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Cardiac and Vascular Atrogin-1 mRNA Expression is Not Associated with Dexamethasone Efficacy in the Monocrotaline Model of Pulmonary Hypertension

Michael L. Paffett, Meghan M. Channell, Jay S. Naik*, Selita N. Lucas, and Matthew J. Campen

Department of Pharmaceutical Sciences, University of New Mexico College of Pharmacy, Albuquerque, NM 87131

*Department of Cell Biology and Physiology University of New Mexico School of Medicine, Albuquerque, NM 87131

Abstract

Atrophic signaling elements of the ubiquitin-proteasome system (UPS) are involved in skeletal muscle wasting as well as pressure overload models of heart failure. In our prior experiments, we demonstrated a transcriptional down-regulation of atrophy-inducing vascular E3 ubiquitin ligases in a toxic model of pulmonary hypertension where pulmonary artery and right ventricle (RV) hypertrophy are evident. Given the numerous reports of glucocorticoid activation of the UPS and the negative regulator of muscle mass, myostatin, we investigated the efficacy of dexamethasone to reverse monocrotaline (MCT)-induced pulmonary hypertension and augment atrogin-1 expression in both pulmonary arteries and myocardium. Dexamethasone caused significant reductions in body weight in combination with MCT. As predicted, MCT-induced pulmonary hypertension was evident by increases in RV systolic pressure, right ventricle to left ventricle plus septal weight ratios (RV/LVS) and arterial remodeling. MCT treatment significantly reduced both RV and PA atrogin-1 expression. Dexamethasone treatment reversed the MCT-induced pathological indices and restored RV atrogin-1 expression, but did not impact atrogin-1 expression in pulmonary arteries. Myostatin was poorly expressed in pulmonary arteries compared to the RV, and dexamethasone treatment increase RV myostatin in controls but not MCT-treated rats. These findings suggest that mechanisms independent of myostatin/atrogin-1 are responsible for glucocorticoid efficacy in this model of pulmonary hypertension.

Keywords

Pulmonary hypertension; vascular smooth muscle; ubiquitin proteasome system; atrogin; glucocorticoid

Introduction

Pulmonary artery remodeling and aberrant vasoconstriction leading to right ventricular hypertrophy are consistent hemodynamic features of pulmonary hypertension, regardless of the etiology [1]. While abnormal growth of the vascular wall is caused by a variety of growth factors and eventual maladaptive hemodynamic compensatory responses [2], investigations of the ubiquitin proteasome system (UPS) involvement in this pathology are

limited, although parallels may be drawn with studies of compensatory left ventricular hypertrophy [3,4]. Within this realm of proteasomal induction, abnormal catabolism of skeletal muscle in syndromes of cachexia [5] and glucocorticoid-induced atrophy [6] are thought to be driven by a class of E3 ubiquitin ligases initially termed as “atrogenes”. Atrogin-1 (a.k.a. FBXO32 or MAFbx) has been identified as a prominent ubiquitin ligase that controls degradation of both skeletal [7] and cardiac muscle [8]; however a paucity of information exists in regard to the role the proteasome influences vascular smooth muscle pathophysiology.

The first physiological role for atrophy-inducing E3 ligases in smooth muscle was recently described where elevated atrogin-1 and muscle ringer finger protein-1 (MuRF-1) expression coincides with post-partum uterine smooth muscle involution [9], prompting questions related to a potential role for the ubiquitin-proteasome pathway in smooth muscle hypertrophy syndromes. Atrogin-1, through distinct molecular interactions and ancillary proteins, conjugates ubiquitin onto muscle-specific proteins for targeted degradation, as well as regulating activity of upstream Forkhead-O (FoxO) transcription factors that potentiate an atrophic phenotype [10,11]. In addition to ubiquitin-dependent transcriptional regulation, atrogin-1 overexpressing mice show resistance to pathologic left ventricular hypertrophy through enhanced ubiquitination and degradation of calcineurin in a pressure overload model of cardiac hypertrophy [12]. Complementary to genetic overexpression of atrophic signaling molecules, glucocorticoids lead to muscle atrophy, primarily by activating the UPS and increasing the expression of atrogin-1 and MuRF-1 [13,14]. Dexamethasone is a potent inducer of growth inhibitory signaling in cardiac and skeletal muscle types mediated by increases in the transforming growth factor β family member, myostatin [15]. Genetic deletion of myostatin prevents glucocorticoid-induced atrophy [16], suggesting that E3 ligase expression may be downstream of myostatin signaling.

