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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 transcript levels in mammals via glucocorticoid response elements (GREs) in the *myostatin* promoter, 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 rainbow trout (*Oncorhynchus mykiss*), two genome duplication events have produced three putatively functional *myostatin* genes, *myostatin-1a*, *-1b* and *-2a*, which are ubiquitously and differentially expressed. In addition, *in silico* promoter analyses of the rainbow trout *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 relationship between this glucocorticoid and *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.

Keywords

rainbow trout; myostatin; cortisol; stress; myoblasts

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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 (Garikipati et al., 2007; Garikipati et al., 2006; Rescan et al., 2001), 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 88-100% identity, and functional studies in fish demonstrate that the myogenic functions are also conserved (Lee et al., 2009; Lee et al., 2010; Medeiros et al., 2009; Rodgers and Garikipati, 2008; Sawatari et al., 2010; Xu et al., 2003).

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; De Santis and Jerry, 2011; Gabillard et al., 2013; Helterline et al., 2007). 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 (Allen and Du, 2008; Funkenstein et al., 2009b; Gabillard et al., 2013; Garikipati et al., 2007; Garikipati et al., 2006; Hu et al., 2013; Li et al., 2012a; Li et al., 2012b; Li et al., 2012c; Ma et al., 2001a; Nadjar-Boger et al., 2012; Nadjar-Boger et al., 2013; Ostbye et al., 2007a; Rodgers and Garikipati, 2008; Spiller et al., 2002; Xue et al., 2012). 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 (Funkenstein et al., 2009b; Gabillard et al., 2013; Garikipati et al., 2007; Garikipati et al., 2006; Ma et al., 2001a; Ma et al., 2003; Ostbye et al., 2007a; Rodgers and Garikipati, 2008). 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 (Gilson et al., 2007; Ma et al., 2001a; Ma et al., 2003; 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 (De Santis and Jerry, 2011; Funkenstein et al., 2009a; Gabillard et al., 2013; Garikipati et al., 2007; 2006; Ostbye et al., 2007b; Roberts and Goetz, 2003; Rodgers and Garikipati, 2008). 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., 2007; Garikipati et al., 2006).

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 make 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.

Materials and Methods

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-liter 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.

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 place 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.

Experiment 2: Effects of cortisol on myoblast myostatin expression in vitro

Following a protocol developed by Rescan and colleagues (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 $2x10^6$ cells/mL. Cultures were incubated at 18 °C in complete media (10% DMEM) under normal atmospheric conditions without CO_2 supplementation. Media was changed daily for the first two days of culture. On day three, cells were treated with media 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 hr, media was removed and cells were harvested for total RNA isolation (RNAzol; Molecular Research Center).

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., 2007; Garikipati et al., 2006). For validation of cortisol action following injections, *Hsp90* mRNA expression changes were analyzed in liver tissue by qPCR using primers previously described (Ings et al., 2011; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). 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 et al., 2001; Meyer et al. 2013).

Plasma glucose analysis

Immediately after euthanasia, blood samples were collected via caudal venipuncture. Blood was temporarily stored in heparinized vacuum tubes on ice. Samples were then centrifuged and plasma was isolated from packed cells. Glucose levels were analyzed in plasma (5 μL) using an Accu-Chek glucose meter (Roche) as previously described (Galt et al., 2013; Meyer et al., 2013).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com. For *in vivo* analyses, plasma glucose and *HPS90* expression were evaluated using one-way ANOVA, while m*yostatin* expression was evaluated using two-way ANOVA treating fish as experimental units. For *in vitro* analyses, all expression levels were analyzed using one-way ANOVA. Tukey's Multiple Comparison Test was conducted to compare among treatment groups as needed. All results were considered significant at $P<0.05$. All qPCR data are reported relative to control as percent-mean ±S.E.M.

