Salicylate Disrupts Interrenal Steroidogenesis and Brain Glucocorticoid Receptor Expression in Rainbow Trout

Amélie Gravel and Mathilikath M. Vijayan
Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

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Varying levels of pharmaceuticals, including salicylate, ibuprofen, and acetaminophen, have been reported in the aquatic environment, but few studies have actually addressed the impact of these drugs on aquatic organisms. We tested the hypothesis that these pharmaceuticals are endocrine disruptors in fish by examining their impact on interrenal corticosteroidogenesis in rainbow trout. Indeed, acute adrenocorticotrophic hormone (ACTH)–mediated cortisol production in trout interrenal cells in vitro was significantly depressed (20–40%) by these pharmaceutical drugs. Furthermore, we investigated whether this interrenal dysfunction involved inhibition of the steroidogenic capacity in rainbow trout. To this end, we fed trout salicylate-laced feed (100 mg/kg body weight) for 3 days and assessed the transcript levels of key proteins involved in corticosteroidogenesis, including steroidogenic acute regulatory protein (StAR), peripheral-type benzodiazepine receptor (PBR), cytochrome P450 cholesterol side chain cleavage (P450scc), and 11β-hydroxylase. Salicylate treatment did not affect the resting plasma cortisol or glucose levels, whereas the acute ACTH-stimulated cortisol production was significantly depressed in the interrenal tissue. This disruption of steroidogenesis by salicylate corresponded to a significant drop in the gene expression of StAR and PBR, but not P450scc or 11β-hydroxylase, compared to the sham-treated fish. Also, brain glucocorticoid receptor (GR) protein content and not GR mRNA level was significantly reduced by salicylate. Taken together, salicylate is a corticosteroid disruptor in trout and the targets include the key rate-limiting step in interrenal steroidogenesis and brain glucocorticoid signaling.

Key Words: fish; Oncorhynchus mykiss; cortisol; StAR; peripheral-type benzodiazepine receptor; glucocorticoid receptor; ibuprofen; acetaminophen; NSAIDs.

Cortisol, the main corticosteroid released in response to stress in teleostean fishes, is thought to play a key role in enabling animals to regain homeostasis after stressor exposure (Barton et al., 2002; Mommsen et al., 1999; Wendelaar Bonga, 1997). The circulating level of this steroid is tightly regulated by the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). For instance, stress stimulates the release of corticotropin-releasing factor (CRF) from the hypothalamus, which in turn stimulates the release of adrenocorticotrophic hormone (ACTH), the main secretagogue for cortisol biosynthesis, from the pituitary. ACTH binds to the interrenal cells (steroidogenic cells), located predominantly in the anterior part of the kidney (head kidney) in fish, to activate the steroidogenic pathway leading to the release of cortisol (Hontela, 2005; Mommsen et al., 1999; Wendelaar Bonga, 1997). However, the circulating cortisol levels are also tightly regulated by a negative feedback loop, including glucocorticoid receptor (GR) signaling in the brain, inhibiting the release of trophic hormones (CRF and/or ACTH) in response to elevated steroid levels (Mommsen et al., 1999; Wendelaar Bonga, 1997). Consequently, any adverse effect on the functioning of the HPI axis would compromise the ability of the animal to respond to additional stressors (Barton et al., 2002; Hontela, 2005). Indeed, xenobiotics disrupt cortisol response to stress by targeting multiple sites along the HPI axis, including impaired steroidogenesis and brain glucocorticoid signaling (Aluru and Vijayan, 2006; Aluru et al., 2004; Hontela, 2005; Vijayan et al., 2005).