Glucocorticoids improve hemodynamic indices of experimental pulmonary hypertension [17] and are potent atrophy inducing agents that have been shown to augment atrogin-1 and MuRF-1 expression [18]. Moreover, we recently observed that atrogin-1 and MuRF-1 are temporally decreased in hypertrophied pulmonary arteries from monocrotaline (MCT)-treated rats, in a manner inversely proportional to the degree of pulmonary hypertension [19], suggesting that downregulation of muscle-specific degradation pathways may contribute to smooth muscle hypertrophy. The aim of the present study was to test the effects of dexamethasone on smooth muscle and striated muscle atrogin-1 expression owing to differing tissue-specific expression of the crucial intermediate and negative regulator of muscle growth, myostatin. Therefore, we tested the efficacy of dexamethasone in reversing established MCT-induced pulmonary hypertension and its impact on atrogin-1 and myostatin levels in cardiac as well as vascular smooth muscle.

Materials and Methods

Animals

Male Sprague-Dawley rats were obtained from a commercial vendor (Charles River Laboratories) and housed in quarantine for 14 days prior to study onset. When the cohort of rats all weighed >300g, rats were dosed with MCT (50 mg/kg, ip) on day 0 then treated with dexamethasone (17.5 or 50 μ g/kg/day, single daily ip injection; n=6/group) from day 28–42 post MCT. We chose to dose over this later period of PAH manifestation 1) to avoid the early inflammatory phase of MCT pathology and 2) to avoid the impact of dexamethasone of biotransformational liver enzymes [20]. Body weights were assessed weekly. At day 42, hemodynamics and tissue weights were assessed, and lungs and pulmonary arteries were isolated and preserved for assessment of atrogin-1 expression.

Hemodynamic and Right Ventricular Hypertrophy Measurements

Right ventricular pressure was obtained under anesthesia, as previously described [19]. Briefly, the right external jugular vein was exposed by blunt dissection and RVSP measurements were made with a saline filled Micro-Renathane catheter (.050 OD $\times$.040 ID). The catheter was advanced until typical RV pressure wave deflections were observed. Pressure data was collected for >1 min. Blood pressure recordings were only taken while heart rates were >300 bpm to minimize the isoflurane-induced cardio-depressant effects. Following mid-line thoracotomy, rats were euthanized by exsanguination and visual confirmation of catheter placement in either right ventricle or PA was examined.

Pulmonary Artery Histopathology

Standard histopathology techniques (i.e. hematoxylin/eosin staining) were performed on fixed paraffin embedded lung sections (4 μ m) as previously described [19]. Digital images of muscularized resistance and intermediate caliber pulmonary arteries were acquired and defined by internal diameters of < 75 μ m and 75–150 μ m, respectively. Measurements included outer medial and luminal circumference using Image J software (NIH, Bethesda, MD). Vessel diameters were calculated from medial circumference and vessel wall area was calculated by subtracting luminal circumference from outer medial circumference.

Quantitative Real-Time PCR Analysis

Quantitative measurement for atrogin-1 and MuRF-1 mRNA was assessed by real-time qPCR. Briefly, PAs were dissected from the heart-lung bloc in ice-cold saline and snap frozen immediately following RVSP measurement. Total RNA was then isolated from PAs using acquisition using RNeasy Fibrous Tissue Mini Kit (Qiagen), and quantified as described previously [19]. Multiplex real-time PCR amplification of cDNA utilizing inventoried and made to order primer/FAM dye/MGB probe sets (TaqMan, Applied Biosystems) for atrogin-1 (Rn01504258_m1) and MuRF-1 (Rn00590197_m1), relative to the housekeeping gene, β -actin (primer limited VIC dye/MGB probe-4352340E), was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative expression ($\Delta\Delta C_T$) was calculated by subtracting the C_T of the endogenous β -actin control gene from the C_T value of the gene of interest where the normalized gene expression method ($2^{-\Delta\Delta C_T}$) was used for relative quantification of gene expression [21].

