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Central and peripheral glucocorticoid receptors are involved in the plasma cortisol response to an acute stressor in rainbow trout

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ABSTRACT

Cortisol, the primary circulating corticosteroid in teleosts, is elevated during stress following activation of the hypothalamus–pituitary–interrenal (HPI) axis. Cortisol exerts genomic effects on target tissues in part by activating glucocorticoid receptors (GR). Despite a well-established negative feedback loop involved in plasma cortisol regulation, the role of GR in the functioning of the HPI axis during stress in fish is still unclear. We used mifepristone (a GR antagonist) to suppress GR signaling in rainbow trout (*Oncorhynchus mykiss*) and assessed the resultant changes to HPI axis activity. We show for the first time that mifepristone caused a functional knockdown of GR by depleting protein expression 40–75%. The lower GR protein expression corresponded with a compensatory up-regulation of GR mRNA levels across tissues. Mifepristone treatment completely abolished the stressor-induced elevation in plasma cortisol and glucose levels seen in the control fish. A reduction in corticotropin-releasing factor (CRF) mRNA abundance in the hypothalamic preoptic area was also observed, suggesting that GR signaling is involved in maintaining basal CRF levels. We further characterized the effect of mifepristone treatment on the steroidogenic capacity of interrenal tissue *in vitro*. A marked reduction in cortisol production following adrenocorticotropic hormone stimulation of head kidney pieces was observed from mifepristone treated fish. This coincided with the suppression of steroidogenic acute regulatory protein, but not P450 side chain cleavage mRNA abundances. Overall, our results underscore a critical role for central and peripheral GR signaling in the regulation of plasma cortisol levels during stress in fish.

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1. Introduction

Cortisol is the principal glucocorticoid in teleost fish, as in many vertebrates, and plays an essential role in a plethora of physiological processes including maintenance of energy balance, immunoregulation, growth, and reproduction [34]. Cortisol also functions as a mineralocorticoid in teleosts, as they lack the capacity to synthesize aldosterone, and is thus important for the maintenance of hydromineral balance [33,47]. Cortisol is synthesized in steroidogenic cells located in the adrenal cortex of tetrapods and in the analogous head kidney interrenal tissue in fish. Diurnal and stress-induced synthesis and secretion of cortisol involves the activation of the hypothalamus–pituitary–interrenal axis (HPI axis) in fish [12].

Briefly, during HPI axis activation corticotropin-releasing factor (CRF), produced in the hypothalamic preoptic area (POA), stimulates the pituitary corticotropes to secrete adrenocorticotropic hormone (ACTH). A specific binding protein for CRF (CRF-BP), also produced in the POA, is likely to play a role in CRF-mediated ACTH regulation during stress [1,28]. Blood-borne ACTH in turn stimulates synthesis and secretion of cortisol into the circulation [12]. Rate limiting steps in cortisol biosynthesis include the transport of cholesterol between the outer and inner mitochondrial membranes by steroidogenic acute regulatory protein (StAR; [42]), and the conversion of cholesterol to pregnenolone by cytochrome P450 side chain cleavage (P450scc; [36]). In teleosts, as in other vertebrates, changes in the transcript abundance of these proteins is reflected in the cortisol production capacity of interrenal tissues [4,6].

During stress, elevated plasma cortisol levels mobilize energy stores primarily through genomic actions [7]. For example, cortisol up-regulates mRNA abundance of hepatic phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting gluconeogenic enzyme, resulting in increased glucose production by hepatocytes [5,39]. The physiological effects of cortisol on target tissues are mediated by glucocorticoid and mineralocorticoid receptors (GR and MR, respectively). One MR and two GR genes (GR1 and GR2) have been cloned and sequenced in several teleosts (e.g. [16,25,32]), with the exception of zebrafish (*Danio rerio*) that has only a single GR in the genome [3,2,40]. As in humans, a splice variant of GR was identified in zebrafish [40] and other teleosts [21,25], adding further complexity to cortisol regulation in fish. Binding characteristics

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of teleostean corticosteroid receptors consistently reveal one receptor with higher cortisol affinity and transactivational capacity. In the carp (*Cyprinus carpio*), GR2 out competes both MR and GR1 for cortisol at low concentrations [43], while in Burton's mouthbreeder (*Haplochromis burtoni* [25] and rainbow trout (*Oncorhynchus mykiss* [16,44]), MR out competes GR at low steroid concentrations. A recent study in rainbow trout demonstrated differential abundance of MR (but not GR1 or GR2) transcripts in the brain of rainbow trout selected for low responsiveness to stress [31], but the functional significance is unclear. This is because the downstream effects of MR signaling in fish are poorly understood. Overall, GR signaling is thought to play a key role in mediating the stress effects of cortisol [16,44]. However, despite the well-established negative feedback regulation of plasma cortisol levels [15,23,22], and the localization of GR in all levels of the HPI axis [16,45], a role for this receptor signaling in the HPI axis functioning during stress has not been confirmed.