Results

Cortisol elevated plasma glucose and Hsp90 expression in vivo, but did not affect myostatin expression

To verify that cortisol treatments initiated a physiological response, both plasma glucose levels and liver *Hsp90* mRNA expression were assessed 12 h post-injection for each treatment group. Plasma glucose levels were elevated after cortisol treatment 144.8% and 154.8% relative to both the control and vehicle control, respectively (Figure 1a). In addition, cortisol treatment significantly increased *Hsp90* mRNA expression 153.9% and 116.7% relative to both the control and vehicle control, respectively (Figure 1b).

To determine if cortisol regulates myostatin expression, we quantified *myostatin-1a, -1b,* and *-2a* mRNA expression in white and red muscle 12 h and 24 h post-injection. In white muscle, cortisol treatment had no statistically significant effects on *myostatin-1a, -1b,* and *-2a* mRNA expression *in vivo,* both 12 h and 24 h post-injection (Figures 2d, -e, -f). In addition, no differences were detected in red muscle *myostatin-1a* and *-1b* expression at either time point (Figures 2a, -b). *Myostatin-2a* expression was significantly upregulated in red muscle in the vehicle and cortisol treatment groups, both 12 h (824.3% and 633.4% over control, respectively; Figure 2c) and 24 h post-injection (455.7% and 581.0% over control, respectively; Figure 2c). However, no difference was detected between these two groups.

Cortisol elevated Hsp90 and myostatin-1b expression in primary myoblasts

To examine whether cortisol acts at the level of the myoblast in skeletal muscle, we quantified *Hsp90* mRNA expression in total myoblast lysates 24 h following treatment. Cortisol, at both 100 ng/mL and 1000 ng/mL, significantly increased *Hsp90* mRNA

expression relative to the no treatment control (98.8% and 116.3% over control, respectively; Figure 3a). No differences were detected between the vehicle control and cortisol treatment at 10 ng/mL relative to the no treatment control.

To determine if cortisol regulates *myostatin* in myoblasts, we quantified *myostatin-1a*, *-1b*, and *2a* mRNA expression in total myoblast lysates 24 h following treatment with increasing concentrations of cortisol. No changes were observed regarding *myostatin-1a* and *-2a* expression following our treatments (Figures 3b, -d). *Myostatin-1b* mRNA responded to cortisol treatment in a dose-dependent manner (Figure 3c). Specifically, expression significantly increased relative to the control at 10 ng/mL (94.5%) and at 100 ng/mL (256.6%). The mRNA abundance at 1000 ng/mL decreased to levels similar to the 10 ng/mL treatment (89.8% and 94.5%, respectively) but was still significantly higher than control levels.

Discussion

Many studies have reported a relationship between GCs and myostatin, but few have described this relationship in fishes. In this study, we conducted single exposure experiments, treating both juvenile rainbow trout and primary rainbow trout myoblasts with cortisol to determine if an interaction between cortisol and *myostatin* mRNA expression exists in this species. The increase in plasma glucose and hepatic *Hsp90* mRNA expression demonstrate that our cortisol treatment induced a response after 12 h *in vivo* and 24 h *in vitro*. Further, the increase in myoblast *Hsp90* mRNA expression was significant at both 100 ng/mL and 1000 ng/mL of cortisol, suggesting a dose-dependent response. These data corroborate previous *in vivo* and *in vitro* studies assessing *Hsp90* mRNA expression patterns following glucocorticoid treatments in rainbow trout (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003).