Statistics

All data were tested for assumptions of normality. A one-way analysis of variance was used to compare groups for most assays, with Newman-Keuls post-hoc comparison tests (GraphPad Prism, v 5.02). For body weight trends a 2-way ANOVA was used for time and treatment factors. Probability values less than 0.05 were considered significant.

Results

MCT and Dexamethasone Induced Significant Reductions in Total Body Weight

As expected, MCT led to a significant reduction in growth, most notably in the first 3 weeks post-injection (Figure 1B). From day 21–42, however, the body weights in MCT-treated rats paralleled saline-treated control rats. Consistent with this finding was an absence of mortality over the 42-day observation period, as the relatively low MCT dose regimen (50 mg/kg) in the somewhat older animals was milder than is often reported for 60 mg/kg. Dexamethasone, administered intraperitoneally from day 28–42, also led to predictable dose-dependent reductions in body weight (Figure 1B). Collectively, all animals (saline controls and MCT-treated) injected with the high dose of dexamethasone exhibited a weight reduction of >30 g over the 2 week treatment period, compared to saline-injected rats that

gained >30g over that time frame. Final body weights showed a significant difference from control for the MCT + Dexamethasone groups (both doses; Figure 1C).

Dexamethasone Partially Reverses MCT-Induced Pulmonary Hypertension

As the primary index of pulmonary hypertension with respect to MCT and indicator of efficacy for dexamethasone within this cohort, we measured RV pressure in anesthetized rats. Anesthesia was maintained at a light level, with heart rate >300 bpm, to minimize cardiovascular depressive effects of the isoflurane. As previously observed [19], rats treated with 50 mg/kg MCT i.p. demonstrated a significant increase in RV systolic pressure (mean $\pm$ SD = 41.7 ± 3.1 mmHg; Figure 2A) compared to saline-treated controls (22.8 ± 2.1 mmHg) and dexamethasone controls (22.5 ± 2.4 mmHg; Figure 2A). At the low dose, dexamethasone failed to significantly resolve the RVSP increase (41.3 ± 9.4 mmHg), but the high dose significantly reduced pressure (32.8 ± 5.2 mmHg). We also examined the right ventricular hypertrophic response to MCT and, as expected, found significant increases in the ratio of RV weight to LVS weight (0.54 ± 0.06 ; Figure 2B) compared to saline-treated controls (0.33 ± 0.03) and dexamethasone controls (0.31 ± 0.02). Furthermore, high dose dexamethasone had a significant effect of partially reversing RV/LVS (Figure 2B) without decreasing left ventricular weights (Figure 2D) in MCT injected rats.

In addition to RV to LVS ratios, all MCT-treated rats exhibit a significant increase in total (RV+LVS) heart weights, normalized to their body weight prior to dexamethasone treatment (Figure 2C). This body weight (at day 28 post MCT injection) was used because of the dramatic global weight loss observed with dexamethasone treatment. Interestingly, dexamethasone in non-pulmonary hypertensive, saline vehicle injected, rats reduced normalized heart weights to a small degree compared control rats that was mostly attributable to LV weight differences (Figure 2D).

MCT-Induced Vascular Hypertrophy Reduced by Dexamethasone

We assessed pulmonary artery wall thickness by measuring the cross-sectional area from resistance (< 75 μ m) and intermediate caliber (75–150 μ m) arteries from the experimental cohorts. We reliably saw that MCT induced a thickening in vessels of all sizes, consistent with numerous previous reports (Figure 3). In the high-dose dexamethasone treated animals, thickening of the small and medium-sized vessels was partially and completely reversed, respectively.