Towards understanding the role of GR in cortisol regulation in teleosts, we used the well-established GR antagonist mifepristone, also known as RU486, to pharmacologically block GR signaling in rainbow trout. Mifepristone blocks the transactivational activity of both GR isoforms in response to dexamethasone in this species [16], and abrogates cortisol-induced transcriptional changes in hepatocytes [5], and is therefore an excellent tool for studying GR actions. We assessed the effects of GR antagonism on HPI axis functioning by quantifying stressor-induced changes in plasma cortisol, target tissue GR transcript and protein expression levels, and hypothalamic mRNA levels of CRF and CRF-BP. We also determined the effect of mifepristone treatment on steroidogenic capacity of the interrenal tissue by quantifying cortisol production and mRNA levels of StAR and P450scc following ACTH stimulation *in vitro*.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada) or Humber Springs Hatchery (Mono Mills, ON, Canada), and transferred to the University of Waterloo Aquatic Facility (Waterloo, ON, Canada). Fish were acclimated for 4–6 weeks in 100 L flow-through tanks at 12 ± 1 °C on a 12-h light and dark cycle. The fish were fed 2% of their body weight once daily (5 days a week) with commercial trout feed (Martin Mills, Elmira), modified as described below. Care and use of animals was approved by the University of Waterloo's Animal Care Committee and followed the guidelines of the Canadian Council for Animal Care.

2.2. Experimental protocol

Six groups of 6 fish (mean body weight 226 ± 2.6 g) were assigned to one of two diets. Fish in 3 of the tanks were fed mifepristone-laced food for 3 days prior to stressor application, while fish in the remaining 3 tanks were fed control food. Each diet was prepared by soaking food pellets in 100% ethanol alone (control) or with 10 mg kg^{-1} body weight mifepristone (*RU486*; Sigma, St. Louis, MO) and allowing the ethanol to evaporate as previously described [8]. On the day of sampling, one tank of fish from each treatment was terminally anesthetised by rapidly netting all six fish and transferring them to a lethal dose of 2-phenoxyethanol (2 ml L^{-1}; Sigma). Fish in the remaining 4 tanks were exposed to a standardized handling disturbance of 1 min of repeating netting and chasing [4]. Fish were allowed to recover for 1 or 24 h and were sampled as described above. Blood was collected by tail excision into heparinized tubes, then centrifuged at 6000*g* for 10 min. Plasma was removed and quickly frozen on dry ice. The brain and liver were excised and snap-frozen on dry ice for determination of gene and protein expressions. All tissues were stored at −80 °C until analysis.

The above feeding trial was repeated also to measure CRF and CRF-BP expression in the pre-optic area (POA) of the brain, and to examine steroidogenic capacity of head kidney tissues to ACTH stimulation *in vitro*, in response to mifepristone treatment. After 3 days of exposure to control or mifepristone diet, fish were subjected to a handling stress (described above) and sampled either prior to stress or 1 h after stressor exposure. POA was sampled and stored exactly as mentioned above. For *in vitro* ACTH challenge, head kidney was removed from six fish in each diet group prior to stress, rinsed in ice-cold L15 medium, followed by finely mincing (~1 mm^3) the tissue. Head kidney pieces from each fish were added to duplicate wells of a 24-well Falcon plate containing 500 μl fresh L15 medium at 13 °C for determination of *in vitro* cortisol production capacity as described previously [4]. Head kidney tissue was equilibrated for 2 h at 13 °C with gentle rocking. The media was replaced with fresh L15 and incubated for 1 h, following this one of the duplicate wells for each kidney was then exposed to either fresh L15 media alone (control) or containing 0.5 IU ml^{-1} ACTH [1–39] (Sigma) [38]. The supernatant was collected after 2 h and frozen for cortisol analysis later. Wet weight of the tissue was recorded before being snap frozen for future determination of gene expression levels.

2.3. Plasma analysis

Cortisol levels in plasma and supernatant (*in vitro* study) were measured using a commercially available radioimmunoassay kit (ImmunoChem Cortisol, MP Biomedicals, CA) exactly as described before [4]. Plasma glucose and lactate levels were measured colorimetrically using commercially available kits [4]. The media cortisol levels for the *in vitro* study were normalized to mg wet tissue weight.

2.4. Analysis of GR protein expression

The tissue total GR protein expression was analysed by immunodetection exactly as described previously [6]. Briefly, protein concentration of brain or liver homogenates was measured using the bicinchoninic acid method with bovine serum albumin as standards. SDS–PAGE and immunodetection of GR were performed with 40 μg protein samples. The primary antibody used for immunodetection was a polyclonal rabbit antibody raised against trout total GR (1:1000; [39]). The secondary antibody was alkaline phosphatase-conjugated goat-anti-rabbit IgG (1:3000). Protein bands were visualized with NBT (0.033 wt/vol%) and BCIP (0.017 wt/vol%) and their intensities quantified using a Chemi Imager and AlphaEase software (Alpha Innotech, Santa Clara, CA).