Overall, our analyses failed to detect significant expression changes in *myostatin-1a* or *-1b* mRNAs in white or red skeletal muscle following intraperitoneal injections of cortisol (relative to all controls). Because red-oxidative and white-glycolytic skeletal muscle exist as distinct tissues in teleost fish, differences in expression patterns can be analyzed easily between the two metabolically different muscle types. Several reports have demonstrated muscle fiber type-specific *myostatin* patterns in several teleosts (Biga et al., 2004; Ostbye et al., 2001; Patruno et al., 2008; Roberts and Goetz, 2003; Roberts et al., 2004) suggesting relevance to fiber specificity. Here we observed an increase in *myostatin-2a* expression specifically in red muscle, 12 h and 24 h post-injection, in both the vehicle and cortisol treatment groups. However, this response is likely attributable to the vehicle, safflower oil, which was chosen for its low viscosity relative to other lipids. The increase in *myostatin-2a* in response to safflower oil was unexpected considering its common use as an inert vehicle for steroids. *Myostatin-2a* did not respond to either treatment in white muscle and therefore suggests tissue-specific sensitivity to this or other lipid sources. Further, trends were observed in both *myostatin-1a* and *-1b* expression in response to the vehicle in both tissues, though not statistically significant. These data, along with our previous work showing changes in *myostatin* expression in response to a high-fat diet, provide evidence that myostatin may be responsive to lipid availability (Galt et al., 2013).

Regulation of myostatin in response to glucocorticoids occurs at many levels in mammals, including direct regulation by cortisol through a GRE located in the 5′ regulatory region of the *myostatin* gene (Gilson et al., 2007; Ma et al., 2001a; Ma et al., 2003; Qin et al., 2013). In this study, the lack of a clear response *in vivo* suggests that exogenous cortisol may not affect *myostatin* gene expression in rainbow trout muscle tissue. These findings are consistent and corroborate previous documentation that the rainbow trout myostatin gene promoters lack putative GREs (Garikipati et al., 2007; Garikipati et al., 2006). In fish, few studies have directly tested this relationship between glucocorticoids and *myostatin* expression. Immersion of tilapia larvae in cortisol resulted in decreased *myostatin* mRNA expression, and dexamethasone (a potent synthetic glucocorticoid) injections decreased *myostatin* expression in channel catfish (Rodgers et al., 2003; Weber et al., 2005). To our knowledge, no study has examined the regulatory regions of *myostatin* for GREs in either tilapia or channel catfish. Typically, the GR positively regulates gene expression, but in some cases negative regulation or repression of expression can occur (Schoneveld et al., 2004). In addition, myogenic regulatory factor (MRF) binding sites appear to be highly conserved in the *myostatin* promoters of fish and mammals. Therefore, the decrease in *myostatin* expression in tilapia and channel catfish may be the result of glucocorticoid-induced degradation and disruption of MRF activity thus decreasing the activation of *myostatin* expression (Jogo et al., 2009; Sun et al., 2008).

Studies examining *myostatin* expression under stress in fish have reported conflicting results. For example, chronic overcrowding of zebrafish results in elevated *myostatin* expression in lateral skeletal muscle (Vianello et al., 2003), while high stocking densities in zebrafish elevated both *myostatin-1* and *-2* in spleen tissue but not muscle (Helterline et al., 2007). The zebrafish *myostatin-2* promoter contains a putative GRE, and this element may be responsible for the detected changes in *myostatin* expression described above (Garikipati et al., 2006). In addition to traditional physical stressors, a 30-day fasting period decreased *myostatin-1* and *-2* expression in rainbow trout muscle, and this decrease was ameliorated after 14 days of refeeding (Johansen and Overturf, 2006). Conversely, no change in *myostatin* expression was detected in adult tilapia after 30 days of fasting, but *myostatin* levels were consistently reduced in tilapia larvae after 9 days of fasting (Rodgers et al., 2003). In Asian sea bass, a 30-day fast differentially regulated *myostatin* isoform expression (De Santis and Jerry, 2011). Two studies have reported correlations between circulating cortisol levels with *myostatin* expression levels in fish in relation to growth hormone and probiotics (Biga et al., 2004; Carnevali et al., 2006). However, none of these studies are definitive, as only zebrafish *myostatin-2* and Asian sea bass *myostatin-1* have putative GREs in their promoters (De Santis and Jerry, 2011; Garikipati et al., 2006). Therefore, the differential expression patterns in response to these stressors could reflect indirect actions of the stress response, the metabolic status of the fish, or divergent adaptations among fishes.