A key rate-limiting step in steroid biosynthesis involves the transport of cholesterol from intracellular sites of storage to the inner mitochondrial membrane. The cholesterol transport process is thought to require at least two key regulatory proteins: the steroidogenic acute regulatory protein (StAR) (Stocco, 2001; Stocco et al., 2005) and the peripheral-type benzodiazepine receptor (PBR) (Delavoie et al., 2003; Hauet et al., 2002; Papadopoulos, 2004). Together, StAR and PBR facilitate the transfer of cholesterol to the active site on cytochrome P450 side chain cleavage (P450scc), thereby initiating the steroid biosynthetic cascade (Culty et al., 1999; Papadopoulos, 2004). The ultimate step for cortisol biosynthesis is catalyzed by 11β-hydroxylase, which was shown not to be a limiting factor in the production of cortisol (Aluru and Vijayan, 2006). Most studies on cholesterol transport proteins in steroidogenic cells involved mammalian cell systems, and very little is known about the regulation of StAR and/or PBR in lower vertebrates. Recently, StAR was cloned and sequenced from trout (Kusakabe et al., 2002), and this protein showed stress-induced elevation similar...
to that seen in mammalian models (Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Hagen et al., 2006; Kusakabe et al., 2002). Also, aryl hydrocarbon receptor signaling was shown to disrupt corticosteroidogenesis by targeting StAR transcript levels (Aluru and Vijayan, 2006; Aluru et al., 2005). Together, these studies highlight StAR as a key target for steroidogenic disruption by toxicants; however, very little is known about the role of PBR expression in fish.

While studies on corticosteroid disruption by xenobiotics have focused on organic contaminants and heavy metals (Hontela, 2005; Vijayan et al., 2005), very little is known about the impact of other toxicants, including pharmaceutical drugs in the aquatic environment. Indeed, human and veterinary prescription and nonprescription drugs have become a matter of concern in aquatic toxicology due to the fact that many of these compounds have been detected in the aquatic environment (Daughton and Ternes, 1999; Halling-Sørensen et al., 1998; Heberer, 2002; Metcalfe et al., 2003a,b; Trudeau et al., 2005). However, most studies on the impact of pharmaceutical drugs in aquatic animals have been restricted to short-term acute toxicity bioassays (Cleuvers, 2004; Webb, 2001), and very little is known about either the sublethal effects of these drugs or their mechanisms of action. A recent study demonstrated that acetylsalicylic acid (ASA) altered plasma thyroid hormone levels and also attenuated the stress-induced cortisol response to stress in tilapia (Oreochromis mossambicus) (van Anholt et al., 2003). However, the mechanism involved in this disruption of cortisol response to stress was not ascertained.

The objective of this study was to investigate whether the mode of action of pharmaceutical drugs involved steroidogenic disruption in fish. For this, we initially screened three drugs, two nonsteroidal anti-inflammatory drugs (NSAIDs: salicylate and ibuprofen) and one analgesic/antipyretic agent (acetaminophen), for their impact on acute ACTH-mediated cortisol production in rainbow trout (Oncorhyncus mykiss) interrenal cells in vitro. Furthermore, we tested the hypothesis that key proteins in the corticosteroid biosynthetic pathway are targets for impact by pharmaceutical drugs. To this end, we fed trout salicylate in vivo, according to the protocol followed by van Anholt et al. (2003), and examined the interrenal capacity for cortisol production, including mRNA abundance of StAR, PBR, P450scc, and 11β-hydroxylase in the interrenal tissue. In addition, brain GR expression was assessed, especially since this protein is crucial for the negative feedback regulation of cortisol to determine if salicylate targets multiple sites along the corticosteroid stress axis.

MATERIALS AND METHODS

Chemicals

Leibovitz’s (L-15) culture medium, porcine ACTH (1–39), acetaminophen, ibuprofen, salicylate (salicylic acid, sodium salt), protease inhibitor cocktail, bicinechonic acid reagent, and 2-phenoxyethanol were purchased from Sigma (St Louis, MO) and collagenase/dispose was from Roche (Montreal, QC, Canada). Multiwell (24-well plate) tissue culture plates were from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ). The electrophoresis reagents and molecular weight markers were from BioRad (Hercules, CA). 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, Canada). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, ON, Canada).

Experimental Animals

Juvenile rainbow trout (~ 150 g body mass) were obtained from Humber Springs Hatchery, Mono Mills, Ontario. Fish were maintained for at least a month in aerated 200-l tanks with constant water flow at 13°C with a 12 h light:12 h dark photoperiod prior to their use for either the in vitro or the in vivo study.

