Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout

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Submitted 30 May 2007; accepted in final form 5 September 2007

Aluru N, Vijayan MM. Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. Physiol Genomics 31: 483–491, 2007. First published September 11, 2007; doi:10.1152/physiolgenomics.00118.2007.—Cortisol, the principal corticosteroid in teleosts, is secreted in rainbow trout (Oncorhynchus mykiss); stress response; intermediary metabolism; cortisol; fish; RU486; cortisol; fish; GR; MR; RU486; cortisol; fish; genomic cortisol signaling in the liver molecular reprogramming that are GR specific in fish (31, 59). To this end, RU486 (a GR antagonist) has been a useful tool to elucidate GR-specific responses in piscine models (7, 15, 23, 51, 54, 61), including inhibition of cortisol-induced glucose release in trout hepatocytes (50).

From a comparative standpoint, unlike mammals, teleosts appear to have multiple isoforms of GR (7, 18, 46), although the physiological role for each isoform is unknown. Also, some of the cortisol effects may be mediated via mineralocorticoid receptor (MR) activation; recently, an MR-specific ligand was identified in trout (46, 56). In addition to genomic signaling mediated by GR and MR, recent studies also point to a nongenomic signaling by corticosteroids involving either membrane receptor activation and/or changes in membrane structure associated with this steroid incorporation (6, 57). Although teleostean GRs have been cloned and sequenced and their transcriptional regulation characterized using bacterial reporter constructs (56), very little is known about the molecular responses to GR activation in a physiologically relevant model system.

Primary culture of hepatocytes has been routinely used as a model system of choice for characterization of hormone signaling and associated metabolic response in teleosts (32, 34, 52). Indeed, with the use of this cell system, studies have established that, as in mammals, cortisol is a gluconeogenic hormone in fish (5, 31, 50, 60). The increased liver capacity for gluconeogenesis is an important stress adaptation, as glucose is the primary fuel to cope with the energy-demanding pathways essential for regaining homeostasis (31). Although studies have focused on changes in tissue metabolic capacity with cortisol treatment, few studies have actually addressed the molecular mechanisms during stress adaptation that are GR specific in fish (31, 59). To this end, RU486 (a steroid analog) has been a useful tool to elucidate GR-specific responses in piscine models (7, 15, 23, 51, 54, 61), including inhibition of cortisol-induced glucose release in trout hepatocytes (50).

The advent of microarray technology has certainly hastened the process of identifying multiple pathways affected by experimental manipulations (3, 17), including stressor-responsive gene expression patterns in piscine models (25, 26, 47, 65). However, little is known about the role of stress hormone cortisol signaling in this adaptive molecular response to stress. Consequently, in this study, we utilized a custom-made low-density rainbow trout (Oncorhynchus mykiss) cDNA array, enriched with genes involved in the endocrine and metabolic responses to stress, to identify the GR-responsive gene expression pattern in trout hepatocytes. We resorted to using a low-density array, as opposed to recently developed high-density arrays, mainly because our focus was on genes that were well characterized in trout as playing a key role in the adaptive responses to regain homeostasis. The GR-specific responses were teased out by treating cells with cortisol and blocking the response with RU486 (50, 61, 62).

CORTISOL, the principal glucocorticoid in teleosts, is secreted by the interrenal tissues (analogous to the adrenal cortex) dispersed in the head kidney region. This steroid is involved in a diverse array of functions, including growth and metabolism, osmoregulation, immune function, and reproduction (31, 63). A well-studied role for cortisol involves tissue-specific metabolic reprogramming to cope with the increased energy demands associated with stress, but the associated molecular responses are far from clear. It is well established that cortisol actions are mediated via the glucocorticoid receptor (GR) belonging to the nuclear receptor family of ligand-bound transcription factors (46, 59).

From a comparative standpoint, unlike mammals, teleosts appear to have multiple isoforms of GR (7, 18, 46), although the physiological role for each isoform is unknown. Also, some of the cortisol effects may be mediated via mineralocorticoid receptor (MR) activation; recently, an MR-specific ligand was identified in trout (46, 56). In addition to genomic signaling mediated by GR and MR, recent studies also point to a nongenomic signaling by corticosteroids involving either membrane receptor activation and/or changes in membrane structure associated with this steroid incorporation (6, 57). Although teleostean GRs have been cloned and sequenced and their transcriptional regulation characterized using bacterial reporter constructs (56), very little is known about the molecular responses to GR activation in a physiologically relevant model system.