Treatment of immortalized mammalian myoblasts (C2C12) with dexamethasone upregulates both *myostatin* mRNA expression and protein levels (Ma et al., 2001b). Therefore, we assessed the effects of cortisol treatment *in vitro* using primary rainbow trout myoblasts to further characterize the relationship between cortisol and *myostatin* expression. While *Hsp90* mRNAs were upregulated with glucocorticoid administration, cortisol treatment had

no effect on *myostatin-1a* or *-2a* expression, but *myostatin-1b* expression increased in a dose-dependent manner from 10 ng/mL to 100 ng/mL of cortisol. Interestingly, expression levels in response to 1000 ng/mL of cortisol were similar to levels at 10 ng/mL and significantly less than at the intermediate cortisol dose of 100 ng/mL. Previous work in primary bovine myoblasts has shown that exogenous myostatin negatively regulates *myostatin* mRNAs, suggests autoregulation of *myostatin* via a feedback loop (Forbes et al., 2006). A similar mechanism could be at play in this study, however, myostatin protein levels were not assessed. Alternatively, cortisol may negatively affect MRF activity at the high dose as previously documented (Jogo et al., 2009; Sun et al., 2008), and changes in MRF activity may indirectly affect *myostatin-1b* expression. While our analysis failed to detect changes in *myostatin-1b* expression in white muscle, it is intriguing that myoblasts isolated from this same tissue upregulated *myostatin-1b* when stimulated with cortisol. However, myoblasts are immature cells, and the bulk of skeletal muscle is constituted by differentiated myofibers. Additionally, the extracellular matrix plays a key role in the regulation of myostatin *in vivo*, and this is largely absent in cell culture-based assays (Kishioka et al., 2008; Miura et al., 2010; Sengle et al., 2011; Zeng et al., 2014). Further, the laminin used in our cell culture system has been shown to bind myostatin (Yasaka et al., 2013), and this may interfere with normal myostatin signaling.

This study provides evidence for a divergence in the GC responsiveness and tissue/cell specific regulation of *myostatin* in rainbow trout. Treatment of juvenile rainbow trout with intraperitoneal injections of cortisol failed to demonstrate a clear and significant effect on *myostatin-1a* and *-1b* mRNA expression. It is unclear whether the increase in red muscle *myostatin-2a* expression can be attributed to cortisol treatment alone, as the vehicle itself has a similar effect. Further, only *myostatin-1b* expression increased in primary myoblasts in response to cortisol treatment. To our knowledge, these data are the first to report that the regulation of *myostatin* by GCs in rainbow trout contrasts with the robust regulation that has been observed in mammals. Further studies are needed to determine if cortisol directly regulates *myostatin* expression in other salmonids, particularly those known to possess putative GREs in *myostatin* promoters.

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Abbreviations

GRE	glucocorticoid response element
CORT	cortisol
VC	vehicle control
NO	no injection control