In Vitro Study—Interrenal Cells

Interrenal cell preparation. Cortisol production in vitro was determined using interrenal cell suspension following the protocol described by Leblond and Hontela (1999), with slight modification. Briefly, fish were anesthetized with 2-phenoxyethanol, and the caudal peduncle was dissected to expose the caudal vein. Initial blood sample was collected after which the caudal vein was perfused with a syringe containing saline solution (0.7% NaCl) to clear blood from the kidney. The interrenal tissue was dissected and placed in fresh L-15 medium supplemented with antimycotic/antibiotic, 5 mM NaHCO₃, and 5 mM glucose, with pH adjusted to 7.6. The tissue was washed and resuspended in fresh supplemented L-15 medium with 2 mg/ml collagenase/dispose and incubated for 60 min at 13°C with gentle agitation in a 15-ml conical centrifuge tube. Following enzymatic digestion, the solution was filtered through a 40-μm mesh, and the filtrate was centrifuged at 250 × g for 10 min. The supernatant was removed and the pellet resuspended in fresh L-15 medium and the cell density adjusted to 75 × 10⁶ cells/ml.

Exposure to drugs. Cells were plated in a 24-well culture plate with 500 μl of the cell suspension per well. Protocol for exposure and ACTH stimulation was similar to that followed by Leblond and Hontela (1999), with slight modification. All incubations were performed at 13°C with slow agitation, and the plated cells were allowed 2-h settling period. The cells were then centrifuged for 3 min at 200 × g, supernatant was removed, and the cells were exposed to either L-15 alone or L-15 containing acetaminophen, ibuprofen, or salicylate (1 μM–1000 μM) for 22 h. Exposure to CdCl₂ (160 μM) was used as a positive control to confirm the effectiveness of this system, as previous studies clearly showed that this concentration of cadmium inhibits cortisol production without affecting cell survival (Lacroix and Hontela, 2004; Leblond and Hontela, 1999). After the incubation, ACTH was added to each well (0.5 IU/ml final concentration) and medium collected 2 h later for cortisol analysis. This concentration of ACTH gave maximal cortisol response with trout interrenal preparations (Aluru and Vijayan, 2004). There was no significant effect of drug treatments on cell viability as determined by measuring lactate dehydrogenase leakage (data not shown).

In Vivo Study—Salicylate Exposure

Groups of five trout each were separated into two aquaria (60-l capacity) and maintained exactly as above for 2 weeks. The fish were fed to satiety with trout chow (five-point sinking food, Martin Mills Inc., Elmira, ON, Canada) once daily 5 days a week. After 2 weeks of acclimation, one group of fish were fed control diet (trout chow at 2% body mass), while the other group was fed the same amount of feed laced with salicylate (100 mg/kg). The drug incorporation into the feed and the feeding protocol was exactly as described before for rainbow trout (Aluru et al., 2005). Briefly, salicylate was dissolved in 100% ethanol and sprayed on the trout chow and the ethanol allowed to evaporate overnight. A control feed was also prepared exactly as the test feed, but without
the drug. The salicylate concentration chosen for this study, as well as the administration route, was based on the methodology followed by van Anholt et al. (2003).

The fish were fed the treatment diet for 3 days and sampled on the fourth day. Sampling consisted of quickly netting all fish from each tank and anesthetizing with an overdose of 2-phenoxyethanol (1:1000). Fish were bled by caudal puncture into heparinized tubes, and plasma collected after centrifugation (10 min at 6000 \( \times \) g) was kept frozen at \(-70^\circ\)C for cortisol and glucose analyses. Pieces of brain and head kidney tissues were collected and immediately frozen on dry ice and stored at \(-70^\circ\)C for mRNA and protein determination later. Also, pieces of fresh head kidney tissue, immediately after sampling, were placed in L-15 media and subjected to an \textit{in vitro} ACTH challenge test (see below).