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The advent of microarray technology has certainly hastened the process of identifying multiple pathways affected by experimental manipulations (3, 17), including stressor-responsive gene expression patterns in piscine models (25, 26, 47, 65). However, little is known about the role of stress hormone cortisol signaling in this adaptive molecular response to stress. Consequently, in this study, we utilized a custom-made low-density rainbow trout (Oncorhynchus mykiss) cDNA array, enriched with genes involved in the endocrine and metabolic responses to stress, to identify the GR-responsive gene expression pattern in trout hepatocytes. We resorted to using a low-density array, as opposed to recently developed high-density arrays, mainly because our focus was on genes that were well characterized in trout as playing a key role in the adaptive responses to regain homeostasis. The GR-specific responses were teased out by treating cells with cortisol and blocking the response with RU486 (50, 61, 62).
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MATERIALS AND METHODS

Chemicals

Cortisol, RU486 (mifepristone), L-15 media, protease inhibitor cocktail, 2-phenoxethanol, bicinechomonic acid (BCA) reagent, and antibiotic and antitumycotic solution were purchased from Sigma (St. Louis, MO). Multiwell (6-well plate) tissue culture plates were obtained from Sarstedt. Electrophoresis reagents and molecular mass markers were from Bio-Rad (Hercules, CA). Primary antibody to trout GR was developed in our laboratory (50), and the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG, was purchased from Bio-Rad. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) were obtained from Fisher Scientific (Nepean, ON, Canada).

Experimental Fish

Juvenile rainbow trout (120 ± 10 g body mass) were obtained from Humber Springs Trout Farm (Mono Mills, ON, Canada) and kept at the University of Waterloo Aquatic Facility at 10 ± 1°C on a 12:12-h light-dark cycle and fed once daily to satiation (5 point sinking feed; Martin Mills, Elmira, ON, Canada). The fish were acclimated for at least 5 wk before the experiments, and the animal protocol was approved by the animal care committee at the University of Waterloo.

Primary Culture of Trout Hepatocytes

An in situ liver perfusion method using collagenase was followed in isolating rainbow trout hepatocytes, as described in detail before (49). Briefly, isolated hepatocytes were washed with modified Hanks' medium (110 mM NaCl, 3 mM KCl, 1.25 mM KHPO4, 5 mM NaHCO3, 0.6 mM MgSO4, 1 mM MgCl2, and 10 mM HEPES; pH 7.63 at room temperature) and then with modified Hanks' medium containing 1.5 mM CaCl2 and 2% defatted bovine serum albumin (BSA), pH 7.63. L-15 media supplemented with antibiotic and antitumycotic agents were used to resuspend the washed hepatocytes. Trypan blue exclusion method was used to determine cell viability, and >95% of cells were viable. Cells were plated in six-well tissue culture plates at a density of 1.5 million cells/well (0.75 million cells/ml) in L-15 media and were maintained at 13°C for 24 h before commencement of the experiment. All the experiments were repeated with hepatocytes isolated from four different fish.

Experimental Treatments

The experimental protocol consisted of replacing the media with either fresh media (control; 0.01% ethanol used as vehicle) or media containing cortisol (100 ng/ml), RU486 (GR antagonist; 1,000 ng/ml), or a combination of both RU486 (1,000 ng/ml) and cortisol (100 ng/ml); cells were maintained at 13°C for 24 h before sampling. The exposure time was chosen based on our previous study (50), which showed maximal response to cortisol stimulation at 24 h in trout hepatocytes. In the combination group, cells were incubated with the drug 30 min before the addition of cortisol. The cortisol concentration used mimicked stressed levels seen in trout, while the RU486 concentration was shown previously to block cortisol-mediated metabolic effects in trout hepatocytes (50). Both cortisol and RU486 were dissolved in ethanol, and the final concentration of ethanol in the incubation medium was maintained at 0.01% in all treatments. At the end of the experimental period, the medium was collected, and the cells were centrifuged (13,000 g for 1 min); supernatants were removed, and the cell pellets were flash frozen in dry ice and stored at −70°C. Media glucose was determined colorimetrically using a commercially available kit (Trinder method; Sigma). The cell viability was determined by measuring the release of lactate dehydrogenase (LDH) into the medium. The LDH activity was determined following standard protocols (4). LDH leakage was minimal (<5% of total LDH) in all groups, and there were no significant difference among treatments (data not shown).

SDS-PAGE and Western Blotting

Sample protein concentrations were determined using the BCA method with BSA as the standard. The procedures followed for SDS-PAGE and Western blotting were according to established protocols (4). Briefly, samples (40 μg of protein) were separated on 8% polyacrylamide gels using the discontinuous buffer system of Laemmli (27). The proteins were transferred onto nitrocellulose membrane (20 V for 30 min) with a semidy transfer unit (Bio-Rad) using transfer buffer [25 mM Tris, pH 8.3, 192 mM glycine, and 20% (vol/vol) methanol]. Equal loading was confirmed by staining the membrane with Ponceau S. The membranes were washed and blocked with 5% skimmed milk in TBS-t (20 mM Tris, pH 7.5, 300 mM NaCl, and 0.1% (vol/vol) Tween 20 with 0.02% sodium azide) for 60 min. Polyclonal anti-trout GR antibody was used at a 1:1,000 dilution, and the secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3,000 dilution). The membranes were incubated in primary antibody for 60 min at room temperature, washed with TBS-t (2 × 5 min), incubated with secondary antibody for 60 min, washed with TBS-t (2 × 5 min), and finally washed with TBS (1 × 15 min). Band detection was carried out with NBT (0.033% wt/vol) and BCIP (0.017% wt/vol), and the bands were quantified with Chemi-imager using AlphaEase software (Alpha Innotech). The molecular mass of the protein was confirmed using prestained low-range molecular mass markers (phosphorylase B, 112 kDa; BSA, 81 kDa; ovalbumin, 49.9 kDa; carbonic anhydrase, 36.2 kDa; soybean trypsin inhibitor, 29.9 kDa; lysozyme, 21.3 kDa).