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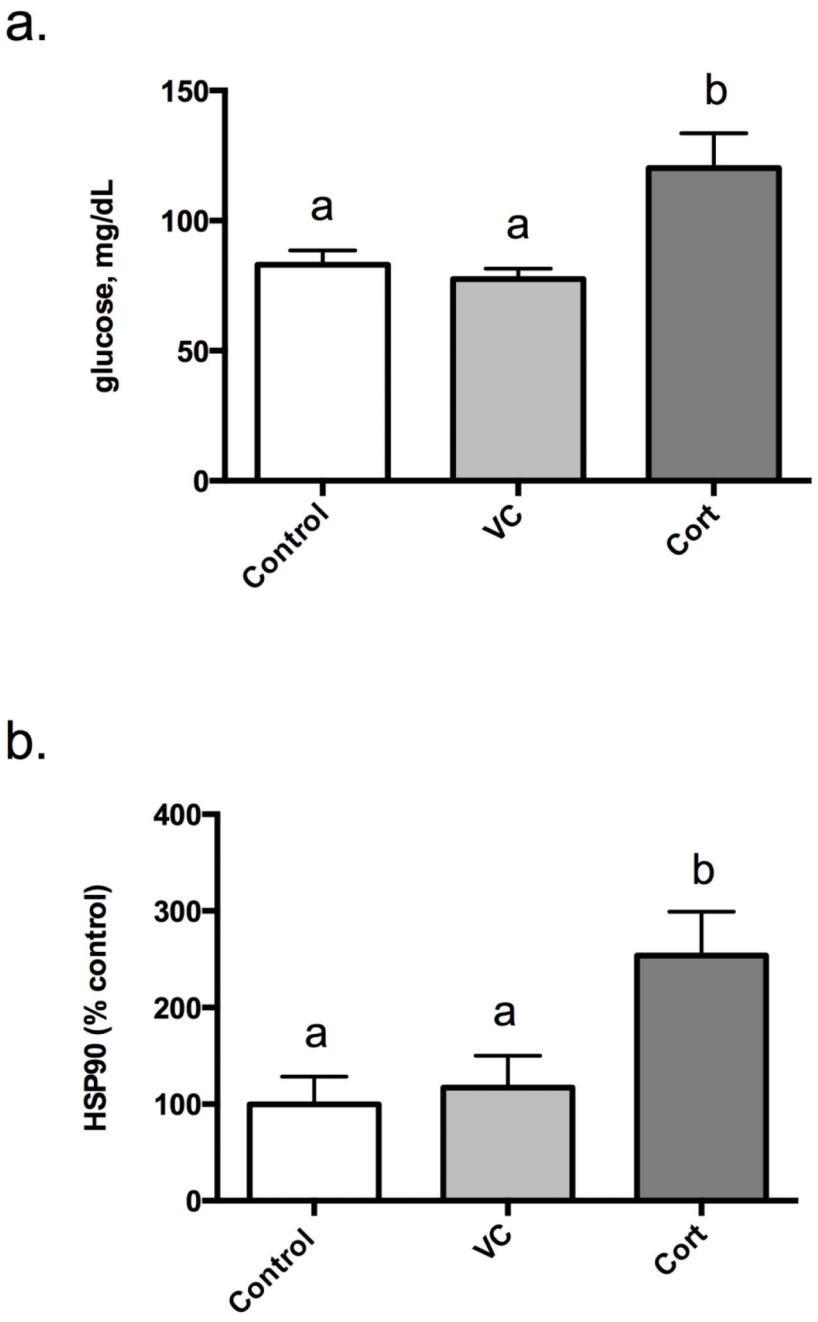

Figure 1.

Circulating blood glucose levels (a) and liver *HSP90* mRNA expression levels (b) 12 h post-cortisol treatment. Results are mean glucose mg/dL ±SEM (a) and percent-mean relative to control ± SEM (b) (*n*=6 fish/trt). Treatment groups include: no injection control (Control), vehicle control (VC; 2 μL/g BW safflower oil), and cortisol (Cort; 2 μg/g BW). Different letters are significantly ($P<0.05$) different.

