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# Cortisol modulates the expression of cytokines and suppressors of cytokine signaling (SOCS) in rainbow trout hepatocytes

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ABSTRACT

Although liver is a key target for corticosteroid action, its role in immune function is largely unknown. We tested the hypothesis that stress levels of cortisol down regulate immune-relevant genes in rainbow trout (*Oncorhynchus mykiss*) liver. Hepatocytes were treated with lipopolysaccharide (LPS) for 24 h either in the presence or absence of cortisol. LPS stimulated heat shock protein 70 expression, enhanced glycolytic capacity, and reduced glucose output. LPS stimulated mRNA abundance of cytokines and serum amyloid protein A (SAA), while suppressors of cytokine signaling (SOCS)-3 was reduced. Cortisol increased mRNA abundances of IL-1β, SOCS-1 and SOCS-2, while inhibiting either basal or LPS-stimulated IL-8, TNF α2 and SAA. These cortisol-mediated effects were rescued by Mifepristone, a glucocorticoid receptor antagonist. Altogether, cortisol modulates the molecular immune response in trout hepatocytes. The upregulation of SOCS-1 and SOCS-2 by cortisol may be playing a key role in suppressing cytokine signaling and the associated inflammatory response.



## 1. Introduction

Cortisol is the major corticosteroid that is released in response to stress in teleosts, and this hormone is important for re-establishing homeostasis (Wendelaar Bonga, 1997; Mommsen et al., 1999; Aluru and Vijayan, 2009). Stressor-induced elevation of plasma cortisol involves the activation of the hypothalamus–pituitary–interrenal (HPI) axis, including the release of corticotropin-releasing factor from the hypothalamus, the adrenocorticotrophic hormone from the pituitary, and the stimulation of steroid biosynthesis in the interrenal tissues (analogous to the adrenal gland in mammals) (Wendelaar Bonga, 1997; Mommsen et al., 1999). This stress steroid has wide ranging effects and the actions are mediated by the corticosteroid receptors in target tissues. There are multiple glucocorticoid receptors (GRs) in teleosts due to whole genome duplication events, with the only exception so far being the zebrafish that has only a single GR in the genome (Prunet et al., 2006; Bury and Sturm, 2007; Alsop and Vijayan, 2009). Teleosts also express a mineralocorticoid receptor (MR); however, the function(s) of activating this receptor remain unclear (Sturm et al., 2005).

Cortisol is a key modulator of physiological processes, including stress response, growth, metabolism and immune response (Mommsen et al., 1999; Vijayan et al., 2010; Tort, 2011). A key response to stress that is cortisol mediated is the enhancement of liver metabolic capacity, including gluconeogenesis and increasing glucose output from this tissue (Mommsen et al., 1999; Aluru and Vijayan, 2007). This metabolic adjustment is essential for restoring homeostasis, as glucose is the preferred fuel for tissues to meet the increased energy demand that is essential to cope with stress (Mommsen et al., 1999; Aluru and Vijayan, 2007). While several studies have examined the role of stressor-induced cortisol in modulating liver metabolic capacity (Aluru and Vijayan, 2009), little is known about liver function in response to stressors eliciting an immune response in fish.

Stress, and the associated plasma cortisol elevation, has immunosuppressive effects in fish (Tort, 2011). For instance, cortisol modulates immune function by inhibiting lipopolysaccharide (LPS)-induced cytokine expression in fish immune cells (Zou et al., 2000; Engelsma et al., 2003; MacKenzie et al., 2006). However, to our knowledge no study has examined the effect of either stress or cortisol on the expression of suppressors of cytokine signaling (SOCS) in fish. Three isoforms of SOCS (SOCS-1, SOCS-2 and SOCS-3) were recently cloned and sequenced in rainbow trout, and they showed differential tissue expression (Wang and Secombes, 2008; Wang et al., 2010). While the functions of these proteins in fish are unclear, SOCS are known to integrate several physiological processes, including immune function, growth and embryonic development in mammals (Kile and Alexander, 2001).

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Against this backdrop we tested the hypothesis that cortisol will suppress expression of immune responsive genes and SOCS genes in rainbow trout hepatocytes. Trout hepatocytes are a model metabolic system to study cortisol effects (Aluru and Vijayan, 2007), but few studies have examined immune response with this cell system. LPS was used as an immune-stimulant in this study because it is a well-established tool to regulate immune responsive genes, including cytokines and acute phase proteins in fish (Engelsma, 2002; MacKenzie et al., 2006; Martin et al., 2010). As a marker of immune response, we measured the expression of interleukin1β (IL-1β), IL-8 and tumor necrosis factor α2 (TNF α2), serum amyloid protein A (SAA) and the three isoforms of SOCS using real-time quantitative PCR in trout hepatocytes. The cellular stress response was ascertained by determining the protein expressions of heat shock protein 70 and glucocorticoid receptor, key markers of disturbance to protein homeostasis (Deane and Woo, 2010) and metabolic response (Aluru and Vijayan, 2007), respectively. A GR antagonist (Mifepristone) was also used to tease out the direct role of cortisol signaling involving GR activation on immune and metabolic responses to an immunostimulant in hepatocytes. The metabolic capacity was ascertained by measuring the activities of glycolytic (hexokinase, glucokinase, pyruvate kinase), citric acid cycle (malate dehydrogenase, isocitrate dehydrogenase) and gluconeogenic (phosphoenolpyruvate carboxykinase) enzymes along with glucose release (Aluru and Vijayan, 2007).