cDNA Microarray

Microarray experiments were designed to comply with minimum information about microarray experiment (MIAME) guidelines. The development and application of the targeted trout cDNA microarray have been described in detail before (65). The microarray platform has been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; accession no. GPL 3713). A common reference standard (RS) was made by pooling equal amounts of RNA from untreated hepatocytes. Each and every sample was cohybridized with the RS, and a total of 16 hybridizations (4 treatments × 4 independent fish hepatocytes) were performed. Dye swapping was carried out so that each treatment had two samples labeled with either cyanine-3 (Cy3) or Cy5.

RNA Isolation, cDNA Synthesis, and Labeling

Total RNA was extracted from the hepatocytes using the RNeasy extraction kit (Qiagen). The quality of the total RNA obtained was checked by gel electrophoresis on a 1% agarose gel containing ethidium bromide, and the concentration was determined spectrophotometrically at 260/280 nm using a Cary 50 UV-Vis spectrophotometer (Varian).

Thirty micrograms of intact total RNA (either treatment sample or RS RNA) were indirectly labeled with Cy5 and Cy3 following The Institute for Genomic Research (TIGR) protocol (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml) (Amersham Biosciences, Piscataway, NJ). In brief, cDNA was synthesized by incorporating aminoallyldUTP (AA-dUTP) at 42°C for 4 h. RNA was hydrolyzed with 10 μl of 1 M NaOH and 10 μl of 0.5 M EDTA for 15 min at 70°C and neutralized by adding 10 μl of 1 M HCl. To remove unincorporated AA-dUTP and free amines, the reactions were purified with Qiagen columns using phosphate wash buffer and phosphate elution buffer. The purified sample was dried in a Speed Vac, and AA-cDNA was resuspended in 0.1 M sodium bicarbonate buffer; coupling to the cyanine dye ester was performed for 90 min in the dark at room temperature. The removal of uncoupled dye was performed using the Qiagen PCR purification kit.
Hybridization and Postprocessing

Before hybridization, the Cy3- and Cy5-labeled samples were combined and dried in a Speed Vac. To the dried sample, 26 μl of hybridization buffer and 2 μl each of calf thymus DNA (10 mg/ml) and yeast tRNA (10 mg/ml) were added. The samples were denatured for 2 min at 95°C, snap cooled on ice for 1 min, and centrifuged for 1 min at maximum speed. The solution was pipetted onto a microarray slide placed in a hybridization chamber, and the coverslip was placed carefully. Hybridization was carried out overnight in a water bath at 37°C.

After hybridization, the coverslip was removed by immersion in a low-stringency wash buffer (1× SSC, 0.2% SDS). The slide was washed for 10 min in low-stringency wash buffer, 5 min in high-stringency wash buffer (0.1× SSC, 0.2% SDS, and MilliQ water), and twice for 2.5 min in 0.1× SSC. Low-stringency washing was carried out at 48°C, whereas the remaining washing steps were at room temperature with shaking. Finally, the slides were quickly dipped in MilliQ water and centrifuged at 1,000 g for 2 min in a 50-ml Falcon tube at room temperature for drying.

Image Analysis

Image analysis was carried out using VersArray ChipReader software version 3.1 (Bio-Rad). For each microarray slide, two images were produced by illuminating the array at 635 nm (excitation of Cy5) and 532 nm (Cy3). For both illuminations, photomultiplier tube (gain and light amplification) settings were at 900, and laser power was set at 90%. All images were captured in a TIFF format.

Spot finding and quantification were carried out with ImaGenie 3.0 microarray image analysis software (BioDiscovery, Los Angeles, CA). Briefly, a grid was created, taking into account the number of spots, the printing pattern, and the size of the spots. Spot finding was performed using a semiautomatic method in which each spot was checked for proper alignment within the grid manually. Finally, spot quantification was performed to obtain the expression level of each gene on the array.

Data Analysis

The data analysis was carried out using the microarray data analysis system (MIDAS) (48). Poor or negative control spots were flagged as unreliable and were excluded from the analysis. For each spot, signal/noise (SN) threshold was calculated using the following formula: [(A or B) + Bkg(A or B)]/Bkg(A or B), where, respectively, I(A) and I(B) denote background-corrected signal intensity for channels A and B and Bkg(A) and Bkg(B) denote background intensity for channels A and B. The SN ratio for each channel was compared with the set threshold value of 2.0, and any spots ≤2.0 were marked as bad and excluded from downstream analysis. Data were then LOWESS normalized before calculation of the gene expression ratios. Data for each treatment (fold change relative to the RS) were calculated as a ratio of normalized gene intensity for the sample to that of the reference RNA on each slide. The raw data set has been deposited into GEO (http://www.ncbi.nlm.nih.gov/geo; series GSE 7671).

