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Effects of copper on CYP1A activity and epithelial barrier properties in the rainbow trout gill

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Abstract

The effects of copper on β-naphthoflavone (βNF)-induced ethoxyresorufin *O*-deethylase (EROD) activity were studied in rainbow trout (*Oncorhynchus mykiss*) gill filaments (after *in vivo* exposure) and in gill cells cultured as both primary cultures and as polarised epithelia, *i.e.* with water in the apical compartment and culture medium in the basolateral compartment. In the *in vivo* study βNF and copper were added to the water, in primary cultures both chemicals were added to the culture medium and in cultured epithelia copper was added to the apical water whilst βNF was added to the basolateral culture medium. In primary cultures this investigation was repeated with and without foetal bovine serum (FBS) supplementation of the culture media. Gill barrier properties, specifically polyethylene glycol (PEG-4000) permeability (*i.e.* paracellular permeability), sodium efflux and transepithelial electrical resistance (TER) were also investigated in cultured gill cell epithelia after apical treatment with copper. Two micromolar copper had no effect on EROD activity in gill filaments *in vivo* irrespective of whether EROD was induced by 0.01, 0.1 or 1.0 μM βNF. Similarly, 0.5–100 μM copper had no effect on EROD induction in cultured epithelia. In primary cultures copper did reduce EROD induction but the effective concentration was dependent on whether the cells were supplemented with FBS, *i.e.* EROD activity was reduced by all copper concentrations of 5 and above if FBS was included, but only by 1000 μM if FBS was omitted. In cultured epithelia PEG-4000 permeability increased, whilst sodium efflux and TER were unaffected following treatment with 75 μM copper. Based on these results we conclude that the branchial monooxygenase system is a less sensitive target for copper than the barrier properties of the gill. Indeed, these data suggest the apical membrane of the gill epithelial cells minimises the uptake of waterborne copper and therefore protects the intracellular environment, including the CYP1A system. This could enable the freshwater fish gill to retain their potential of first-pass metabolism of waterborne organic compounds whilst simultaneously being exposed to waterborne copper.

Keywords: Gill; Copper; EROD activity; Barrier properties; Sodium efflux; PEG-4000 permeability; Transepithelial electrical resistance (TER)

1. Introduction

The gill epithelium is the primary site for respiratory gas exchange, osmoregulation, acid–base regulation and nitrogenous waste excretion in fish (*e.g.* reviewed by Evans et al., 1999; Claiborne et al., 2002; Marshall, 2002; Perry and Gilmour, 2002; Wilkie, 2002). The gill epithelial cells have a well-developed apical–basolateral polarisation with an apical membrane facing the water and a basolateral membrane in contact with body fluids. In addition, multi-stranded tight junctions seal the paracellular spaces in the gill epithelium (Sardet et al., 1979) and membrane-bound ion-transporting ATPases in the epithelial cells balance passive gains and losses of water and ions by absorption or excretion of ions (Marshall, 2002). These features make the gill epithelium an effective barrier against either a hypotonic (freshwater) or hypertonic (seawater) environment.

However, the intimate contact with ambient water makes the gill epithelium a major route of uptake of environmental pollutants, such as metals and polycyclic aromatic hydrocarbons (PAH). Generally metals interfere with ion regulation in the gill (Lock et al., 1981; Verbost et al., 1989; Wood et al., 1996) and in this respect copper is one of the most toxic (Lauren and McDonald, 1987a,b). In both seawater and freshwater fish cop-

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per interferes with sodium uptake through non-competitive inhibition of Na/K ATPase (Stagg and Shuttleworth, 1982; Evans, 1987; Lauren and McDonald, 1987a,b). In freshwater fish copper, at high concentrations, opens the paracellular pathway in the gill by displacing calcium from the tight junctions, resulting in an increased loss of body electrolytes (Lauren and McDonald, 1985). The fish die when 20–40% of the whole body sodium content is lost (Taylor et al., 2003).

Among the most widespread organic pollutants in the aquatic environment are the aryl hydrocarbon receptor (AhR) agonists, *i.e.* polychlorinated dibenzo-*p*-dioxins and dibenzofurans, coplanar polychlorinated biphenyls and PAH. Several AhR agonists are toxic to vertebrates causing a wide array of effects, *e.g.* disturbances of development, growth, reproduction and immune response, and cancer promotion (Poland and Knutson, 1982; Poellinger, 2000). Fish exposed to AhR agonists show an increased expression of cytochrome P4501A (CYP1A) in various organs, including the gills. The CYP1A protein has been localised to respiratory cells, mucus cells and pillar cells, but not chloride cells of the gill (Miller et al., 1989; Smolowitz et al., 1991; Husøy et al., 1996; Van Veld et al., 1997; Jönsson et al., 2004). CYP1A induction is a widely used biomarker of AhR agonist exposure that can be measured as an increase of the ethoxyresorufin *O*-deethylase (EROD) activity. By means of a recently developed gill filament-based EROD assay low concentrations of AhR agonists in ambient water can be detected in fish (Jönsson et al., 2002; Jönsson, 2003). This assay has been used to demonstrate the presence of CYP1A-inducing compounds in Scandinavian freshwater and marine environments (Jönsson et al., 2002, 2003, 2004; Jönsson, 2003).