2.5. Quantification of gene expression

Total RNA was isolated from liver, brain, preoptic area, and head kidney pieces using an RNeasy Mini kit (Qiagen, Mississagua, ON). First strand cDNA was synthesized from 1 μg of DNase-treated total RNA using a cDNA synthesis kit (MBI Fermentas, Burlington, ON) according to manufacturer's instructions. Transcript levels of GR [21], StAR, P450scc, CRF, CRF-BP and β-actin were measured in triplicate using gene-specific primers (Table 1) as previously described [2]. Relative standard curves were constructed for each gene and used to quantify mRNA level in each sample relative to β-actin exactly as described previously [4]. The β-actin Ct values

Table 1
Gene-specific primer sequences used for real-time PCR. Forward (F) and reverse (R) primers are listed for each gene, as is the corresponding GenBank Accession Number. GR, glucocorticoid receptor; StAR, steroidogenic acute regulatory protein; P450scc, P450 side-chain cleavage enzyme; CRF, corticotropin-releasing factor; CRF-BP, CRF-binding protein.

Gene	Primer sequence (5′–3′)	GenBank Accession No.
β-Actin	F: AGA GCT ACG AGC TGC CTG AC R: GCA AGA CTC CAT ACC GAG GA	AF157514
GR	F: AGA AGC CTG TTT TTG GCC TGT A R: AGA TGA GCT CGA CAT CCC TGA T	Z54210
StAR	F: CGC TGG CAT CTC CTA CA R: GGG ACT TCG TTA GTG TTC G	AB047032
P450scc	F: GAG GAG GGT AGG AGC CA R: CCT TGT GGG ACT CTG GT	S57305.1
CRF	F: ACA ACG ACT CAA CTG AAG ATC TCG R: AGG AAA TTG AGC TTC ATG TCA GG	AF296672
CRF-BP	F: CAT CAC CCA GCC ATC AAA CAC R: GAG TAT GAC AGC GTT GAC ATC GA	AY363677

were similar across all samples and, therefore used as the normalizing gene.

2.6. Statistical analysis

A two-way analysis of variance (ANOVA) followed by a Holm-Sidak test for multiple comparisons was used to determine the effect of mifepristone and stress on plasma variables, gene, and protein expressions in the brain and liver, before and after a handling stressor. Data are presented as mean ± SE. A *t*-test was used to determine the effect of mifepristone on ACTH-stimulated cortisol secretion from head kidney pieces *in vitro* (stimulated vs. unstimulated; normalized to tissue weight). A two-way ANOVA followed by a Holm-Sidak test for multiple comparisons was also used to determine the effect of mifepristone and ACTH-stimulation on StAR and P450scc gene expression. All analyses were performed using SigmaStat 3.0 ($p < 0.05$).

3. Results

3.1. Plasma levels

Prior to the handling stressor, plasma cortisol levels in control (4.76 ± 0.197 ng/ml) and mifepristone (3.25 ± 0.350 ng/ml) fish were not statistically different ($p > 0.05$). In fish fed the control diet, plasma cortisol levels were 6.5-fold higher 1 h post handling stress ($p < 0.001$), but returned to pre-stress levels by 24 h ($p > 0.05$). In fish fed mifepristone, there were no statistical differences in plasma cortisol levels at any time point post-stress (Fig. 1A; $p > 0.05$). Plasma glucose levels in fish fed either diet were not statistically different pre-stress (12.28 vs 12.57 ng ml^{-1}; $p > 0.05$). In fish fed the control diet, plasma glucose rose 1.3-fold 1 h post-stress ($p < 0.05$), then decreased 1.6-fold below pre-stress levels at 24 h ($p < 0.05$). In fish fed mifepristone there was no change in plasma glucose 1 h post-stress, but a 1.8-fold decrease at 24 h ($p < 0.05$). Plasma lactate levels responded identically to the handling stressor regardless of diet. At 1 h post-stress there was a 2.0-fold increase in plasma lactate in control and mifepristone-fed fish ($p < 0.05$), and a return to pre-stress levels by 24 h ($p > 0.05$).