**Cortisol production.** The \textit{in vitro} incubation and ACTH challenge were conducted exactly as described before by Aluru and Vijayan (2004). Briefly, head kidney pieces from each fish were finely minced (~1-mm\(^3\) size) and rinsed with cold L-15 media and then distributed into two wells (10 fish \( \times \) 2 wells each = 20 wells) of a 24-well Falcon tissue culture plate. The tissue slices were allowed to recover for 2 h at 13\(^\circ\)C with gentle shaking. Media were collected after the recovery period for determination of basal cortisol production, after which the tissue slices (one set each) were exposed to either fresh L-15 media alone (control wells) or fresh L-15 media containing 0.5 IU/ml ACTH. The incubation lasted for 2 h, and the media were collected and stored frozen at \(-70^\circ\)C for later determination of cortisol concentration. The tissue wet weight was measured in order to normalize the cortisol production rates.

**Plasma Cortisol and Glucose Analyses**

Plasma cortisol concentration was measured using a commercially available ImmunoChem 125I RIA kit (MP Biomedicals, Irvine, CA) that was validated for was measured in order to normalize the cortisol production rates.

**Immunodetection of GR**

Tissue homogenization and sample preparation for immunodetection was described in detail previously (Boone et al., 2002). Briefly, samples in Laemmli’s buffer were loaded (40 \( \mu \)g protein/sample) onto 8% SDS-PAGE and proteins separated using a discontinuous buffer (Laemmli, 1970). The proteins were transferred onto nitrocellulose membranes using a semidry transfer unit (BioRad). The membranes were blocked for 1 h with 5% skim milk in TTBS (20mM Tris, pH 7.5, 300mM NaCl, and 0.1% [vol/vol] Tween 20 with 0.02% sodium azide) buffer, probed for 1 h at room temperature with polyclonal rabbit anti-trout GR (1:1000) in the blocking solution, washed with TTBS, and incubated for 1 h with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:3000 dilution). The protein bands were detected with NBT and BCIP, and the intensities of the scanned bands were quantified using the AlphaEase software (AlphaEase Innotech, San Leandro, CA).

**Transcript Levels—Quantitative Real-Time PCR**

**RNA isolation and cDNA synthesis.** Total RNA (DNase treated) was isolated from tissues using the RNeasy Mini Kit protocol (Qiagen, Mississauga, ON), and the RNA was quantified by spectrophotometry at 260 nm. The first-strand cDNA was synthesized from 1 \( \mu \)g total RNA using First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON). Briefly, total RNA was heat denatured (70\(^\circ\)C) and cooled on ice, and the sample was used in a 20-\( \mu \)l reverse transcriptase reaction using 0.5 \( \mu \)g oligo (dT) primers and 1\( \mu \)M each dNTP, 20 U ribonuclease inhibitors, and 40 \( \mu \)M MuLV reverse transcriptase. The reaction was started by incubating at 37\(^\circ\)C for 1 h and stopped by heating at 70\(^\circ\)C for 10 min.

**Primers.** The primers (Table 1) were designed using rainbow trout GR, StAR, P450scc, 11\(\beta\)-hydroxylase, PBR, and \(\beta\)-actin cDNA sequences (GenBank accession nos. Z54210, AB047032, S57305, AF179894, AY029216, and AF157514, respectively). The primer pairs amplified either a ~100-bp fragment for GR and \(\beta\)-actin or a ~500-bp fragment for all the other genes used in quantitative real-time PCR (qPCR) (iCycler, BioRad).

**Data analysis.** Quantification of transcript levels was performed as previously described (Sathiyaa and Vijayan, 2003) by qPCR using Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Briefly, cDNA samples were used as template for amplification of the housekeeping gene (\(\beta\)-actin) and target genes (GR, StAR, P450scc, 11\(\beta\)-hydroxylase, and PBR) using appropriate primers and their respective annealing temperatures (Table 1). A relative standard curve for each gene was constructed using either serial dilutions of cDNA or plasmid vector with inserted target sequences and subjected to the same qPCR cycles and conditions as test samples: 95\(^\circ\)C for 3 min; 40 cycles of denaturation at 95\(^\circ\)C for 20 s, annealing for 20 s, and extension at 72\(^\circ\)C for 20 s; and then cool at 4\(^\circ\)C hold. The iCycler IQ real-time detection software (BioRad) was used to determine the threshold values (\(C_T\)) for every sample. A standard curve with log input amount and \(C_T\) was established for each gene and used to calculate the transcript levels in samples. The target gene transcript levels were normalized by taking a ratio of target gene to housekeeping gene. This normalized value was standardized using an internal calibrator (sham group) and transcript levels expressed as percent sham.

