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Resveratrol reverses monocrotaline-induced pulmonary vascular and cardiac dysfunction: A potential role for atrogin-1 in smooth muscle

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ABSTRACT

Arterial remodeling contributes to elevated pulmonary artery (PA) pressures and right ventricular hypertrophy seen in pulmonary hypertension (PH). Resveratrol, a sirtuin-1 (SIRT1) pathway activator, can prevent the development of PH in a commonly used animal model, but it is unclear whether it can reverse established PH pathophysiology. Furthermore, atrophic ubiquitin ligases, such as atrogin-1 and MuRF-1, are known to be induced by SIRT1 activators but have not been characterized in hypertrophic vascular disease. Therefore, we hypothesized that monocrotaline (MCT)-induced PH would attenuate atrophy pathways in the PA while, conversely, SIRT1 activation (resveratrol) would reverse indices of PH and restore atrophic gene expression. Thus, we injected Sprague–Dawley rats with MCT (50 mg/kg i.p.) or saline at Day 0, and then treated with oral resveratrol or sildenafil from days 28–42 post-MCT injection. Oral resveratrol attenuated established MCT-induced PH indices, including right ventricular systolic pressure, right ventricular hypertrophy, and medial thickening of intrapulmonary arteries. Resveratrol also normalized PA atrogin-1 mRNA expression, which was significantly reduced by MCT. In cultured human PA smooth muscle cells (hPASMC), resveratrol significantly inhibited PDGF-stimulated proliferation and cellular hypertrophy, which was also associated with improvements in atrogin-1 levels. In addition, SIRT1 inhibition augmented hPASMC proliferation, as assessed by DNA mass, and suppressed atrogin mRNA expression. These findings demonstrate an inverse relationship between indices of PH and PA atrogin expression that is SIRT1 dependent and may reflect a novel role for SIRT1 in PASMCs opposing cellular hypertrophy and proliferation.

1. Introduction

Abnormal hypertrophy and hyperplasia of smooth muscle cells can lead to anatomical narrowing of lumens, both in the vasculature and airways. Numerous pathologies manifest smooth muscle cell hypertrophy and proliferation, including idiopathic and secondary forms of pulmonary arterial hypertension (PAH), atherosclerosis, and even in reactive airway diseases such as chronic asthma (Newby and Zaltsman, 2000; Siddiqui and Martin, 2008). Luminal narrowing results in increased resistance and, in the instance of PAH, an increase in right ventricular afterload and eventual maladaptive hypertrophy and ultimately right heart failure ensues (Humbert et al., 2004). There is general consensus that arterial remodeling is among the principal causes of increased resistance that, along with adventitial fibroblasts and endothelial cells transitioning to a mesenchymal phenotype (Stenmark et al., 2002) and increased endothelial proliferation (Budhiraja et al., 2004), contribute to a flow-restricted state in the diseased lung. While much is known regarding PASMC hypertrophic and proliferative pathways in PAH syndromes, the recently characterized molecular atrophy pathways, largely driven by the ubiquitin–proteasome system, have yet to be characterized in syndromes of abnormal vascular growth.

Atrophy-mediating E3 ubiquitin ligases, atrogin-1 and MuRF-1, play a significant role in regulating cardiac and skeletal muscle growth by selective proteasomal degradation of key proteins such as calcineurin (CnA), MyoD, and eIF3 (Lagirand-Cantaloube et al., 2009; Li et al., 2004; Csibi et al., 2009). Expression and activity of these E3 ubiquitin ligases oppose cellular hypertrophy and are enhanced during calorie restriction and muscle wasting disorders (Hepple et al., 2008; McFarlane et al., 2006). Upstream regulators of atrogin-1 expression, such as F-box (Fox)-O transcription factors, induce atrophy and apoptosis in a number of muscle types (McLoughlin et al., 2009), but their role in smooth muscle atrophy is undocumented.

Interestingly, the polyphenolic compound, resveratrol, enhances transcriptional activity of FOXO1 through a sirtuin-1 dependent deacetylation mechanism in cardiomyocytes (Ni et al., 2006) and inhibits vascular smooth muscle proliferation (Poussier et al., 2005). Furthermore, atrogin-1 has been shown to attenuate pathological hypertrophy in cardiomyocytes driven by agonist-induced calcineurin (CnA)-dependent activation of NFATc4, whereas silencing of atrogin-1 with siRNA enhanced CnA/NFAT signaling and hypertrophy in cardiomyocytes (Li et al., 2004). Currently, there is a lack of information

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regarding the role of atrogin-1 or other E3 ubiquitin ligases in pathological vascular smooth muscle hypertrophy.

Resveratrol has recently been shown to prevent monocrotaline (MCT)-induced pulmonary hypertension, in part, by having anti-proliferative, anti-inflammatory and anti-oxidant effects in pulmonary vascular smooth muscle cells (Csiszar et al., 2009). Endothelial function was also improved in small intrapulmonary arteries where endothelial nitric oxide synthase (eNOS) expression was similarly enhanced by chronic resveratrol treatment. Although this study examined the preventative effects of resveratrol on MCT-induced vascular hypertrophy, information regarding the effect of resveratrol *reversing* established PAH is lacking (Chicoine et al., 2009). Furthermore, no information exists regarding pulmonary artery (PA) transcriptional regulation of the ubiquitin ligases, atrogin-1 and MuRF-1, during the development and persistence of experimentally induced pulmonary hypertension. Therefore, we investigated 1) whether chronic resveratrol administration could reverse established MCT-induced increases in right ventricular pressure and remodeling; 2) the temporal expression of ubiquitin ligases in pulmonary arteries from MCT-pulmonary hypertensive rats; 3) the effect of resveratrol on arterial atrogin-1 expression and vascular function in the MCT model; and 4) the effect of resveratrol to stimulate atrogin expression in vascular smooth muscle cells.

