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The effects of exogenous cortisol on *myostatin* transcription in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Glucocorticoids (GCs) strongly regulate myostatin expression in mammals via glucocorticoid response elements (GREs), and bioinformatics methods suggest that this regulatory mechanism is conserved among many vertebrates. However, the multiple *myostatin* genes found in some fishes may be an exception. *In silico* promoter analyses of the three putative rainbow trout *(Oncorhynchus mykiss) myostatin* promoters have failed to identify putative GREs, suggesting a divergence in myostatin function. Therefore, we hypothesized that *myostatin* mRNA expression is not regulated by glucocorticoids in rainbow trout. In this study, both juvenile rainbow trout and primary trout myoblasts were treated with cortisol to examine the effects on *myostatin* mRNA expression. Results suggest that exogenous cortisol does not regulate *myostatin-1a* and *-1b* expression *in vivo*, as *myostatin* mRNA levels were not significantly affected by cortisol treatment in either red or white muscle tissue. In red muscle, *myostatin-2a* levels were significantly elevated in the cortisol treatment group relative to the control, but not the vehicle control, at both 12 h and 24 h post-injection. As such, it is unclear if cortisol was acting alone or in combination with the vehicle. Cortisol increased *myostatin-1b* expression in a dose-dependent manner *in vitro*. Further work is needed to determine if this response is the direct result of cortisol acting on the *myostatin-1b* promoter or through an alternative mechanism. These results suggest that regulation of *myostatin* by cortisol may not be as highly conserved as previously thought and support previous work that describes potential functional divergence of the multiple *myostatin* genes in fishes.

1. Introduction

Myostatin, a member of the transforming growth factor-ß superfamily, is a well-characterized inhibitor of muscle growth in mammals (McPherron et al., 1997; Rodgers and Garikipati, 2008). In fishes, *myostatin* has been cloned and expression patterns have been characterized in a number of fishes: zebrafish (McPherron and Lee, 1997; Xu et al., 2003), brook trout (Roberts and Goetz, 2001, 2003), gilthead seabream (Maccatrozzo et al., 2001), Atlantic salmon (Ostbye et al., 2001), rainbow trout (Rescan et al., 2001; Garikipati et al., 2006; Garikipati et al., 2007), channel catfish (Kocabas et al., 2002), sea perch (Ye et al., 2007), and orange-spotted grouper (Ko et al., 2007). In addition, the *myostatin* sequences are well-conserved among vertebrates, with the bioactive domain ranging from 88 to 100% identity, and functional studies in fish demonstrate that the myogenic functions are also conserved (Xu et al., 2003; Rodgers and Garikipati, 2008; Lee et al., 2009; Medeiros et al., 2009; Lee et al., 2010; Sawatari et al., 2010).

Genome duplication events have produced multiple myostatin genes in salmonids. Three of these genes, *mystatin-1a, -1b* and *-2a,* are putatively functional, while a premature stop codon in the open reading frame of *myostatin-2b* prevents the production of mature transcripts in rainbow trout and Atlantic salmon (Garikipati et al., 2007; Ostbye et al., 2007b). Interestingly, these genes appear to be ubiquitously expressed and differentially regulated during development and in response to various physiological changes (Biga et al., 2004; Helterline et al., 2007; De Santis and Jerry, 2011; Gabillard et al., 2013). Such expression patterns are suggestive of a functional divergence among fishes, as the single mammalian *myostatin* ortholog is predominately expressed in muscle. To identify potential mechanisms responsible for changes in spatial and temporal expression patterns, numerous studies have investigated the promoter region of *myostatin* in mammals and fish to characterize putative transcription factor binding sites and hormone response elements (Ma et al., 2001a; Spiller et al., 2002; Garikipati et al., 2006, 2007; Ostbye et al., 2007a; Allen and Du, 2008; Rodgers and Garikipati, 2008; Funkenstein et al., 2009b; Li et al., 2012a,b,c; Nadjar-Boger et al., 2012;

Abbreviations: GRE, glucocorticoid response element; CORT, cortisol; VC, vehicle control; NO, no injection control.

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Xue et al., 2012; Gabillard et al., 2013; Hu et al., 2013; Nadjar-Boger et al., 2013). Putative E-box protein, myogenic regulatory factor (MRF), and myocyte enhancing factor (MEF) binding motifs and glucocorticoid response elements (GREs) appear to be highly conserved components of the *myostatin* promoter in vertebrates (Rodgers and Garikipati, 2008). In this study, we were specifically interested in the presence or absence of GREs as a potential mediator in the divergence of *myostatin* expression among vertebrates.

In mammals, *myostatin* expression is highly regulated by glucocorticoids (GCs), and promoter analyses suggest that the regulation by GCs may be highly conserved among vertebrates (Ma et al., 2001a, 2003; Garikipati et al., 2006, 2007; Ostbye et al., 2007a; Rodgers and Garikipati, 2008; Funkenstein et al., 2009b; Gabillard et al., 2013). The actions of GCs appear to be mediated by the binding of the glucocorticoid receptor (GR) to GREs in the regulatory region of the myostatin gene to upregulate gene expression (Ma et al., 2001a, 2003; Gilson et al., 2007; Qin et al., 2013). However, most of the research describing GC regulation of myostatin has been done in mammals, and currently little empirical data are available regarding this relationship in other vertebrates, including fishes. The current evidence suggests that the putative GREs are not present in all of the *myostatin* promoters within fish species. Different approaches for identifying consensus sequences have yielded somewhat contradicting results and it is not clear if glucocorticoids directly regulate myostatin expression in teleosts (Roberts and Goetz, 2003; Garikipati et al., 2007, 2006; Ostbye et al., 2007b; Rodgers and Garikipati, 2008; Funkenstein et al., 2009a; De Santis and Jerry, 2011; Gabillard et al., 2013). Interestingly, *in silico* promoter analyses have failed to identify putative GREs in the promoters of any *myostatin* paralogs in rainbow trout, and no study has tested a relationship between glucocorticoids and *myostatin* expression in this species (Garikipati et al., 2006, 2007).