3.2. GR expression

Expression of GR was significantly affected by diet. In the brain (Fig. 2A) and liver (Fig. 2B), GR mRNA levels were 1.3-fold higher

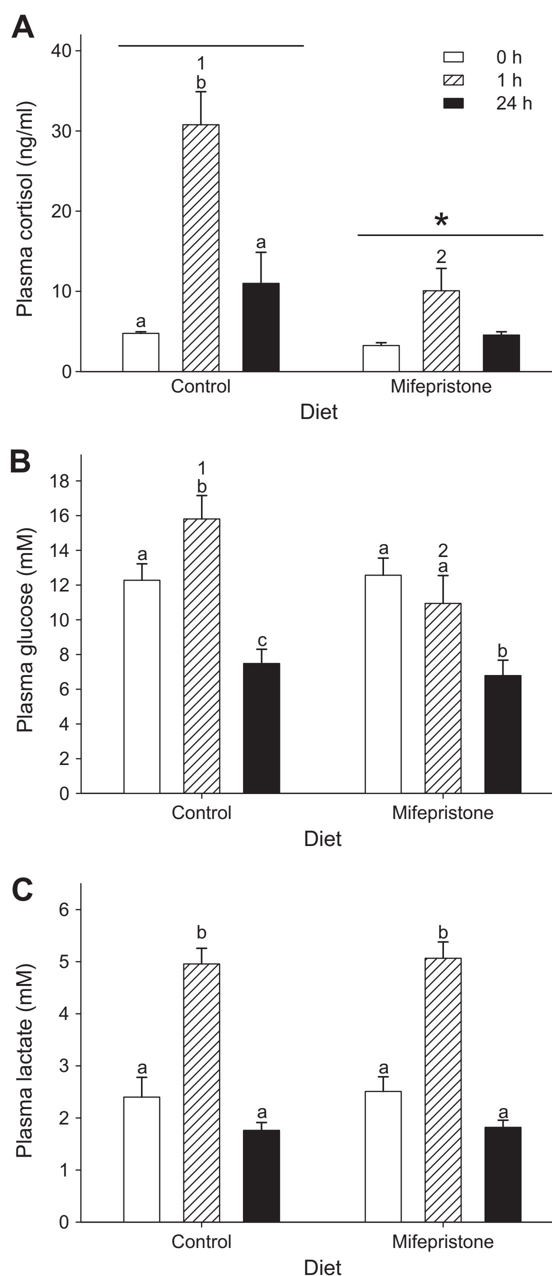

Fig. 1. Changes in plasma (A) cortisol, (B) glucose, and (C) lactate levels in fish fed one of two diets (control or mifepristone) in response to a handling stressor. One group of fish from each diet was sampled prior to stressor application to determine pre-stress values (0 h, white bars). Remaining fish were sampled either 1 h (hatched bars) or 24 h (black bars) after application of a 1 min handling stressor. Plasma cortisol was measured by radioimmunoassay while plasma glucose and lactate were measured colorimetrically using commercially available kits. Data are presented as mean ± SE (n = 5–6). Significant differences are indicated by letters (between time points within a given diet), numbers (between diets within a given time point), or * (overall between diets), and the absence of a symbol indicates no difference ($p < 0.05$; two-way ANOVA followed by a Holm-Sidak test for multiple comparisons).

overall in mifepristone-fed fish than in control-fed fish ($p < 0.01$). Following the handling stressor, there was no change in brain GR mRNA in fish on either diet ($p > 0.05$). Conversely, liver GR mRNA was 1.6-fold lower than pre-stress levels in control-fed fish at 24 h post-stress ($p < 0.01$), and this effect was not observed in mifepristone-fed fish ($p > 0.05$).

Overall, brain GR protein levels were 1.6-fold lower in mifepristone-fed fish relative to controls (Fig. 2C and E; $p < 0.001$). GR