Dexamethasone Restores Cardiac, but not Vascular Atrogin-1 Transcripts

To assess whether dexamethasone improved a key element linked to muscle atrophy pathways to baseline levels, we quantified the message for atrogin-1 in both pulmonary arteries and right ventricles. As previously observed [19], MCT induced a significant decrease in pulmonary artery atrogin-1, to 65% of control levels ($p < 0.001$; Figure 4A). Dexamethasone treatment did not alter pulmonary artery atrogin-1 mRNA levels in control rats and failed to increase atrogin-1 significantly at either dosage in MCT-treated rats. In right ventricles, MCT also induced a decrease in atrogin-1 mRNA, to 75% of control values (Figure 4B). In contrast to pulmonary artery atrogin expression, dexamethasone elevated right ventricular atrogin-1 levels in MCT-treated rats compared to those receiving MCT and saline injections ($p < 0.05$).

Myostatin is Differentially Expressed in Ventricles and Pulmonary Artery

Because glucocorticoids are potent inducers of skeletal muscle atrophy [6] and the negative regulator of muscle mass [15,16], myostatin, we first examined myostatin mRNA expression in pulmonary artery and cardiac tissues. Transcript levels of myostatin in LV and RV control

samples were comparable to each other, while the levels in the PA samples were less than 5% of the cardiac levels (Figure 5A), suggesting by large C_t values (37.24 ± 0.883) that this regulator of striated muscle mass may not be relevant in smooth muscle. For this reason and because atrogin-1 was significantly up-regulated in the right ventricle but not in the pulmonary arteries, we then compared changes in cardiac myostatin within the context of our study design and found that in control rats, dexamethasone induced a substantial increase in myostatin levels in the right ventricle (Figure 5B). In MCT-pulmonary hypertensive rats, myostatin levels were unchanged in right ventricles compared to saline controls. Surprisingly, dexamethasone failed to augment myostatin mRNA expression in MCT injected rats and were significantly decreased compared to controls.

Discussion

In the present study, we found that dexamethasone could partially reverse hemodynamic and vascular morphological changes induced in the MCT model of pulmonary hypertension. While we hypothesized a transcriptional role for atrogin-1, which is clearly involved in glucocorticoid-induced skeletal muscle atrophy, the crucial intermediate myostatin that links glucocorticoid receptor activity to FoxO-mediated pathways was not highly expressed in vascular smooth muscle. Although not intensively studied, the anti-proliferative effects of dexamethasone were more likely to play in the resolution of vascular hypertrophy.

Consistent with previous work, we found pulmonary artery atrogin-1 to be downregulated in the MCT model after 42 days. This attenuated transcriptional pattern between day 7 and 14 after MCT injection, paralleled the time course for increased RV pressure in which atrogin-1 expression as well as a normotensive phenotype was restored with the administration of resveratrol in an identical study design [19]. Furthermore, we report for the first time that cardiac atrogin-1 is also downregulated in the hypertrophied right ventricles of MCT-pulmonary hypertensive rats. Interestingly, dexamethasone had no significant effect on vascular atrogin-1, but did normalize atrogin-1 levels in the right ventricle. Compared to mRNA expression of myostatin in the right ventricle, vascular expression of myostatin was negligible, potentially explaining the inability of dexamethasone to induce pulmonary artery atrogin-1 expression. This inability of dexamethasone to increase atrogin-1 expression is consistent with the findings by Hwee et al. [22], where MuRF-1 null mice exhibited an increase in proteasomal activity in response to dexamethasone. We did, however, observe a strong induction of right ventricular myostatin transcript levels compared to saline injected rats, consistent with atrophy and concomitant increases in skeletal myostatin expression following administration of dexamethasone [23]. Surprisingly, myostatin transcript levels in cardiac tissues of MCT-treated rats were not significantly increased by dexamethasone and it is unclear why there would be a normalization of atrogin-1. It is possible that higher doses of dexamethasone or a more continuous administration thereof, may be required to induce these effects; however, with close to a 10% loss in total body mass over the two week regimen, any therapeutic effects of higher dose in terms of therapeutic resolution are likely to be complicated by anorectic outcomes.