The current study was conducted to determine the effects of cortisol on *myostatin* expression in rainbow trout, using both *in vivo* and *in vitro* approaches. Based on *myostatin* promoter analyses, we hypothesize that *myostatin* mRNA expression is not affected by cortisol treatments. The presumed loss of GREs in the promoters of the rainbow trout myostatin genes makes this species an excellent model system for studying the divergence in glucocorticoid regulation of myostatin. Our experiments failed to identify a clear cortisol response *in vivo* and only detected a significant increase in *myostatin-1b* expression *in vitro*, suggesting a change in GC regulation of *myostatin* relative to mammals. Although our experimental design did not directly test for the presence of GREs, our results do illustrate a potential divergence in the regulation of myostatin among the vertebrates, specifically by cortisol.

2. Materials and methods

2.1. Animal care

Juvenile rainbow trout used in the experiments detailed below were obtained from the United States Fish and Wildlife Service's Garrison National Fish Hatchery, Riverdale, North Dakota, and housed at North Dakota State University. All fish were maintained in 800-L flow-through tanks with a 12L:12D photoperiod and were fed AquaMax Grower (PMI Nutrition International, Inc., Brentwood, MO, USA) to apparent satiation twice daily, except 24 h before experimentation. All experiments conducted with animals were approved in advance of experimentation by the Institutional Animal Care and Use Committee at North Dakota State University, Fargo.

2.2. Experiment 1: effects of cortisol on myostatin expression in vivo

Juvenile rainbow trout (70–100 g) were randomly assigned to three experimental tanks (60 L, 12 °C, 4 h flow-through, 12 fish per tank, ~20 kg/m^3 maximum stocking density) and allowed to acclimate for one week. The three treatment groups consisted of injections of cortisol (CORT), vehicle control (VC), and no injection control (Control). Prior to injections, fish were anesthetized with buffered tricaine methylsulfonate (MS-222; 100 mg/L). The fish received either an intraperitoneal injection of cortisol (Janzen et al., 2012) dissolved in safflower oil or an injection of safflower oil at a volume consistent with the CORT group (2 μL/g BW). Post-injection, fish were placed in a recovery tank for five and then returned to their appropriate experimentation tank. At 12 h and 24 h post-injection, six fish per treatment group were euthanized by overdose of MS-222 (>300 mg/mL; AVMA Guidelines for the Euthanasia of Animals, 2013) and blood plasma samples were immediately collected for glucose measurements. Additionally, tissues (skeletal muscle: red and white; and liver) were flash-frozen and stored at −80 °C.

2.3. Experiment 2: effects of cortisol on myoblast myostatin expression in vitro

Following a protocol developed by Rescan et al. (1995), primary myoblasts were isolated from juvenile rainbow trout (1–2.5 g). Following mechanical dissociation, white muscle tissue was washed, enzymatically digested (collagenase type IV and trypsin) and cells were filtered (100 and 40 μm). Isolated cells were counted using a hemocytometer and the trypan blue exclusion method. Isolated cells were plated on poly-L-lysine-treated (Sigma), laminin-coated (BD Biosciences) plates at a density of 2×10^6 cells/mL. Cultures were incubated at 18 °C in a complete medium (10% DMEM) under normal atmospheric conditions without CO_2 supplementation. The medium was changed daily for the first two days of culture. On day three, cells were treated with a medium containing cortisol or ethanol (vehicle control). All treatments were run in triplicate on duplicate plates and consisted of increasing concentrations of cortisol: CORT 0, 10, 100, and 1000 ng/mL. After 24 h, the medium was removed and cells were harvested for total RNA isolation (RNAzol; Molecular Research Center).

2.4. Quantitative real-time PCR

Total RNA was isolated from red muscle, white muscle, liver, and myoblast cell cultures using RNAzol (Molecular Research Center, Inc.) according to the manufacturer's instructions. Total RNA concentrations were quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) and 1 μg of total RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega). Quantitative PCR (qPCR) was performed using PerfeCTa SYBR Green SuperMix (Quanta Biosciences) according to the manufacturer's recommendations using the Mx3000P system (Stratagene). All reactions contained 2 μL sample cDNA (produced from 1 μg total RNA and diluted 1:10) or 1 μL vector at desired concentrations for standard curve. All primers used were specific for each of the three putatively functional myostatin isoforms (*myostatin-1a*, *-1b*, and *-2a*) and used at 300 nM. Myostatin primer sequences were used as previously described (Garikipati et al., 2006, 2007). For validation of cortisol action following injections, *Hsp90* mRNA expression changes were analyzed in liver tissue by qPCR using primers previously described (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003; Ings et al., 2011). Standard curves were generated by serial dilution of plasmids (pGEM-T Easy Vector, Promega) containing the amplicon of interest. Briefly, 1:10 serial dilutions of stock constructs were performed, resulting in final concentrations of 1.0×10^1 copies/μL to 1.0×10^8 copies/μL. The PCR cycling parameters were as follows: 94 °C (2 min) followed by 40 cycles at 94 °C for 20 s, 60 °C for 30 s, and 68 °C for 1 min. A dissociation curve was performed for each assay to ensure primer specificity by running a single cycle as follows: 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. All data were analyzed using Mx3000P system software (Stratagene). All assays utilized a comparative baseline strategy using the ΔC_q method that standardized raw data to starting input cDNA quantity (Bustin et al., 2009; De Santis and Jerry, 2011; Meyer et al., 2013).