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Salicylate impacts the physiological responses to an acute handling disturbance in rainbow trout

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Abstract

While salicylates (non-steroidal anti-inflammatory drugs) have been detected in the aquatic environment, few studies have focused on the mechanism of action of these pharmaceuticals on aquatic organisms. We reported previously that salicylate disrupted the acute trophic hormone-stimulated corticosteroidogenesis in rainbow trout (*Oncorhynchus mykiss*) interrenal tissue *in vitro*. Here, we tested the hypothesis that this drug will inhibit the adaptive plasma cortisol response and the associated metabolic response to an acute stressor in trout. Fish were fed salicylate-laced feed (100 mg/kg body weight) for 3 days, subjected to an acute (5 min) handling disturbance and sampled 1, 4 and 24 h after the stressor exposure. Salicylate treatment attenuated the stressor-induced plasma cortisol but not glucose or lactate elevations. The disruption of cortisol response corresponded with a significant reduction in transcript levels of the steroidogenic acute regulatory protein (StAR), but not peripheral-type benzodiazepine receptor, cytochrome P450 side-chain cleavage or 11β-hydroxylase. Salicylate did not modify the stressor-induced elevation of brain glucocorticoid receptor (GR) protein expression, while liver GR protein content was reduced. Salicylate impact on liver metabolic capacity involved depressed liver glycogen content, whereas no significant changes in liver hexokinase, glucokinase, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate carboxykinase, aspartate aminotransferase and alanine aminotransferase activities were observed. Taken together, salicylate impairs the stressor-mediated plasma cortisol response and the associated liver metabolic capacity in trout. The mode of action of salicylate involves disruption of StAR and liver GR, two key proteins critical for cortisol production and target tissue responsiveness to this steroid, respectively.

Keywords: Fish; Salmonid; *Oncorhynchus mykiss*; Acute stress response; Cortisol; Glucocorticoid receptor; Intermediary metabolism; Endocrine disruptor

1. Introduction

A key indicator of fish stress performance is the activation of the hypothalamus–pituitary–interrenal (HPI) axis and the associated metabolic changes (Mommsen et al., 1999; Iwama et al., 2006). Cortisol, the main corticosteroid in teleosts, is released in response to stressor-mediated stimulation of interrenal tissue by adrenocorticotropic hormone (ACTH) (Wendelaar Bonga, 1997; Mommsen et al., 1999). The rate-limiting step in corticosteroid biosynthesis is thought to be the transport of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR; Stocco et al., 2005; Miller, 2007) and the peripheral-type benzodiazepine receptor (PBR; Papadopoulos, 2004). In addition, cytochrome P450 side-chain cleavage (P450scc) and 11β-hydroxylase are also two enzymes critical for cortisol biosynthesis (Payne and Hales, 2004).

The stressor-induced acute cortisol response is a key signal orchestrating the metabolic adjustments critical to cope with the enhanced energy demand to stress (Mommsen et al., 1999; Iwama et al., 2006). For instance, cortisol enhances liver metabolic capacity, including higher amino acid catabolism, gluconeogenesis and glycogenolysis, leading to increased glucose production (Wendelaar Bonga, 1997; Mommsen et al., 1999; Iwama et al., 2006). The rise in plasma cortisol levels after an acute stressor is quickly regulated by a negative feedback loop, which involves glucocorticoid receptor (GR) signaling in the brain (Mommsen et al., 1999; Aluru et al., 2004; Vijayan et al., 2005). Also, modulation of target tissue responsiveness to cortisol during stressor exposure may involve ligand-mediated GR autoregulation in trout (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Collectively, the functioning of the HPI axis, including GR dynamics, is a key aspect of the highly conserved adaptive stress response to regain homeostasis.

Recent studies suggest that this adaptive cortisol response in fish is impacted by environmental pollutants (Hontela, 2005;

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Aluru and Vijayan, 2006; Gravel and Vijayan, 2006). While most of those studies focused on aryl hydrocarbon receptor (AhR) agonists and metals, very little is known about the impact of pharmaceutical drugs in modulating the stress axis in fish. Indeed the detection of pharmaceutical drugs in the aquatic environment in μg/L concentrations has raised concerns about the potential adverse effects of these drugs on aquatic organisms. Non-steroidal anti-inflammatory drugs (NSAIDs), due to their high consumption, are among the most important group detected in surface waters (Heberer, 2002; Metcalfe et al., 2003a,b). While toxicity studies with pharmaceutical drugs have been restricted to acute toxicity studies (Webb, 2001; Cleuvers, 2004; Trudeau et al., 2005), little is known about either the mechanism of action of these drugs or their impact on fish stress performance. A recent study showed that acetylsalicylic acid treatment attenuated the stressor-mediated cortisol response in Mozambique tilapia, *Oreochromis mossambicus* (van Anholt et al., 2003). Also, we demonstrated that StAR and PBR are targets for salicylate impact in rainbow trout (*Oncorhynchus mykiss*) interrenal tissue leading to the proposal that this NSAID is a corticosteroid disruptor (Gravel and Vijayan, 2006).