## 2. Materials and methods

### 2.1. Experimental fish

Immature rainbow trout (*Oncorhynchus mykiss*; 150 ± 10 g body mass) were obtained from Alma Research Station (Alma, ON, CAN), and maintained at the University of Waterloo Aquatic Facility, at 12 ± 1 °C on a 12:12-h light/dark cycle. The fish were fed once daily to satiety with commercial trout pellet (Martin Mill, Elmira, Ontario). The fish were acclimated for 2 weeks before the experiments.

### 2.2. Primary culture of trout hepatocytes

Trout hepatocytes were isolated by *in situ* perfusion of liver with collagenase (Sigma, St. Louis, MO, USA) exactly as described before (Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007). Trypan blue dye exclusion method was used to confirm hepatocyte viability and >95% cells were viable. Cells were plated in six-well tissue culture plates (Sarstedt Inc., NC, USA) 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. After 24 h, the L-15 media was replaced with fresh L-15 and cells were incubated at 13 °C for 2 h prior to experimental treatments.

### 2.3. Experimental treatments

The experimental protocol for this study consisted of treating hepatocytes from each fish with either 0 or 30 μg/mL LPS (*Escherichia coli*, 055:B5; Sigma) along with either control media (0.01% ethanol used as vehicle) or media containing cortisol (100 ng/mL; Sigma), Mifepristone (1000 ng/mL; Sigma) or a combination of Mifepristone and cortisol. In the combination group, cells were incubated with Mifepristone 30 min before the addition of cortisol. The cells were then maintained at 13 °C for 24 h before sampling. The LPS concentration used was shown previously to elicit pro-inflammatory cytokine expression in fish cells (Stolte et al., 2008). The cortisol concentration used represents a typical stress level for trout, while the Mifepristone concentration used was shown previously to block cortisol-mediated metabolic effects in trout hepatocytes (Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007). 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 on dry ice and stored at −70 °C. There were six wells in total for each of the eight treatments. Two were pooled for glucose and enzyme analysis, two for protein expression analysis (immunodetection) and the remaining two for quantitative real-time PCR (qPCR). The experiment was repeated with hepatocytes isolated from seven independent fish.

### 2.4. Glucose release and cell viability

Media glucose was determined colorimetrically using a commercially available kit (Raichem, Cliniqa Corporation, CA, USA). The cell viability in culture was determined by measuring the leakage of lactate dehydrogenase (LDH) into the medium (Aluru and Vijayan, 2007). The LDH activity was determined as described below.

### 2.5. Enzyme activity

The enzyme activities were measured in 50 mM imidazole-buffered enzyme reagent (pH 7.4) at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax, Molecular Devices Corp., Palo Alto, CA, USA) exactly as described before (Vijayan et al., 2006). The following assay conditions were used:

#### 2.5.1. Hexokinase (HK: EC 2.7.1.1)

1 mM glucose, 5 mM $MgCl_2$, 0.24 mM NADH, 2 mM phosphoenolpyruvate (PEP), 20 U/mL lactate dehydrogenase (LDH) and 5 U/mL pyruvate kinase; reaction started with 4 mM ATP.

#### 2.5.2. Glucokinase (GK: EC 2.7.1.2)

20 mM glucose, 5 mM $MgCl_2$, 0.24 mM NADH, 2 mM phosphoenolpyruvate (PEP), 20 U/mL lactate dehydrogenase (LDH) and 5 U/mL pyruvate kinase; reaction started with 4 mM ATP.

#### 2.5.3. Pyruvate kinase (PK: EC 2.7.1.40)

30 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 20 U/mL LDH; reaction started with 2.5 mM PEP.

#### 2.5.4. Lactate dehydrogenase (LDH: EC 1.1.1.27)

0.12 mM NADH and reaction initiated with 1 mM pyruvic acid.

#### 2.5.5. Malate dehydrogenase (MDH: EC 1.1.1.37)

0.12 mM NADH; reaction started with 0.5 mM oxaloacetate.

#### 2.5.6. Isocitrate dehydrogenase (IDH: EC 1.1.1.42)

4 mM $MgCl_2$, 0.4 mM NADP; reaction started with 0.6 mM isocitrate.

#### 2.5.7. Phosphoenolpyruvate carboxykinase (PEPCK: EC 4.1.1.32)

20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM phosphoenolpyruvate, and 0.12 mM NADH; reaction started with 0.2 mM deoxyguanosine diphosphate.

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute per gram protein.

### 2.6. SDS–PAGE and immunodetection

Sample protein concentrations were measured with bicinchoninic acid (BCA) reagents using BSA as the standard. SDS–PAGE and immunodetection were carried out exactly as described before (Aluru and Vijayan, 2007. Briefly, sample protein (40 μg protein