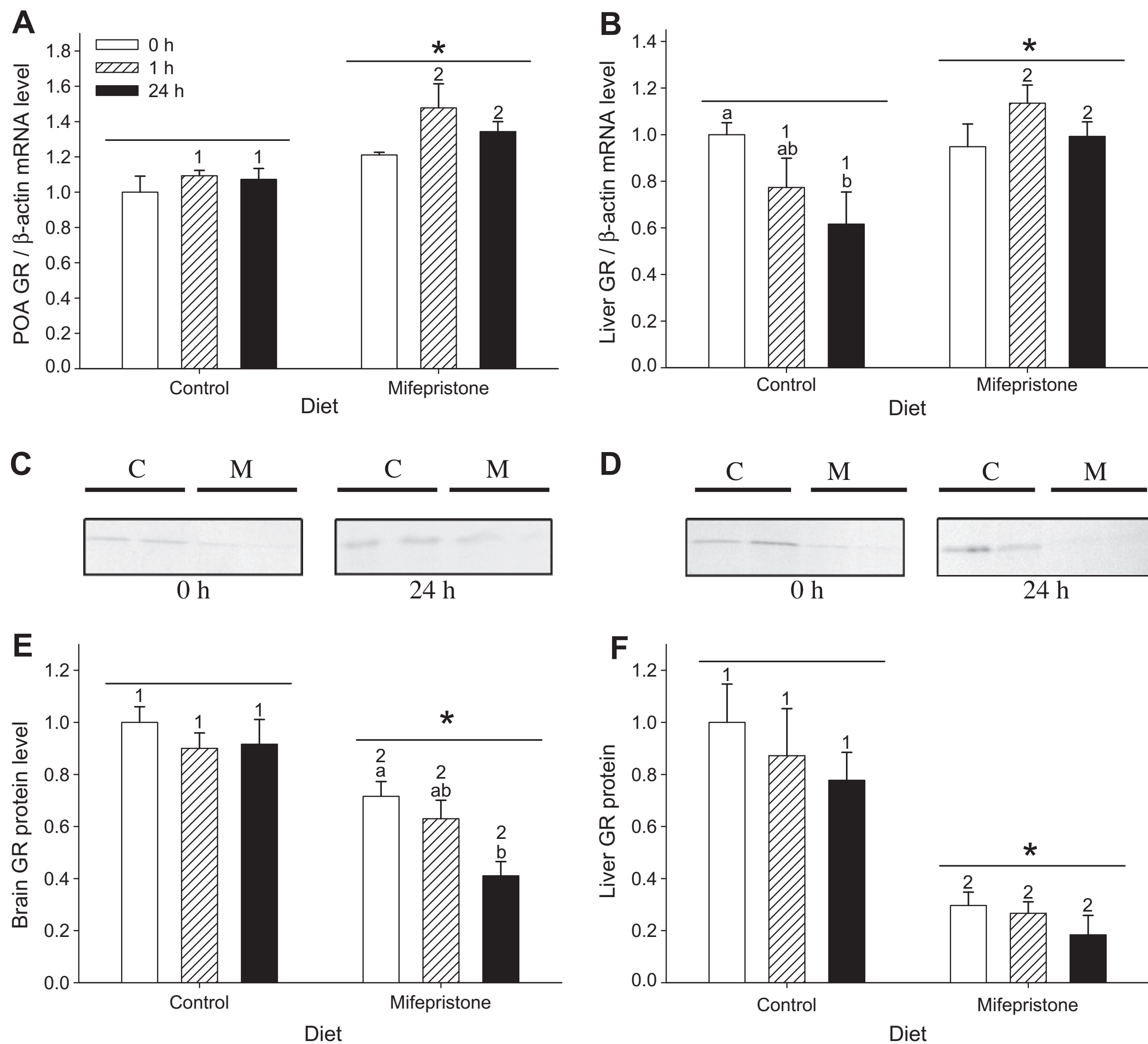

Fig. 2. Changes in glucocorticoid receptor (GR) gene and protein expression levels in fish fed one of two diets (control or mifepristone) in response to a handling stressor. One group of fish from each diet was sampled prior to stressor application to determine pre-stress mRNA and protein levels (0 h, white bars). Remaining fish were sampled either 1 h (hatched bars) or 24 h (black bars) after application of a 1 min handling stressor. Transcript abundance of GR in the (A) brain and (B) liver were quantified by real-time PCR and normalized to β-actin mRNA levels. Representative Western blots of GR protein level in the (C) brain and (D) liver at 0 h and 24 h post-stress in control (C) and mifepristone (M) fed fish. Protein content in the (E) brain and (F) liver were quantified by densitometry following Western blot. Data are presented as mean ± SE (n = 4–6) relative to control 0 h. Significant differences are indicated by letters (between time points within a given diet), numbers (between diets within a given time point), or * (overall between diets), and the absence of a symbol indicates no difference ($p < 0.05$; two-way ANOVA followed by a Holm-Sidak test for multiple comparisons).

protein levels were lowest in mifepristone-fed fish 24 h after the handling stressor (2.2-fold below pre-stress levels; $p < 0.01$). As observed in the brain, liver GR protein levels were 3.5-fold lower overall in mifepristone-fed fish compared to control-fed fish (Fig. 2D and F; $p < 0.001$); however, the handling stressor did not affect liver GR protein content for either diet ($p > 0.05$).

3.3. CRF and CRF-BP transcript levels

Preoptic area transcript abundances of CRF and CRF-BP were not significantly affected by the stressor after 1 h recovery. CRF mRNA levels were significantly reduced by mifepristone treatment in unstressed fish (0.090 ± 0.036 vs 0.025 ± 0.005, respectively; Fig. 3A). While this difference was no longer significant at 1 h post-stress, the overall effect of the mifepristone diet was a significant 2.5-fold reduction in POA CRF mRNA ($p < 0.05$). Conversely, CRF-BP transcript abundance in the POA was not affected by diet (Fig. 3B; $p > 0.05$).

3.4. Stimulated cortisol production

Basal, unstimulated cortisol production in head kidney tissue from fish on the control or mifepristone diet was not significantly different (15.6 ± 1.92 and 41.1 ± 26.7 pg mg^{-1} h^{-1}, respectively; $p > 0.05$). Addition of ACTH increased cortisol production in head kidney tissue relative to unstimulated tissue in both control and mifepristone-fed fish (540 ± 146 and 274 ± 73.4 pg mg^{-1} h^{-1}, respectively), but the magnitude of the response was significantly lower in tissue from mifepristone-fed fish (6.7 vs 34.6-fold; $p < 0.05$; Fig. 4A).