Just recently dexamethasone has been shown to prevent or lessen the degree of MCT-induced PAH presumably by modulating immune and secondary inflammatory responses. In a similar study design to ours, Price LC et al. [24] demonstrated that dexamethasone reversed MCT-induced PAH which was associated with a reduction in IL-6 overexpression and inflammatory cell infiltration common to this model of PAH. They also found that dexamethasone also restored whole lung BMPR2 expression and inhibited rat pulmonary artery smooth muscle cell proliferation *in vitro*. In addition to these findings, Wang, W et al. [25] examined the effects of dexamethasone in preventing MCT-induced PAH associated with differential T-helper 1 and 2 cell numbers as well as differential perivascular CD4+ and

CD8⁺ T cells. In addition, lung fractalkine mRNA was elevated, but CX3CR1 mRNA expression was downregulated. The findings of these studies clearly suggest that dexamethasone plays a complex role immune-modulation which plays a role in attenuating MCT-induced PAH, but a specific mechanism has yet to be identified. Therefore, we cannot rule out the therapeutic effects of dexamethasone related to its anti-inflammatory properties in the current study. However, the focus of this study was aimed at identifying myostatin as an effector between dexamethasone and upregulating atrophy pathways in pulmonary artery smooth muscle and right ventricular cardiac muscle. It is also possible that certain elements of atrophic signaling may have been activated by dexamethasone and we can only speculate to what degree or what other proteasomal pathways may be upregulated without further biochemical measures.

Cardiac atrogen-1, and likely related components of the ubiquitin proteasome system, may have an important role in modulating the severity of cardiomyopathies. Li et al. [8] found that upregulating atrogen-1 could reduce cardiomyocyte growth in culture and mice lacking atrogen-1 demonstrated enhanced cardiac growth following a 3 wk exercise regimen. Cardiac hypertrophy induced in an obesity model similarly led to reductions in myocardial FoxO activity and atrogen-1 levels [26]. Therapeutic upregulation of specific components of the ubiquitin proteasome pathway, such as atrogen-1, may attenuate growth pathways and delay the onset of pathology. However, this strategy may be counterproductive in more advanced heart failure scenarios as a thinner, albeit more efficient ventricular wall may not be able to maintain optimal cardiac output. Recently, it was shown that controlled upregulation of a related E3 ubiquitin ligase, MuRF-1, led to exacerbated ventricular wall thinning and diminished cardiac function in a model of pressure-induced cardiac failure [27]. In the present study, the relatively mild pulmonary hypertension related cardiac manifestations may be treatable by increasing the atrophic gene expression, but more advanced right heart failure would likely be exacerbated. Dexamethasone did exhibit modest improvements in RVSP and pulmonary artery remodeling as it has well established anti-proliferative effects in vascular and airway smooth muscle [28, 29]. Furthermore, it is likely that modest improvements in right ventricular atrogen-1 expression by dexamethasone arises from signal transduction independent from myostatin considering that myostatin expression was not significantly elevated in MCT injection rats regardless of dexamethasone treatment.

A slight reduction in right ventricular hypertrophy was observed with dexamethasone. The more substantial dexamethasone-induced improvement in RV/LVS reported by Hilliker and Roth [30] may relate to differences in the treatment regimen, which in their seminal study began 3 days prior to injection of the MCT pyrrole, whereas in the present study it was initiated 28 days after MCT injection. We had hypothesized that the well-documented effect of dexamethasone on myostatin would lead to atrophic changes in both the vascular and cardiac tissues. Part of this rationale is based on *in vitro* studies that demonstrate that dexamethasone induces myostatin through enhanced glucocorticoid receptor transcriptional activity [31]. In Sprague-Dawley rats with doses comparable to the concentrations utilized here, dexamethasone led to significant increases in gastrocnemius myostatin mRNA after only 5 days [23]. Further *in vivo* evidence documented in mice with the myostatin gene disrupted, dexamethasone treatment failed to reduce the mass of gastrocnemius, soleus or tibialis anterior muscles, while wild-type mice exhibited a 10–15% reduction with the same regimen [16]. In addition, skeletal muscle atrogen-1 and MuRF-1 levels were also relatively unresponsive in the myostatin-null mice treated with dexamethasone, while these enzymes were upregulated 50–70% in wild-type mice. Interestingly, myostatin overexpression has been shown to dephosphorylate Akt, thus activating the transcription factor FoxO [32] and increasing protein ubiquitination which in separate studies glucocorticoid-induced skeletal muscle atrophy has been shown to increase FoxO3a mediated atrogen-1 mRNA expression [33].