To further explore the role of NSAIDs as endocrine disruptors in fish, we tested the hypothesis that salicylate disrupts stressor-induced plasma cortisol level and the associated metabolic response in rainbow trout. To this end, we fed trout salicylate for 3 days exactly as described before (Gravel and Vijayan, 2006) and subjected the fish to a standardized handling disturbance (Aluru and Vijayan, 2006). Plasma cortisol, glucose and lactate levels were measured as indicators of organismal stress response, while liver glycogen and glucose content and activities of several enzymes involved in intermediary metabolism were determined to assess the liver metabolic capacity. We also evaluated the cortisol biosynthetic capacity of trout interrenal tissue during recovery from an acute stressor by quantifying the mRNA abundance of StAR, PBR, P450scc and 11ß-hydroxylase, while brain and liver GR content was determined to investigate the impact of salicylate on cortisol feedback regulation and target tissue signaling, respectively.

2. Material and methods

2.1. Chemicals

Sodium salicylate, protease inhibitor cocktail, bicinchoninic acid (BCA) reagent, and 2-phenoxyethanol were purchased from Sigma (St. Louis, MO). Costar 96-well EIA/RIA flat bottom microplates were from Corning Inc. (Corning, NY), while the electrophoresis reagents, molecular weight markers and alkaline phosphatase-conjugated goat anti-rabbit IgG were from BioRad (Hercules, CA). The polyclonal rabbit anti-trout GR antibody was developed in our laboratory (Sathiyaa and Vijayan, 2003). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (StressGen, Victoria, BC). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, Ontario).

2.2. Animal

Juvenile rainbow trout (∼40 g body mass) were obtained from Rainbow Springs Hatchery, Thamesford, Ontario. Groups of 14 trout each were maintained for a month in four aerated tanks (40 L capacity) with constant water flow at 13 °C with a 12 hL:12 hD photoperiod prior to the experiment. The fish were fed to satiety with trout chow (five-point sinking food, Martin Mills Inc., Elmira, ON) once daily for 5 days a week.

2.3. Experimental design

After acclimation, fish in two aquaria were fed control diet (trout chow at 2% body mass), while the other two aquaria were fed the same amount of feed as sham group but laced with salicylate (100 mg/kg body weight) exactly as described before (Gravel and Vijayan, 2006). Briefly, sodium salicylate was dissolved in 100% ethanol and sprayed on the trout chow, while control diet received only ethanol. The fish were fed the treatment diet for 3 days and sampled on the fourth day. Sampling consisted of quickly netting seven fish each from the sham or salicylate-fed tanks and anaesthetizing with an overdose of 2-phenoxyethanol (1:1000). These fish were the unstressed fish, after which fish in all four tanks were subjected to a 3 min handling disturbance according to Aluru and Vijayan (2006) and sampled at 1, 4 and 24 h after stressor exposure. Fish were bled by caudal puncture into heparinized tubes and plasma was collected after centrifugation (10 min at 6000 × *g*) and kept frozen at −70 °C for cortisol, glucose and lactate analyses. Pieces of liver, brain and head kidney tissues were collected and immediately frozen on dry ice and stored at −70 °C for glycogen, enzyme activity, mRNA and protein determination later. The experimental protocol was in accordance with the Canadian Council for Animal Care guidelines and approved by the animal care committee at the University of Waterloo.

2.4. Plasma analyses

Plasma cortisol concentration was measured using a commercially available ImmuChem^{TM} ^{125}I radioimmunoassay kit (MP Biomedicals, CA). Plasma glucose (modified Trinder method; Rainchem, San Diego, CA) and lactate (Trinity Biotech, St. Louis, MO) levels were measured using commercial kits.

2.5. Liver glycogen, glucose and enzyme activities

Livers were homogenized (Ultra Turrax; IKA Works, Wilmington, NC), followed by sonication (Microson, Farmingdale, NY), in a homogenization buffer (50% glycerol, 21 mM Na_2HPO_4, 0.5 mM EDTA-Na_2, 0.2% BSA, 5 mM β-mercaptoethanol and protease inhibitors, pH adjusted to 7.5) exactly as described before (Vijayan et al., 2006). Liver glycogen content was analyzed by measuring glucose content before and after amyloglucosidase hydrolysis according to Vijayan et al. (2006). The glycogen content is shown as micromoles glucosyl units per gram protein in the homogenate. The enzyme activities were measured in 50 mM imidazole-buffered enzyme reagent