Most CYP1A induction studies deal with single compounds whereas in reality in the aquatic environment AhR agonists most often occur together with other pollutants including metals, such as copper. Metals may interfere with biotransformation enzymes (Förlin et al., 1986; Lemaire and Lemaire, 1992; Stien et al., 1997; Viarengo et al., 1997; Fent et al., 1998). A stronger hepatic EROD induction was observed in bass (*Dicentrarchus labrax*) injected with PAH than in bass injected with PAH plus copper (Stien et al., 1997; Viarengo et al., 1997). Copper also appears to interfere with NADPH cytochrome reductase and CYP1A activities as well as increasing the breakdown of CYP1A (Stien et al., 1997; Kim et al., 2002). Copper and other metals have also been reported to increase CYP1A mRNA in cell cultures (Korashy and El-Kadi, 2004). These effects could lead to misjudgement when CYP1A induction is used as a biomarker for exposure to AhR agonists in the environment. Therefore, more data are required on the interaction between environmentally important metals, such as copper, and EROD induction in specific cell types.

Some aspects of the gill physiology can be studied using gill cell cultures (Wood et al., 2002). Gill epithelial cells (respiratory cells) can be grown either as primary cultures in culture plates (Pärt et al., 1993) or as cultured epithelia on permeable inserts (Wood and Pärt, 1997). These epithelial culture techniques have also been modified enabling preparations of cell cultures composed of both respiratory cells and chloride cells (Fletcher et al., 2000). Irrespective of cellular composition, the cultured gill epithelium develops a polarisation and tight junction structure similar to that of the gill *in vivo*. This is best demonstrated by their ability to tolerate water on the apical side for prolonged periods and their high transepithelial electrical resistance (TER; Wood and Pärt, 1997; Fletcher et al., 2000; Wood et al., 2002). Tight junction integrity and sodium leakage across the cultured gill epithelium can be studied by measuring the movement of ^{3}H-polyethelene glycol-4000 (PEG-4000; a paracellular permeability marker) and ^{22}Na, from the basolateral to the apical compartment of the cultures, respectively. The relationship between TER and PEG-4000 permeability in cultured gill epithelia is described by a declining exponential curve (Smith et al., 2005). Sodium efflux, PEG-4000 permeability and TER of the cultured gill epithelia are comparable to those in the gill *in vivo* (Wood and Pärt, 1997). In addition, protein synthesis rates and intracellular sodium concentrations in cultured gill epithelia and the whole fish gill are virtually identical (Smith et al., 2001). The similarity of these parameters *in vitro*, when compared with whole gill physiology, makes the cultured gill epithelium a useful model with which to study copper toxicity (Smith et al., 2001) and EROD induction by various AhR agonists in gill cells (Carlsson and Pärt, 2001).

The objective of this study was to examine effects of copper on branchial EROD activity *in vivo* and *in vitro*. Furthermore, since tight junctions are suggested targets for copper in the gill epithelium, the aim was to relate the effects of copper on EROD activity and barrier properties in gill cells.

2. Materials and methods

2.1. Fish husbandry

Juvenile rainbow trout (weighing approximately 50 g) were purchased from Persbo-Klotens fiskodling AB, Kloten, Sweden and Näs fiskodling AB, By Kyrkby, Sweden. The fish were held in tanks in the aquarium facility at the Evolutionary Biology Centre, Uppsala University, at 8–12 °C and a stocking density of 50–150 fish per m^3. The day/night cycle was continuously and automatically adjusted to the diurnal variations at latitude 51°N. The fish were fed commercial pellets (EWOS ST40, Astra-EWOS, Sweden) once daily, at a fixed ration of 1% of the body mass.

2.2. Chemicals and materials

Dimethyl sulfoxide (DMSO), β-naphthoflavone (βNF), copper sulphate, 7-ethoxyresorufin, dicumarol, 7-hydroxy-3H-phenoxazin-3-one sodium salt (resorufin), ethylenediaminetetraacetic acid (EDTA), gentamicin, bovine serum albumin (BSA) and deoxycholic acid (DOC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fungizone, Leibowitz L-15 medium with L-glutamine (L15), penicillin–streptomycin and trypsin were from Gibco (Paisley, UK). Foetal bovine serum (FBS) was obtained from Statens Veterinärmedicinska Anstalt (Uppsala, Sweden). Tissue culture plates (12-well) and cyclopore polyethylene terephthalate permeable inserts, with a 0.9-cm^2-culture area, a 0.45-μm-pore size and with