Of the genes that were significantly different among the treatments, six were picked for gene quantification using quantitative real-time PCR (iCycler, Bio-Rad) to validate the reliability of gene expression patterns observed with the microarray.

Quantitative Real-Time PCR

cDNA synthesis. The first-strand cDNA was synthesized from 1 μg of total RNA using the First-Strand cDNA synthesis kit (MBI Ferments). Briefly, total RNA was heat denatured (70°C) and cooled on ice. The sample was used in a 20-μl reverse transcriptase (RT) reaction using 0.5 μg of oligo(dT) primers and 1 mM each dNTP, 20 U of ribonuclease inhibitors, and 40 U of Moloney murine leukemia virus RT. The reaction was incubated at 37°C for 1 h and stopped by heating at 70°C for 10 min.

Relative standard curve. A relative standard curve for all of the genes was constructed using serial dilutions of plasmid vector stock with inserted target sequences according to established protocols (50). The primers were designed using rainbow trout sequences, and the details of the gene accession numbers, primer sequences, melting temperatures, and sizes of the amplified products are given in Table 1. The Platinum Quantitative PCR SuperMix-uracil DNA glycosylase (UDG) (Invitrogen) used was 2× concentrated, and every 25-μl reaction had 1.5 U of Platinum Taq DNA polymerase, 20 mM Tris·HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2, 200 μM dGTP, 200 μM dATP, 200 μM dCTP, 400 μM dUTP, and 1 U of UDG; the reaction also contained 0.2 μM forward and reverse primers, fluorescein calibration dye (1:2,000; Bio-Rad), and SYBR Green I nucleic acid gel stain (1:100,000; Roche). Master mixes, to reduce pipetting errors, were prepared at every stage for triplicate reactions (3 × 25 μl) for each standard.

Quantification. One microliter of cDNA sample was used as a template for every 25-μl reaction of target genes, and 0.5 μl of cDNA was used as a template for β-actin. Each sample was set up in triplicate for quantitative real-time PCR (qPCR). The reaction components were exactly the same as described above, and, for every single test sample, a qPCR for both the target and the housekeeping gene (β-actin) was performed. The following PCR cycle was used for gene amplification: 95°C, 3 min, and 40 cycles of denaturing at 95°C, 30 s; annealing (Table 1), 30 s; and extension, 72°C, 30 s, followed by

Table 1. Oligonucleotide primers used in quantitative real-time PCR and their melting temperatures

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Accession No.</th>
<th>$T_m$ (°C)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Sense 5'-AGAAGCTGCTGACTCAAAGGAT-3'</td>
<td>AF157514</td>
<td>49°C</td>
<td>100 bp</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GCAAGACCTCACTAACGAGAGA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>Sense 5'-GAGACCTGCTGACTCAAAGGAT-3'</td>
<td>Z54210</td>
<td>49°C</td>
<td>100 bp</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-AGATGAGCTGCTGACATCTTCGAT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp90</td>
<td>Sense 5'-CGTGCGGAGACCTCTGACATTCA-3'</td>
<td>AB196458</td>
<td>55°C</td>
<td>500 bp</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GAGCTTCTTCGCCCTCTGTAA-3'</td>
<td>AB196457</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Sense 5'-GTGGCTGTACAGAGCAGATCC-3'</td>
<td>U90321</td>
<td>55°C</td>
<td>500 bp</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-CTGTACCGCTGCTCACATTCA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS-1</td>
<td>Sense 5'-GAGGCGACACGGTGTCACCTGGAT-3'</td>
<td>AF390021</td>
<td>60°C</td>
<td>500 bp</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GAGCGGCAATCGCTGCTGTG-3'</td>
<td>AF246149</td>
<td>49°C</td>
<td>100 bp</td>
</tr>
<tr>
<td>PEPCCK</td>
<td>Sense 5'-GACACGTGCTGTACGAGAAGG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GACACGTGCTGTACGAGAAGG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>Sense 5'-GACTTGTGGAGAAGTGGTCCCTTCT-3'</td>
<td>AJ242740</td>
<td>60°C</td>
<td>500 bp</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GACTTGTGGAGAAGTGGTCCCTTCT-3'</td>
<td></td>
<td></td>
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</tbody>
</table>

GR, glucocorticoid receptor; hsp90, heat shock protein-90; GS-1, glutamine synthetase-1; PEPCCK, phosphoenolpyruvate carboxykinase; $T_m$, melting temperature.
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a 4°C hold at the end of the PCR reaction. The transcript abundance for each gene was calculated from the threshold cycles (Ct), using their respective standard curves, followed by normalization with β-actin exactly as described before (50). The transcript abundance of β-actin was unchanged with different treatments and therefore used in the normalization.

Statistical Analysis

Data are shown as means ± SE. One-way ANOVA was used to compare the effect of different treatments on media glucose production, GR protein content, and transcriptional responses observed with either microarray or qPCR. All data were transformed for homogeneity of variance, but nontransformed values are shown. A post hoc (Bonferroni test) test was used for pairwise comparison whenever differences were observed. A probability level of P < 0.05 was considered significant. Statistical analyses were performed with SPSS version 12.0.1 (SPSS, Chicago, IL).