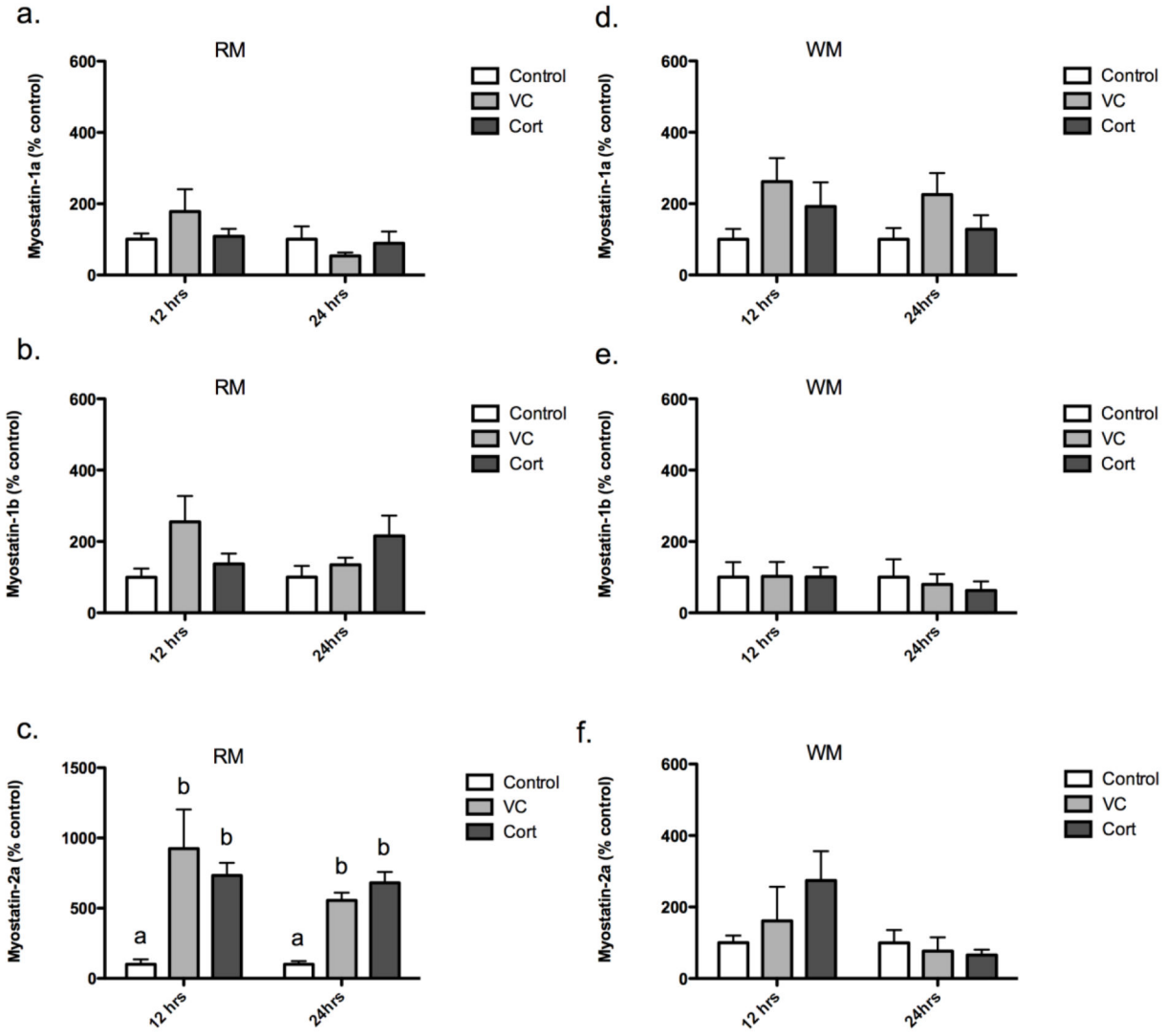

Figure 2.

Red muscle *myostatin-1a* (a), *-1b* (b), and *-2a* (c) mRNA levels 12 h and 24 h post-injection. White muscle *myostatin-1a* (d), *-1b* (e), and *-2a* (f) mRNA levels 12 h and 24 h post-injection. Results are percent-mean relative to control ± SEM (n=6 fish/trt). Treatment groups include: no injection control (Control), vehicle control (VC; 2 μL/g BW safflower oil), and cortisol (Cort; 2 μg/g BW). Different letters are significantly (P<0.05) different.