**Statistical Analyses**

The differences in cortisol production \textit{in vitro} in response to different concentrations of drugs were compared statistically using one-way ANOVA followed by Tukey test. For the \textit{in vivo} study with salicylate, significant differences among the sham and treated groups were ascertained using an unpaired Student \(t\)-test. Data were log transformed, wherever necessary, for

### TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Annealing temperature ((^\circ)C)</th>
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<tbody>
<tr>
<td>GR</td>
<td>Forward: 5'-AGAAGCCTGTTTTTGGCCCTGTA-3'</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGATGAGCTCGACATCCCTGAT-3'</td>
<td></td>
</tr>
<tr>
<td>StAR</td>
<td>Forward: 5'-CGCTGGACATCCTCTACA-3'</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGGACTTTCTGTAAGTTTC-3'</td>
<td></td>
</tr>
<tr>
<td>P450scc</td>
<td>Forward: 5'-AGGGAGGGTTAGAGGACCA-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AAGTTGAGGACTCTCTGT-3'</td>
<td></td>
</tr>
<tr>
<td>11(\beta)-Hydroxylase</td>
<td>Forward: 5'-AAGCTTCTGCGCTTC-3'</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGTGAGCCTGGCGCAAGA-3'</td>
<td></td>
</tr>
<tr>
<td>PBR</td>
<td>Forward: 5'-GGCAGGGACTCTCTGGTCTT-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCCATGCAAGTTAGGTTGTCAGGA-3'</td>
<td></td>
</tr>
<tr>
<td>(\beta)-Actin</td>
<td>Forward: 5'-AGAAGCTACAGGCTGGCTGAC-3'</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCAAGACTCCCATACGGAGGA-3'</td>
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homogeneity of variance, while nontransformed data are shown in the tables and figures. A significance level of $\alpha = 0.05$ was considered statistically significant.

**RESULTS**

**In Vitro Study**

Salicylate, ibuprofen, and acetaminophen, at the concentrations tested, were not cytotoxic to interrenal cells as determined by lactate dehydrogenase leakage (0.5 U/ml per 75 × 10^6 cells). Salicylate and acetaminophen at a concentration of 10^{-5} M and above significantly depressed cortisol production compared to the control group (Figs. 1A and 1B). Ibuprofen exposure significantly decreased cortisol production at concentrations of 10^{-7} M and above, except for a lack of significant difference at 10^{-6} and 10^{-5} M concentrations compared to the control group (Fig. 1C). The maximal suppression of cortisol production with salicylate (10^{-3.5} M) and ibuprofen (10^{-4.5} and 10^{-3.5} M) was around 40% and acetaminophen (10^{-5}–10^{-3} M) was 20% (Figs. 1A–1C).

**In Vivo Study**

**Cortisol and glucose concentration.** Salicylate treatment did not significantly affect either plasma cortisol or glucose concentrations in rainbow trout (Table 2). However, salicylate significantly inhibited (~ 50%) the acute ACTH-mediated cortisol production compared to the sham group (Fig. 2). There was no effect of salicylate treatment on unstimulated cortisol production by trout head kidney slices.

**StAR, PBR, P450scc, and 11β-hydroxylase transcript abundance.** Transcript abundance of genes encoding proteins involved at different stages of the cortisol biosynthetic pathway was measured in the interrenal tissue (Fig. 3). Transcript levels of StAR and PBR, two key proteins involved in cholesterol transport to the inner mitochondrial membrane, were significantly depressed in salicylate-treated fish compared to the sham fish (Figs. 3A and 3B). There was no significant difference in 11β-hydroxylase and P450scc transcript levels between the salicylate-treated and sham groups (Figs. 3C and 3D).

**Glucocorticoid receptor.** GR mRNA abundance in the brain of the salicylate group was not significantly different from the sham group in the present study (Fig. 4A). However, GR protein content in the brain was significantly reduced (~ 50%) with salicylate treatment compared to the sham fish (Fig. 4B).