2. Materials and methods

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Lovelace Respiratory Research Institute and the University of New Mexico. Rats were obtained from a commercial vendor (Charles River Laboratories) and were allowed to acclimate for 2 wks prior to experimentation, with food and water available *ad libitum*.

2.1. Experimental design

In order to study a relatively stable and established remodeled vasculature with minimal contribution from inflammatory pathways, which peak around 14–21 days post-injection (Wilson et al., 1989), rats were treated with MCT 28 days prior to initiation of therapeutics. Pulmonary hypertension was generated in adult, 8–10 wk old male Sprague–Dawley rats by a single injection of MCT (50 mg kg^{-1} i.p.; Sigma-Aldrich) or an equivalent weight-based volume of sterile saline. Rats weighing >300 g in this age range were utilized for this study due to a decrease in mortality throughout the study. Additionally, this moderate dose of MCT we saw minimal mortality (<2%) and only modest morbidity over the 42-day regimen. For the resveratrol efficacy study, rats were randomly assigned and administered resveratrol (3 mg kg^{-1}; Sigma-Aldrich) or sildenafil (as a clinical reference standard; 175 μg kg^{-1}; AK Scientific) via drinking water beginning at 28 days post MCT injection and continuing for an additional 2 wks. Tap water served as vehicle group for an equivalent duration (28–42 d). Drinking water concentrations of resveratrol and sildenafil were calculated on an observed H_2O consumption of 50 ml/day. As water uptake increases with weight gain, the concentrations were not adjusted and remained constant throughout the course of the study. For time course data, rats ($n = 8$–12/group) were euthanized at 0, 7, 14, 28, and 42 days post-injection.

2.2. Hemodynamic and Right Ventricular Hypertrophy Measurements

Rats were anesthetized with isoflurane 1–2.5% at 6 mL/min and the right external jugular vein was exposed by blunt dissection. Right ventricular systolic pressure (RVSP) measurements were made via a fluid-filled pressure transducer (Becton Dickinson, DTXplus) were a heparinized (0.01%), saline filled Micro-Renathane catheter (.050 OD X .040 ID) was placed in the right jugular vein. Catheter advancement proceeded until strong positive–negative deflections were observed, indicating catheter placement in the right ventricle, and secured with 4–0 silk suture. Stable RV pressure tracings were collected for 1 min. Blood pressure recordings were obtained at consistent heart rates to minimize variation from isoflurane-induced cardio-depressant effects. Following mid-line thoracotomy, rats were euthanized by exsanguination and the catheter placement in either right ventricle or PA was confirmed. All instrumentation was calibrated prior to experimentation and data (HR, systolic and diastolic RV pressures and $\pm dP/dT$) were collected with a digital acquisition system (Gould Ponemah 7700) connected to a personal computer.

Right ventricular hypertrophy was assessed as the ratio of the right ventricular free wall to the left ventricle plus septum weight (RV/LV + S). Additionally, under light anesthesia, echocardiographic measures of right ventricular performance were obtained one day prior to catheterization using an Acuson Sequoia 512 Ultrasound (Torres et al., 2010). M-Mode measures were used to capture systolic and diastolic dimensions and fractional shortening was calculated as [(end-diastolic − end-systolic)/end-diastolic] × 100 (%).

2.3. Pulmonary artery remodeling

Standard histopathology techniques (i.e. hematoxylin/eosin staining) were performed on fixed, paraffin-embedded lung sections (4 μm) to determine a qualitative measure of vascular remodeling that occurs in the MCT model of PAH. Digital images of pulmonary arteries in three size ranges (<75 μm, 75–150 μm and >150 μm; n = 8 vessels per size range per rat) were acquired from H/E stained 4 μm sections. Measurements included outer medial and luminal circumference using Image J software (NIH, Bethesda, MD). Vessel diameter was calculated from medial circumference and vessel wall area was calculated by subtracting luminal circumference from outer medial circumference. Furthermore, oblique vessel sections were excluded from analysis.

2.4. Vascular function studies

Left and right pulmonary arteries from saline or MCT-injected rats receiving oral (28–42 d) resveratrol, sildenafil or tap water were carefully dissected in ice-cold HEPES buffered saline solution (HBSS) containing 150 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, titrated to pH 7.4 with NaOH and mounted on a wire myograph (DMT). Once mounted, PA rings were bathed in a physiological salt solution (PSS) containing (in mmol) 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, 0.03 EDTA, 5.5 glucose and aerated with a 95% O_2: 5% CO_2 gas mixture at 37 °C. Isometric tension was applied (2 mN) and allowed to equilibrate for 30 min prior pre-contraction with 60 mM KPSS solution (equimolar NaCl substitution). Acetylcholine (ACh) concentration response curves were performed after KCl-induced contraction stabilized (20 min) to assess endothelial function and expressed as % reversal of KCl-induced contraction.

2.5. Quantitative Real-Time PCR Analysis

PAs were rapidly 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 RNeasy Fibrous Tissue Mini Kit (Qiagen) and quantified by real-time RT-PCR. cDNA was generated using a high capacity reverse transcriptase (ABI) step from 100 ng of RNA using a Peltier thermal cycler (PTC-200, MJ Research). Absolute quantification for dynamic range (serial dilutions of RNA) was performed independently for each primer/dye/probe set to determine amplification efficiency prior to relative PCR analysis. Multiplex real-time PCR amplification of inventoried and custom primer/FAM dye/MGB probe sets (TaqMan, Applied Biosystems) for atrogin-1 (Rn01504258_m1 and Hs01041408_m1), MuRF-1