Compared to fish on the control diet, head kidney tissue from fish fed mifepristone had 1.2-fold lower StAR mRNA levels overall ($p < 0.05$; Fig. 4B). There were no observed differences in basal gene expression levels of P450scc between head kidney tissue from the control and mifepristone-fed groups ($p > 0.05$; Fig. 4C); similarly, there were no differences in mRNA levels of StAR and P450scc after exposure to ACTH ($p > 0.05$; Fig. 4).

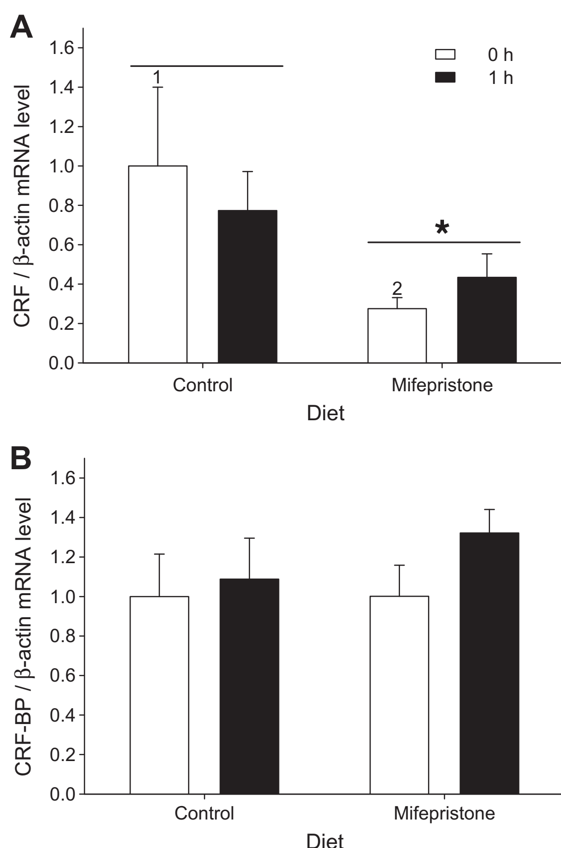

Fig. 3. Changes in preoptic area (A) corticotropin-releasing factor (CRF) and (B) CRF-binding protein (CRF-BP) mRNA expression in fish fed one of two diets (control or mifepristone), before (0 h; white bars) and 1 h after a 1 min handling stressor (1 h; black bars). Transcript abundance was quantified by real-time PCR and was normalized to β-actin mRNA levels. Data are resented as mean ± SE ($n = 6$) normalized to 0 h control for each gene, and statistical differences were detected with a two-way ANOVA ($p < 0.05$). Within a given time point, bars that do not share the same number are significantly different, and bars without numbers are not different. *Denotes a significant difference in mRNA level between diets.

