	1
Cu–L	5	5
Cu–M	25	23
Cu–H	50	44

[a] Waterborne Cu concentrations were analyzed by the UW Trace Organics Laboratory using inductively coupled plasma-mass spectrometry (ICP-MS) using US Environmental Protection Agency (EPA) method 6020A EPA (2008).