RESULTS

Glucose Release

Cortisol treatment increased glucose production (~50%) compared with the control group, whereas RU486 inhibited this cortisol-induced glucose production in trout hepatocytes (Fig. 1). RU486 alone had no effect on glucose production compared with the control group.

GR Protein and mRNA Abundance

GR protein expression was lower in all of the treatment groups compared with the control group (Fig. 2, A and B). No difference in GR protein content was observed between RU486- and cortisol-exposed groups, whereas in the combination group, GR protein content was lower compared with cortisol-treated but not RU486-treated groups (Fig. 2B). Cortisol treatment elevated GR mRNA abundance compared with the control group (Fig. 2C). This cortisol-mediated GR transcript response was abolished by RU486 (combination group). An increase in GR mRNA abundance was also observed with RU486 alone compared with the control group (Fig. 2C).

Gene Expression

Microarray. On the basis of the statistical significance level of P < 0.05, a total of 23 genes were affected by the various treatments compared with the control group. Of the 23 genes, 18 genes are altered by cortisol treatment alone compared with control and are considered GR dependent, as combination treatment (cortisol + RU486) inhibited this cortisol-induced transcriptional response (Tables 2 and 3). Among them, phosphoenolpyruvate carboxykinase (PEPCK), glutamine synthetase-1 (GS-1), GS-2, GS-4, arginase, glutamate dehydrogenase (GAD65), cysteine-rich protein, heat shock protein-90 (HSP90), cytochrome P-450 2K5 (CYP2K5), GR, and cathepsin D transcript levels were elevated by cortisol (Table 2), whereas thyroid hormone receptor-α, cytochrome P-450 2M1 (P4502M1), metallothionein, fibroblast growth factor (FGF), lipoprotein receptor, aryl hydrocarbon receptor (AHR), and vitelline envelope protein-β (VEP-β) transcripts were reduced by cortisol treatment (Table 3).

Treatment with RU486 alone elevated GR, cytochrome P4501A3 (CYP1A3), fushitarazu factor-1 (FGR-1), and insulin receptor-c transcript levels (Tables 2 and 4), whereas
cathepsin D, AhR, and VEP-β transcripts were reduced compared with the control group (Tables 3 and 4). In the combination group (cortisol + RU486), transcript levels of glucokines were higher, whereas total estrogen receptor (ERα and ERβ) was lower compared with the control group (Table 4).

qPCR. We validated the reliability of microarray results by quantifying the transcription abundance of select genes using qPCR. Six genes that showed differences with microarray were chosen, and they were GR, PEPCK, cathepsin D, GS-1, and hsp90. Transcript abundance of all the genes measured showed similar profiles with both qPCR and microarray (Fig. 3). As seen with the array, cortisol significantly increased PEPCK, cathepsin D, GS-1, and hsp90 mRNA abundance compared with all other groups, whereas RU486 inhibited this cortisol-mediated transcriptional response in rainbow trout liver. RU486 treatment alone showed an increase in cathepsin D transcript levels compared with the control group, and this was also seen with the microarray (Fig. 3D). Total ER mRNA abundance was reduced in all treatment groups compared with the control group and was similar to that seen with the array (Fig. 3E).

DISCUSSION

Our results demonstrate for the first time that several genes encoding proteins critical for metabolic adjustments to cope with stressor insults are glucocorticoid responsive in rainbow trout. This finding underscores a mechanistic link between stressor-induced elevations in plasma cortisol concentration and the adaptive liver molecular programming evident during stress recovery in fish (65).

Table 2. List of genes that are upregulated with cortisol treatment and are GR dependent

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>GO Accession No.</th>
<th>GenBank Accession No.</th>
<th>Control</th>
<th>Cortisol</th>
<th>RU486</th>
<th>RU486 + Cortisol</th>
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<tbody>
<tr>
<td>PEPCK</td>
<td>GO:0006094</td>
<td>AF246149</td>
<td>1.75±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.49±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.15±0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.42±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>GS-1</td>
<td>GO:0045213</td>
<td>AF390021</td>
<td>0.99±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.28±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.69±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.28±0.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GS-2</td>
<td>GO:0045213</td>
<td>AF390022</td>
<td>1.93±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.74±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.52±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36±0.73&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>GS-4</td>
<td>GO:0045213</td>
<td>AF390023</td>
<td>0.75±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.27±0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33±0.52&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Arginase</td>
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<td>AY056477</td>
<td>0.75±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.49±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.17±0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93±0.77&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>GAD65</td>
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<td>AF503210</td>
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<td>5.27±0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.15±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.62±0.86&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Cysteine-rich protein</td>
<td>GO:0008283</td>
<td>Y17313</td>
<td>1.75±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.21±0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.64±0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.66±0.46&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Hsp90</td>
<td>GO:0006986</td>
<td>AB196458</td>
<td>0.92±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.23±2.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12±0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.19±0.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CYP2K5</td>
<td>GO:0043390</td>
<td>AF151524</td>
<td>0.92±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.45±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80±0.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GR</td>
<td>GO:0042921</td>
<td>Z54210</td>
<td>1.36±0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.77±0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.61±0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.82±1.53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>GO:0006508</td>
<td>U90321</td>
<td>0.95±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.42±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.17±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.29±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as average fold change (log<sub>2</sub> ratios) from reference standard. All values represent means ± SE; n = 4 independent fish. GR and cathepsin D are also affected by RU486 in addition to cortisol. GAD65, glutamate decarboxylase; CYP2K5, cytochrome P-450 2K5; GO, Gene Ontology. Different letters represent significant differences between treatments (1-way ANOVA; P < 0.05).