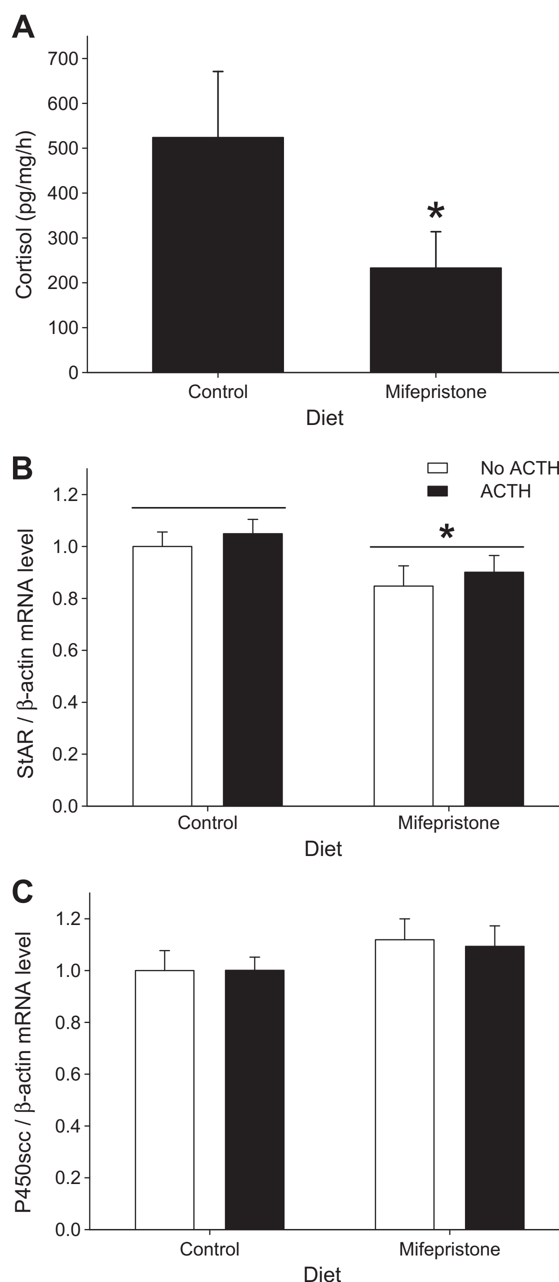

Fig. 4. Effects of adrenocorticotropic hormone (ACTH) stimulation on head kidney (A) cortisol production and (B and C) gene expression levels *in vitro*. Head kidney tissue from fish fed one of two diets (control or mifepristone) was removed, equilibrated in L15 media, then incubated in media alone (No ACTH; white bars) or with 0.5 IU ml^{-1} ACTH (black bars). After 1 h incubation, cortisol content of the media was measured by radioimmunoassay, and mRNA levels of (B) steroidogenic acute regulatory protein (StAR), and (C) P450 side-chain cleavage (scc) were quantified by real-time PCR and normalized to β-actin mRNA levels. Data in A is presented as the magnitude of change in cortisol production (ACTH minus no ACTH; pg mg^{-1} wet tissue weight h^{-1}). All data is mean ± SE ($n = 6$). *Denotes a significant difference between diets [$p < 0.05$; either *t*-test (A) or two-way ANOVA followed by a Holm-Sidak test for multiple comparisons (B and C)].

4. Discussion

This study presents novel data supporting a multi-level role for the glucocorticoid receptor (GR) in the endocrine stress response, highlighting the genomic effects of cortisol on HPI axis regulation. By pharmacologically antagonizing GR with mifepristone, we provide *in vivo* and *in vitro* evidence of reduced basal and post-stress expression of key genes involved in the endocrine stress response. The down-regulation of these genes corresponded with an attenuated cortisol response to handling stress *in vivo*, and reduced interrenal tissue cortisol production following ACTH stimulation *in vitro*. Overall, our results demonstrate for the first time that GR signaling is critical for the activation of the HPI axis in response to an acute stress in fish.

Most teleosts studied to date have two isoforms of GR [16,25,32], except for zebrafish [2], and mifepristone was shown to block the transactivational activity of both isoforms in response to dexamethasone treatment in rainbow trout [16]. In the present study, we reveal for the first time a novel function for this antagonist in teleosts – functional knockdown of GR. In fish fed mifepristone, GR protein levels are drastically reduced in the brain (∼40%) and liver (∼70%) (Fig. 2C and D). We also observed a sharp decline in gill GR protein levels (data not shown), suggesting a generalized response to mifepristone across tissue types. A compensatory transcriptional response to reduced protein levels was observed in both the brain and liver, which is in agreement with the GR autoregulation seen in response to cortisol stimulation in trout liver *in vivo* and *in vitro* [39,46]. In the present study, we used primers based on the first GR isoform to be characterized in trout [21] and, therefore, did not discriminate the GR1 and GR2 isoforms. While some teleost GRs have different transactivational activities [16,43], numerous studies have demonstrated that the distribution

and transcript levels of both isoforms are highly similar in trout (e.g. [30,31,48]. Moreover, although carp express more GR1 in the brain than GR2 [43], a functional role associated with the differential expression have not been established.

Lacing food with mifepristone provides a simple, non-invasive means to antagonize GR signaling and chronically suppress GR protein levels across tissues. Previous studies have typically employed intraperitoneal (ip) implants of mifepristone dissolved in oil in order to antagonize GR. The inherent problem with this invasive technique is that absorption and distribution of the drug cannot be controlled. By feeding mifepristone to fish, the drug is absorbed from the digestive tract directly into the bloodstream, facilitating quick distribution throughout the body. As with ip implants, the actual circulating level of the antagonist cannot be determined with this method: however, a clear and consistent physiological response was observed. The functional relevance of mifepristone-induced GR antagonism and protein knockdown is apparent when comparing the stressor-induced plasma glucose levels of fish on control and mifepristone diets. Given that cortisol up-regulates gluconeogenic enzyme activities in the liver and leads to enhanced glucose output during stress [5,39,46], the absence of a stressor-induced increase in plasma glucose in mifepristone-fed fish (Fig. 1B) supports a reduction in cortisol signaling and the involvement of GR in this function [34].