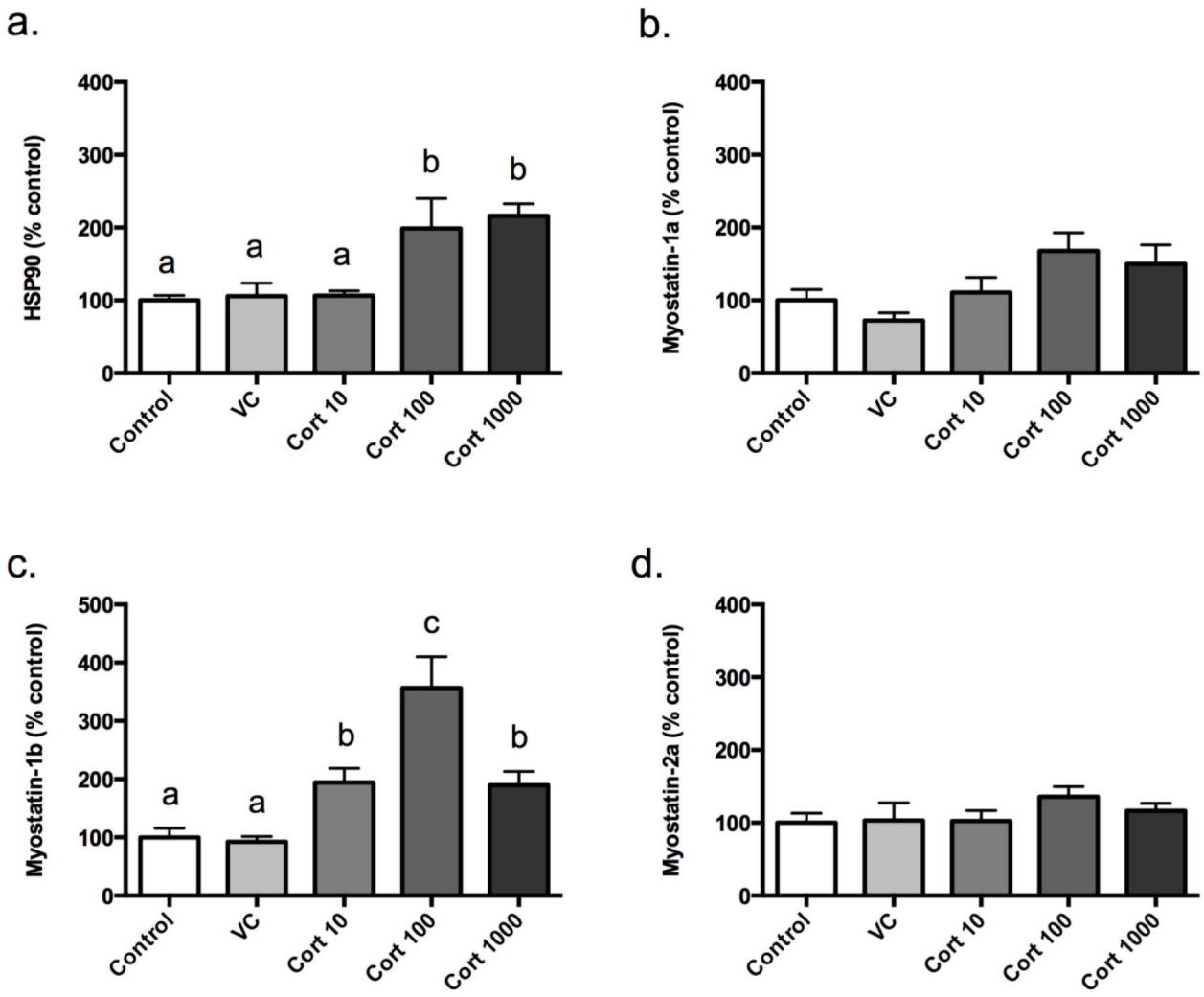

Figure 3.

Myoblast *HSP90* mRNA (a), *myostatin-1a* (b), *-1b* (c), and *-2a* (d) mRNA levels expression levels 24 h post-treatment. Results are percent-mean relative to control ± SEM (n=6). Treatment groups include: Control, vehicle control (VC; ethanol), cortisol at 10 ng/mL (Cort 10), cortisol at 100 ng/mL (Cort 100), and cortisol at 1000 ng/mL (Cort 1000). Different letters are significantly ($P<0.05$) different.