Table 3. List of genes that are downregulated with cortisol treatment and are GR dependent

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>GO Accession No.</th>
<th>GenBank Accession No.</th>
<th>Control</th>
<th>Cortisol</th>
<th>RU486</th>
<th>RU486 + Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid hormone receptor-α</td>
<td>GO:0006366</td>
<td>AF132752</td>
<td>1.5±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.26±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P4502M1</td>
<td>GO:0006118</td>
<td>U16657</td>
<td>1.5±0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-4.71±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5±0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.99±0.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>GO:0046686</td>
<td>M18103</td>
<td>1.05±0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.13±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.68±0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59±1.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FGF</td>
<td>GO:0008283</td>
<td>Y16850</td>
<td>3.51±0.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-8.11±3.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.08±0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.25±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipoprotein receptor</td>
<td>GO:0008150</td>
<td>AJ41787</td>
<td>0.5±1.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-4.51±0.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.61±1.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69±1.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AhR-α + β</td>
<td>GO:0042221</td>
<td>AF065138</td>
<td>1.25±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-7.98±3.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.05±0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.38±0.78&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VEP-β</td>
<td>GO:0007339</td>
<td>AF231707</td>
<td>2.0±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.03±1.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.35±0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as average fold change (log<sub>2</sub> ratios) from reference standard. All values represent means ± SE; n = 4 independent fish. Aryl hydrocarbon receptor (AhR) and vitelline envelope protein-β (VEP-β) are also affected by RU486 in addition to cortisol. P4502M1, cytochrome P-450 2M1; FGF, fibroblast growth factor. Different letters represent significant differences between treatments (1-way ANOVA; P < 0.05).

GR Regulation

While RU486 has been used as a tool to elucidate GR-specific signaling in fish (15, 23, 51, 54), the mode of action of this drug was thought to involve specific binding to GR and inhibition of cortisol signaling (41). Our results suggest GR degradation as an additional novel mechanism for GR silencing by RU486 in fish. As seen before, cortisol also reduced GR protein content in trout hepatocytes, and the proteasome is involved in this ligand-mediated GR protein degradation (5, 50). However, the greater reduction in GR protein with cortisol in the presence of RU486 leads us to propose that an additional pathway(s) may also be involved in RU486-mediated GR degradation.

Cortisol-mediated GR degradation was also shown to stimulate GR mRNA accumulation in trout hepatocytes, leading to the proposal of a negative feedback regulation of GR protein content (50). A similar response to cortisol treatment was also seen in this study, supporting the concept of GR autoregulation in trout hepatocytes (50). We hypothesized that this enhanced GR turnover may be a mechanism for maintaining tissue responsiveness to cortisol stimulation (50, 60). Interestingly, despite a pronounced decrease in GR protein content with RU486, the mRNA response was only minimal, suggesting that inhibition of cortisol signaling by this drug may also involve altered GR turnover in trout hepatocytes. Indeed, disruption of the cortisol-mediated mismatch between GR mRNA and protein content by RU486 (in the combination group) abolished this steroid-induced glucose release. Taken together, these results implicate a key role for cortisol-mediated GR autoreg-

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ulation in maintaining the target tissue responsiveness to cortisol stimulation in trout hepatocytes.

**Metabolic Adjustments**

A major target tissue response to elevated plasma cortisol levels involves modulation of metabolic pathways in fish (31). The hallmark of stress is disturbance to homeostasis, resulting in upregulation of energy-demanding pathways, including protein synthesis, to restore internal fluid balance (63). Indeed, this is reflected in a higher metabolic demand in stressed animals, including fish (63). Although the role of cortisol in regulating energy-demanding pathways is not clearly known, studies have shown an enhancement of liver gluconeogenic capacity leading to elevated glucose production in fish (4, 31, 50). Indeed, this metabolic response is a key aspect of the stress recovery process, as glucose is the preferred fuel, especially for tissues such as gills and brain, to meet the increased energy demand (33). This glucose response with cortisol stimulation corresponded to an upregulation of PEPCK transcript levels, supporting enhanced gluconeogenic capacity (31). The inhibition of this glucose elevation and PEPCK mRNA abundance by RU486 (Figs. 1 and 3) clearly underscores the significance of genomic cortisol signaling in the glucose response, critical for metabolic adjustments to stressor exposure in fish (65).