Given that fish fed mifepristone displayed an errant cortisol response to the handling stress, we then wanted to determine if the steroidogenic capacity of the interrenal cells was altered in these fish. Indeed, head kidney tissue from fish fed mifepristone produced significantly less cortisol in response to ACTH stimulation compared to control-fed fish. The reduced capacity to synthesize cortisol may be attributed to a reduction in StAR mRNA level. As the rate-limiting protein in steroidogenesis, a smaller pool of StAR mRNA, if accompanied by reduced protein levels, would reduce the steroidogenic capacity of the interrenal cells. Indeed studies have clearly shown that up-regulation of StAR and/or P450scc transcripts correspond with enhanced cortisol production, while a suppression of StAR mRNA level coincided with attenuation of cortisol production in response to ACTH stimulation [4,6].

In mammals, ACTH leads to a rapid increase in the transcription of steroidogenic genes, including StAR and P450scc, resulting in cortisol synthesis and release [41]. In teleosts, ACTH-stimulated cortisol release is well established, but its role in the regulation of steroidogenic genes is less clear. For example, while our study and that of Geslin and Auperin [24] saw no change in either StAR or P450scc mRNA in response to ACTH stimulus, both Aluru and Vijayan [6] and Hagen et al. [26] observed an increase in these genes. Methodological differences, including timing and dose, may have played a role, warranting further investigation into the mechanism of ACTH-stimulated cortisol production in teleosts. Also, a StAR-specific antibody would help to address from a functional stand-point some of these inconsistencies seen with mRNA abundances.

Our results also support a role for GR signaling in maintaining CRF mRNA levels in trout POA. Consistent with its role in initiating the HPI axis in response to stress, CRF mRNA levels increase in the POA of teleosts exposed to chronic stressors [11,17,18,28,36]. However, in response to acute stressors, this response is not always seen. In Doyon et al. [19] for example, a 1 h handling and confinement stressor did not elicit an increase in POA CRF gene expression despite clear activation of the HPI axis (elevated plasma cortisol). A similar scenario was observed in common carp (*C. carpio*) exposed to a 30 min restraint stress [28]. Huising and colleagues [28] argue that since the rapid HPI axis activation following a stressor is achieved by stored neuropeptides from the POA, mild stressors of short duration may not be sufficient to deplete these supplies to an extent requiring a compensatory transcriptional increase.

In fish fed mifepristone, we show changes in transcript abundance for CRF but not CRF-BP, consistent with diminished HPI axis competence. Mifepristone treatment reduced CRF transcript abundance ∼60% below control levels, suggesting a role for GR signaling in maintaining this peptide gene expression. This is consistent with a previous study showing that stressors and/or cortisol treatment significantly elevated CRF expression in trout [20]. Assuming the sustained depletion of CRF mRNA also reduced peptide stores, this result would contribute to the abrogated cortisol response to stress in the mifepristone-fed fish. The extent to which GR is involved in this transcriptional change is difficult to ascertain. In mammals, suppression of CRF transcription by high levels of glucocorticoids is mediated directly by GR (see review by Yao and Denver [49]). In teleosts, exogenous cortisol treatment reduces POA CRF mRNA levels in unstressed fish [10,9], and impairs the transcriptional response to some stressors [20], indicating a conserved negative feedback role of cortisol in CRF regulation. As either elevated cortisol [39,46] or mifepristone treatment (present study) down-regulates GR in fish, our results lead us to propose that classic genomic signaling mediated by GR may be important for maintaining the basal rate of CRF transcription, while the suppression of this peptide during negative feedback regulation by excess cortisol may involve other pathways yet to be determined. We hypothesize that non-genomic signaling by cortisol may also be involved in the negative feedback regulation of CRF in trout, as was observed for cortisol-mediated suppression of prolactin release from tilapia pituitaries [14,29].

In mammals, a specific binding protein for CRF, CRF-BP, is proposed to inhibit CRF signaling by limiting the bioavailability of CRF to its receptors [37]. Direct evidence for an inhibitory function in non-mammalian vertebrates is scarce, but several reports of transcriptional changes in CRF-BP expression in response to stress in amphibians [13] and fish [1,19,28] are in line with this function. Results from the present study did not reveal any effect of mifepristone treatment on POA CRF-BP, suggesting GR is not involved in maintaining basal CRF-BP mRNA levels. Altogether it appears that GR signaling is essential for the functioning of the HPI axis during stress in fish.

5. Conclusions

We demonstrate for the first time that mifepristone, a well-established antagonist of GR, also knocks down GR protein expression in rainbow trout. This led to an attenuation in stressor-induced plasma cortisol level suggesting that both central and peripheral GR signaling may be involved in HPI axis functioning. Specifically, we provide evidence that GR signaling is required for maintaining basal CRF and StAR transcript levels in the POA and head kidney, respectively, both of which are critical components of the endocrine stress response. A similar study that incorporates a more severe and/or chronic stressor is needed to fully elucidate the impact of GR knockdown and antagonism on the endocrine stress axis. Overall GR signaling is essential for the functioning of HPI axis and resultant elevation of plasma cortisol levels, a highly conserved response to stress in vertebrates, and any impact on this receptor function may lead to diminished stress performance.

Acknowledgments

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