Perhaps the most intriguing, yet unexplained finding from the present study was that myostatin mRNA was only elevated the RV in saline controls treated with dexamethasone and not in MCT-treated hearts. Notably, this was the only group that exhibited even a slight trend towards a reduction in heart weight. Dexamethasone inexplicably did not have a significant effect on RV myostatin mRNA in MCT-treated rats. In fact, there appeared to be a dose-dependent reduction in myostatin mRNA in both the RV and LVS samples. This may explain, in part, the lack of resolution in the cardiac manifestations of pulmonary hypertension seen in the present study.

In summary, while our earlier studies demonstrate an attenuation of vascular atrogin-1 in the MCT model of pulmonary hypertension and a therapeutic reversal of hemodynamic indices along with associative enhancements in vascular atrogin-1 expression in resveratrol-treated rats, dexamethasone improved hemodynamic indices with an improvement in atrogin-1 expression limited to the myocardium. In addition, dexamethasone induction of myostatin does not appear to be relevant in the vasculature nor in hypertrophied RVs, suggesting that myostatin/atrogin-1 independent mechanisms are responsible for glucocorticoid efficacy in this setting.

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Figure 1.

MCT and dexamethasone alter total body weights in treated animals. A. Body weights for all saline- and MCT treated rats over the 42-day timecourse. B. Net change in body weights from day 28 to 42 in all saline- and dexamethasone-treated rats (regardless of MCT treatment). Concentrations of dexamethasone used were 17.5 $\mu\text{g/kg/day}$ (low) or 50 $\mu\text{g/kg/day}$ (high). C. Final body weights between groups for saline ($n = 4$), saline dexamethasone high ($n = 4$); MCT ($n = 4$), MCT dexamethasone low ($n = 6$) and MCT dexamethasone high ($n = 6$). Mean $\pm$ SEM are shown. Asterisks denote significant difference ($P < 0.05$) by two-way ANOVA (A) or one-way ANOVA with Newman-Keuls *post hoc* test.

Figure 2.

Pulmonary hypertension outcomes in MCT rats treated with dexamethasone. Changes in right ventricular systolic pressure (A) and RV/LVS ratio (B) show significant increases by MCT with slight, but significant reductions at the high dexamethasone dose (50 μ g/kg/day). Total heart weight was significantly elevated by MCT (C), but this change was largely right-sided, and left ventricular weights (D) did not significantly change. Asterisks indicate significant ($P < 0.05$, by ANOVA) difference from control; pound symbols (#) indicate significant difference from MCT alone.

Figure 3.

H&E stained micrographs of pulmonary arteries in MCT rats with and without dexamethasone treatment. Significant vascular hypertrophy was induced by MCT at 42 days in both small (<75 μm diameter) and intermediate (75–150 μm diameter) caliber arteries. Dexamethasone at 50 μg/kg/day from days 28–42 attenuated this remodeling. Data were obtained from 8–10 vessels per size group for all subjects. Asterisks indicate significant ($P < 0.05$, by ANOVA) difference from saline control.

Figure 4.

Quantitative PCR for atrogin-1 levels in the pulmonary artery (A) and right ventricle (B). MCT treatment induced a clear reduction in atrogin-1 expression in the pulmonary artery and RV. Dexamethasone had no impact on pulmonary artery reductions in atrogin-1 mRNA but returned those in the RV to control levels. Asterisks denote significant difference ($P < 0.05$) from saline controls by one-way ANOVA with Newman-Keuls post hoc test.

Figure 5.

Relative myostatin expression in control cardiac tissue (both LV and RV) compared to PA samples (A). Right ventricular myostatin mRNA expression across treatment groups (B). Asterisks denote significant difference ($P < 0.05$) from all other groups by one-way ANOVA with Newman-Keuls *post hoc* test. Dagger (†) indicates a significant downward linear trend in a subgroup analysis of MCT-treated only groups.