![Fig. 3. qPCR results of the randomly chosen genes [phosphoenolpyruvate carboxy kinase (PEPCK), cathepsin D, glutamine synthetase-1, heat shock protein-90 (hsp90), and estrogen receptor] to test the validity of microarray results. Microarray results (log2 ratios) are expressed as %control and shown along with qPCR results for comparison. All values represent means ± SE; n = 4 independent fish. Bars with different letters are significantly different (1-way ANOVA; P < 0.05).](image-url)

<table>
<thead>
<tr>
<th>Gene</th>
<th>GO Accession No.</th>
<th>GenBank Accession No.</th>
<th>Control</th>
<th>Cortisol</th>
<th>RU486</th>
<th>RU486 + Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A3</td>
<td>—</td>
<td>AF059711</td>
<td>1.6±0.21a</td>
<td>1.16±0.08a</td>
<td>4.26±0.21b</td>
<td>1.75±0.73a</td>
</tr>
<tr>
<td>fZFR1</td>
<td>—</td>
<td>AB006153</td>
<td>0.93±0.24a</td>
<td>1.39±0.13a</td>
<td>3.96±0.52b</td>
<td>1.35±0.24a</td>
</tr>
<tr>
<td>Insulin receptor-c</td>
<td>—</td>
<td>AF062498</td>
<td>1.2±0.26a</td>
<td>0.51±0.21a</td>
<td>3.39±0.29b</td>
<td>0.80±0.23a</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>GO:0006096</td>
<td>AF053331</td>
<td>−1.48±1.37a</td>
<td>−4.39±3.31a</td>
<td>−1.88±1.51a</td>
<td>4.34±0.44b</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>GO:0030520</td>
<td>AJ242740/AJ242741</td>
<td>2.75±0.53a</td>
<td>0.44±0.08b</td>
<td>−0.43±0.11b</td>
<td>−0.28±0.5b</td>
</tr>
</tbody>
</table>

Values are expressed as average fold change (log2 ratios) from reference standard. All values represent means ± SE; n = 4 independent fish. Estrogen receptor is significantly downregulated with all the treatments compared with control. CYP1A3, cytochrome P-450 1A3; fZFR1, fushitarazu factor-1. Different letters represent significant differences between treatments (1-way ANOVA; P < 0.05).
The C3 precursor for gluconeogenesis is thought to come from amino acids from peripheral sources including muscle proteolysis (31). However, the upregulation of liver transcripts for genes involved in protein metabolism (GS and arginase), including catabolism (cathepsin D and GAD65), supports a key role for genomic cortisol signaling in the molecular programming of liver protein metabolism. The higher transcript abundance of cathepsin D by cortisol clearly leads us to propose that this steroid increases protein breakdown capacity in the liver. Although the role of cathepsin D in fish muscle proteolysis has been studied to a limited extent (30, 36), little is known about its role in liver protein degradation. Indeed, a corticosteroid response element has been identified in the mammalian cathepsin D promoter sequence and agrees well with the modulation of this gene by dexamethasone in muscle (11). Although the cathepsin D promoter has not been characterized in a piscine model, we hypothesize that genomic cortisol signaling leads to increased proteolytic capacity in fish liver in response to stressor exposure. These susceptible hepatic proteins may serve as transient sources of amino acids for hepatic oxidation as well as gluconeogenesis to fuel extrahepatic tissues during stress recovery (31). The upregulation of liver PEPCK (a key gluconeogenic enzyme) and GAD65 (glutamate metabolism) by cortisol in the present study supports the above contention.

A novel finding from this study is the suppression of lipoprotein receptor (LPR) mRNA content by cortisol in fish. LPRs are a highly conserved group of proteins thought to be involved in the uptake and metabolism of lipoproteins. For instance, vitellogenin receptor belongs to the LPR family, and uptake of vitellogenin (Vtg) into oocytes occurs through cell surface LPR in both invertebrates and vertebrates, including fishes (43). Little is known about LPR activation in fish, but the well-established role of stress and/or cortisol treatment in enhancing lipid metabolism in the liver (31) leads us to propose that LPR regulation by cortisol is part of the metabolic adjustments during stress in fish. Indeed, glucocorticoids are known to influence these receptors in mammals (55). Taken together, the recent observation that some of these glucocorticoid-responsive transcripts (Tables 2 and 3) were also transiently affected during recovery from an acute handling stressor supports a key role for genomic cortisol signaling in the liver metabolic reprogramming critical for stress adaptation (65).

Cellular Stress Response

In addition to its role in energy repartitioning associated with stress, our results also highlight a role for GR signaling in modulating the cellular stress response. Specifically, we show for the first time that the cellular chaperone hsp90, but not hsp70, is modulated by GR signaling in fish. Although previous studies did suggest a role for cortisol in regulating hsp90 expression in fish (12, 49, 59, 60), the mode of action was not ascertained. The close association of hsp90 with GR and the role of this chaperone in maintaining GR stability and signaling are well established in mammalian models (44, 45). Recently, we also confirmed that hsp90 is critical for GR stability in trout hepatocytes (50). The GR-specific hsp90 response observed here, coupled with our earlier report that the inhibition of hsp90 binding to GR enhances this steroid receptor protein degradation (50), implicates a key role for cortisol in modulating this chaperone. We hypothesize that the enhanced tissue responsiveness to cortisol stimulation during stress involves upregulation of hsp90 expression in fish.