**DISCUSSION**

We demonstrate that pharmacological levels of salicylate, ibuprofen, and acetaminophen disrupt interrenal capacity for...
cortisol production in rainbow trout. The interrenal cell preparation used in this study has been extensively used to test the impact of xenobiotics on cortisol biosynthetic capacity in teleostean fishes (Hontela, 2005). Those studies have identified several organic contaminants and heavy metals as adrenotoxicants in fish (Hontela, 2005), but this is the first report of pharmaceutical drugs impacting steroidogenesis in fish.

Recently, ASA administration in vivo was shown to attenuate the stressor-induced cortisol response in Mozambique tilapia, while the mode of action of the drug was not ascertained (van Anholt et al., 2003). The impaired acute ACTH-mediated cortisol response with drugs, including salicylate, in the present study (Fig. 1) leads us to propose steroidogenic disruption as a possible mechanism for the muted cortisol response to stress in tilapia. In support of this notion, salicylate exposure in vivo depressed acute ACTH-mediated interrenal corticosteroidogenesis by 50% supporting the interrenal steroidogenic pathway as a target for salicylate impact in fish. The absence of any significant changes in resting plasma cortisol or glucose levels with either salicylate (Table 2) or ASA (van Anholt et al.,

<p>| Table 2: Plasma Cortisol and Glucose Concentrations in Trout Fed Salicylate for 3 Days |
|----------------------------------------|----------------------------------------|</p>
<table>
<thead>
<tr>
<th>Cortisol (ng/ml)</th>
<th>Glucose (mM)</th>
</tr>
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<tr>
<td>Sham</td>
<td>2.38 ± 0.12</td>
</tr>
<tr>
<td>Salicylate</td>
<td>2.75 ± 0.50</td>
</tr>
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Note. Values represent means ± SEM (n = 5 fish).

**FIG. 2.** Acute ACTH-mediated cortisol production by head kidney slices obtained from trout fed salicylate for 3 days. Data shown as magnitude of cortisol production (unstimulated levels subtracted from the ACTH-stimulated cortisol production) in both groups; values represent means ± SEM (n = 5 fish); *indicates statistical significance from the sham group (Student t-test; p < 0.05).

**FIG. 3.** StAR (A), PBR (B), P450scc (C), and 11β-hydroxylase (D) transcript levels in the head kidney slices obtained from trout fed salicylate for 3 days. Values represent means ± SEM (n = 5 fish); * indicates statistical significance from the sham group (Student t-test; p < 0.05).
Our results for the first time highlight StAR and PBR as targets for salicylate-mediated disruption of steroidogenesis in trout. StAR protein was shown before to be a target for xenobiotics-mediated endocrine disruption in animals (reviewed in Harvey and Everett, 2003; Mutoh et al., 2006), including fish (Aluru and Vijayan, 2006; Aluru et al., 2005), but few studies have investigated the toxic impact on PBR levels (Boujrad et al., 2000; Gazouli et al., 2002). The depressed StAR and PBR transcript levels along with a reduced steroid output from interrenal cells points to an impairment of the cholesterol transport process. This notion finds support from a recent study that showed impaired mitochondrial cholesterol transport as a limiting factor for the steroidogenic disruption by β-sitosterol in male goldfish (Carassius auratus) gonads (Leusch and MacLatchy, 2003). Also, the lack of a significant change in P450scc and 11β-hydroxylase transcript levels argues against pathways downstream of cholesterol transport to be a limiting factor in the inhibition of cortisol production by salicylate.

While our study showed only a marginal reduction in StAR and PBR transcript levels by salicylate (~15–20%), the interrenal tissue capacity for acute cortisol production was substantially lower (~50%). This is in agreement with other studies clearly showing that despite marginal, but significant, transcript level changes in StAR and P450scc with either stressor or ACTH stimulation, the inhibition of cortisol output was of a much greater magnitude relative to transcript changes (Aluru and Vijayan, 2006; Aluru et al., 2005; Geslin and Auperin, 2004; Hagen et al., 2006). Indeed, we showed that abolishment of this minimal transcript response completely reverted the cortisol production capacity of interrenal tissue reflecting the important role for the rate-limiting step in modulating steroidogenesis in fish (Aluru and Vijayan, 2006). The reason for the minor changes in the transcript levels of key steroidogenic proteins in teleostean studies compared to mammalian studies (Boujrad et al., 2000; Gazouli et al., 2002; Walsh et al., 2000a,b) is unclear. Nonetheless, it appears likely that salicylate-mediated disruption of steroidogenesis contributes to the impaired cortisol response to stressor seen in fish (van Anholt et al., 2003), and the mode of action involves reduction in StAR and PBR mRNA levels in fish.