Modulation of Estrogen-Responsive Genes

Elevated plasma cortisol levels were shown to have a negative impact on reproduction, including reduced plasma Vtg, gonadal steroids, and gonadotropin levels in fish (8, 9, 42). Vtg synthesis occurs in liver in response to ER activation by estradiol-17β in fish (16). Cortisol was shown to inhibit hepatic ER and Vtg expression, leading to impaired vitellogenesis in salmonids (28). Although cortisol inhibited hepatic ER but not Vtg mRNA abundance in the present study, it is unclear whether this was GR specific; as RU486 also reduced ER transcript levels in trout. This agrees with results from another trout study demonstrating that RU486 did not inhibit the cortisol-mediated downregulation of ER (28). Indeed, vitellogenin envelope protein (a constituent of extracellular envelope around the growing oocyte) transcript levels, which are regulated by ER signaling (53), were also downregulated by cortisol and RU486. While these results clearly suggest a role for cortisol in modulating estrogen-responsive gene expression, our results highlight for the first time that RU486 may also interfere with the estrogen signaling pathway. Overall, stress-induced impairment of reproductive processes may involve multiple pathways, including cortisol-mediated suppression of transcripts encoding key proteins (ER and VEP) involved in reproduction.

Xenobiotic-Responsive Genes

In addition to the role of glucocorticoids in energy catabolism, this steroid also modulates the expression of several proteins important in the cellular defense against pollutants. For instance, metallothionein (MT), a key protein involved in sequestering metals that also has antioxidant properties, is synthesized in response to metal exposure and was shown to be regulated by glucocorticoids in vertebrates, including fishes (20, 37). The higher MT expression in response to glucocorticoids is mediated via binding of activated GR to glucocorticoid response elements on the promoter region of the MT gene and initiating transcription (20). Interestingly, our results show a reduction in MT transcript levels by cortisol in trout hepatocytes. Although the reason is unclear, one possibility for the difference may be the pharmacological level of cortisol used in the earlier study (20), whereas our study used a steroid concentration representative of a stressed fish.

Glucocorticoids also modulate the cytochrome P-450 (CYP) family of proteins, which are involved in the metabolism and clearance of endogenous steroids and xenobiotic compounds (35). Several isoforms of CYP genes are transcriptionally modulated by glucocorticoids in a GR-dependent manner, and the presence of glucocorticoid response elements in the promoter region of these genes is also established (40). For instance, dexamethasone (GR agonist) induced CYP3A4 and CYP2C9 mRNA expression in human hepatocyte culture by binding to glucocorticoid response elements in the promoter region, and this was inhibited by RU486 (39). Our results show for the first time that liver CYP2K5 and CYP2M1 transcripts are modulated by cortisol in trout hepatocytes. Both CYP2K5 and CYP2M1 are constitutively expressed in trout liver and important for xenobiotic metabolism (29). Although there is no previous evidence on the regulation of these two genes by
steroids in fish, induction of CYP1A1, the most widely studied CYP enzyme in fish, by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was potentiated by cortisol in a GR-dependent fashion. The CYP1A1 induction is mediated by the activation of AhR, a ligand-activated transcription factor belonging to the family of nuclear receptors, and transcript levels of this receptor were reduced by corticosteroids in a mammalian model (1). Our results support the reduction of AhR mRNA abundance by cortisol, although the specificity of the signaling pathway was unclear, as RU486 also suppressed the AhR transcript levels in trout. Because both AhR and GR, along with other steroid nuclear receptors, share a similar complement of accessory proteins for their signaling, it is likely that there is competition for the limited pool of accessory proteins on activation of any particular signaling pathway (44). Indeed, TCDD is shown to inhibit steroid receptor-mediated transcription in cell lines transfected with steroid-responsive elements (21). In addition, sex steroid hormones also inhibit TCDD-induced CYP1A1 enzyme activity via receptor-mediated pathways (21). While similar cross talk has not been established between AhR and GR, the negative relationship between AhR and GR mRNA abundance with TCDD suggests a link between these two receptors (2).

Conclusions

In conclusion, using a pharmacological approach together with targeted cDNA microarray, we identified several glucocorticoid-responsive genes in trout hepatocytes. The majority of these genes are involved in liver metabolic reprogramming, critical for coping with the enhanced energy demand in response to stressor exposure. This is further supported by results from our recent study showing that a majority of the genes that were impacted by cortisol were also stress responsive in trout (65). Also, our results highlight a key role for cortisol in modulating the adaptive responses to xenobiotic exposure and reproduction. Collectively, this study demonstrates for the first time a key role for genomic cortisol signaling, via GR, in the molecular reprogramming of liver function, which may be essential to cope with stress.

GRANTS

Support for this study was provided by the Natural Sciences and Engineering Research Council (NSERC) of Canada discovery grant to M. M. Vijayan and an NSERC postdoctoral fellowship to N. Aluru.

REFERENCES

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