Although the mechanism involved in steroidogenic disruption by salicylate is not clear, few likely scenarios emerge. For instance, salicylate inhibits cyclooxygenases (Borne, 1995; Brooks and Day, 1991) and, therefore, will reduce the formation of arachidonic acid metabolites, including prostaglandins (PG). It is well known that PG modulate the functioning of the hypothalamus-pituitary-adrenal axis in mammals (Cavagnini et al., 1979; Hockings et al., 1993; Nye et al., 1997) and even in fish (Gupta et al., 1985; Wales, 1988).
Also, administration of ASA to tilapia lowered plasma PG\textsubscript{E\textsubscript{2}} concentration (van Anholt et al., 2003), and this coincided with a lowered cortisol response to stress. Together, these results point to a role for PG as a link for salicylate impact on cortisol response in fish. However, recent studies also point to a PG-independent action of arachidonic acid signaling in modulating the cortisol response to stress in fish (van Anholt et al., 2004a,b). It has also been demonstrated that several NSAIDs, including salicylate, exhibit anti-inflammatory effects independent of cyclooxygenase activity (Amann and Peskar, 2002; Tegeder et al., 2001). This raises the possibility that other pathways, in addition to PG, may be involved in salicylate-mediated disruption of cortisol biosynthesis in fish. Specifically, the finding that NSAIDs can also affect the activation of transcription factors, including the peroxisome proliferator-activated receptors (PPARs), a key regulator of steroidogenesis (Komar, 2005), suggests a molecular mechanism for salicylate-mediated inhibition of the rate-limiting step in steroidogenesis. Indeed, this argument finds support from the fact that PPAR alpha stimulation depressed PBR transcript levels in a mammalian cell model (Gazouli et al., 2002), leading to our hypothesis that PPARs activation by salicylate may be a mechanism for inhibition of steroidogenesis in fish.

Although elevated plasma cortisol levels poststressor is associated with enhanced interrenal steroidogenesis, a key player in the cortisol response also involves activation of the hypothalamus-pituitary axis. For instance, elevation of cortisol levels is tightly regulated in part by a negative feedback loop, whereby cortisol levels, via GR activation, reduce CRF and ACTH release from the hypothalamus and pituitary, respectively (Bernier and Peter, 2001; Huisings et al., 2004; Mommsen et al., 1999; Wendelaar Bonga, 1997). Indeed reduction in brain GR protein content with PCBs was accompanied by a disturbed plasma cortisol profile in response to stressors in arctic char (Salvelinus alpinus: Aluru et al., 2004), suggesting impaired feedback regulation. While we did not assess the cortisol response to acute stressors in the present study, the reduced GR protein content (~50%) in the salicylate-treated fish leads us to hypothesize that this drug also disrupts the negative feedback regulation of cortisol in rainbow trout. However, this lower GR protein content was not accompanied by changes in GR mRNA content in the salicylate group, suggesting increased GR protein breakdown. Considered together, the disruption of the cortisol response to stress by pharmaceutical drugs, including NSAIDs, may involve multiple targets along the corticosteroid stress axis in fish.

In conclusion, salicylate, acetaminophen, and ibuprofen are endocrine disruptors in fish and have the potential to impair the adaptive cortisol response to stressors. We further demonstrate that the inhibition of the acute cortisol production capacity seen with salicylate involves depressed StAR and PBR transcript levels, two proteins crucial for cholesterol transport to the inner mitochondria membrane for its utilization by steroidogenic enzymes. In addition, salicylate also reduced brain GR protein content in rainbow trout. Altogether, StAR, PBR, and GR, key proteins involved in the activation of corticosteroid stress axis, are targets for salicylate-mediated impairment of the adaptive cortisol response to stress in fish.

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