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Pulmonary Allergic Reactions Impair Systemic Vascular Relaxation In Ragweed Sensitive Mice

Surovi Hazarika, Michael R. Van Scott, Robert M. Lust, and Christopher J. Wingard

Department of Physiology, Brody School of Medicine at East Carolina University, Greenville, NC 27834.

Abstract

Asthma is often associated with cardiovascular complications, and recent observations in animal models indicate that induction of pulmonary allergic inflammation increases susceptibility of the myocardium to ischemia and reperfusion injury. In this study, we used a murine model of allergen sensitization in which aspiration of allergen induces pulmonary and systemic inflammation, to test the hypothesis that pulmonary exposure to allergen alters vascular relaxation responses. BALB/C mice were sensitized by intra-peritoneal injection of ragweed and challenged by intratracheal instillation of allergen. Airway hyperreactivity and pulmonary inflammation were confirmed, and endothelium-dependent and - independent reactivity of thoracic aorta rings were evaluated. Ragweed-sensitization and challenge induced airway hyperreactivity to methacholine and pulmonary inflammation, but did not affect constrictor responses of the aortic rings to phenylephrine and K depolarization. In contrast, maximal relaxation of aortic rings to acetylcholine and sodium nitroprusside decreased from $87.6 \pm 3.9\%$ and $97.7 \pm 1.2\%$ to $32 \pm 4\%$ and $51 \pm 6\%$, respectively ($p < 0.05$). The sensitivity to acetylcholine was likewise reduced ($EC_{50} = 0.26 \pm 0.05 \mu M$ vs. $1.09 \pm 0.16 \mu M$, $p < 0.001$). The results demonstrate that induction of allergic pulmonary inflammation in mice depresses endothelium-dependent and -independent vascular relaxation, which can contribute to cardiovascular complications associated with allergic inflammation.

Keywords

Inflammation; vascular function; endothelial response

1. Introduction

Both clinical and basic studies have established that inflammation plays a vital role in the initiation and progression of cardiac and vascular complications (Gonzalez, M.A. et al, 2003; Hansson, G.K., 2005; Pearson, T.A. et al, 2003; Schram, M.T. et al, 2005; Willerson, J.T. et al, 2004; Williams, M. et al, 2002). Considering the role of inflammation in the development of these complications, it is possible that underlying diseases associated with systemic inflammation predispose one to cardiovascular complications. Epidemiological studies show a significant association of asthma with cardiac and vascular complications (Cakir, B. et al,

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Corresponding author: Dr. Christopher J. Wingard, Ph.D, Department of Physiology, Brody School of Medicine at East Carolina University, 600 Moye Blvd, Room 6N-98, Greenville, NC 27834, Ph: 252-744-2804, Fax: 252-744-3460, wingardc@ecu.edu.

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2003; Drislane, F.W. et al, 1987; Jazayeri, M.R. et al, 1983; Rhatigan, R.M. et al, 1970; Rosero, S.Z. et al, 1999; Toren, K. et al, 1996; van, d.B.-K. et al, 1986). Most of these complications have been attributed to hypoxia, pulmonary hypertension, and medications associated with bronchoconstriction; but recent observations indicate that systemic inflammation associated with asthma (Jousilahti, P. et al, 2002; Kips, J.C., 2001; Romagnani, S., 2002; Togias, A., 2004) may adversely affect cardiovascular function (Dogra, S. et al, 2007; Schanen, J.G. et al, 2005). In our previous studies in both rabbit and mouse models of allergic asthma, aerosol allergen challenge enhanced infarction associated with myocardial ischemia and reperfusion, and increased neutrophil infiltration into the area at risk (Hazarika, S. et al, 2007). In addition airborne allergen activated circulating neutrophils and coronary endothelium, which may explain in part the increased myocardial damage (Hazarika, S. et al, 2004; Hazarika, S. et al, 2007).

Altered systemic vascular reactivity may be a contributor to an increased susceptibility of the myocardium to ischemia-reperfusion injury in models of allergic asthma. Yildiz et al. have shown that flow mediated dilatation of the brachial artery [Endothelial Dependent Dilatation, (EDD)] was impaired in patients with asthma, and the depressed EDD correlated with the severity of asthma (Yildiz, P. et al, 2004). However, the systemic vascular responses have not been rigorously studied in allergic asthma, but work with ambient particulate matter indicates that inflammation originating in the lungs is associated with altered reactivity of systemic vessels (Bagate, K. et al, 2004; Nurkiewicz, T.R. et al, 2004). Under normal conditions, a balanced release of biological mediators regulates vascular homeostasis. If this balance is disrupted, endothelial cell dysfunction ensues and predisposes the vessel wall to vasoconstrictors, leukocyte adherence, platelet activation, thrombosis and vascular inflammation. Several cytokines are elevated in asthma including IL-4, IL-5, IL-6 and TNF- α , which cause leukocyte activation resulting in a feed-forward cycle of further release of cytokines and chemokines. This feed-forward effect could ultimately affect the vascular endothelium or smooth muscle, altering systemic vascular responsiveness.

Using a murine model of allergen sensitization in which aspiration of allergen induces pulmonary and systemic inflammation, we tested the hypothesis pulmonary allergen exposure leads to altered vascular relaxation responses. To test this hypothesis, we investigated the relaxation response of isolated endothelium-intact and -denuded thoracic aorta segments to phenylephrine, acetylcholine, sodium nitroprusside, forskolin and adenosine.

2. Materials and methods

2.1 Animals

Male, 5–6 week old BALB/C mice were purchased from Harlan (Indianapolis, IN) housed in micro-isolation on a 12/12 light dark cycle at 22 °C and provided food and water *ad libitum*. All animal procedures conformed to the standards in the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the East Carolina University Institutional Committee on the Care and Use of Laboratory Animals.

2.2 Allergic sensitization and challenge of BALB/C mice

Mice were sensitized to ragweed allergen as described previously (Justice, J.P. et al, 2001). Briefly, the animals were divided into two treatment groups, and subsequently sensitized and challenged with ragweed (RW-challenged group) or vehicle (control group). The RW-challenged group received intraperitoneal (i.p) injections of 83 μ g of ragweed adsorbed to Alum on days 0, 4 and 11, followed by intratracheal instillation of 8 μ g of ragweed, in 100 μ l sterile normal saline, on days 14, 15 and 16. The control group received intraperitoneal

injections of normal saline with alum and intratracheal instillation of normal saline at the same time points. On day 17, aortas were harvested from both groups.

2.3 Pulmonary function testing

In a subset of the RW-challenged mice, functional assessment of airway hyper-responsiveness was determined by pulmonary function test 24 hours after the last challenge. Airway reactivity was assessed using a whole body plethysmograph system (Buxco Electronics, Sharon, CT). Conscious animals were placed in the plethysmograph chamber, and activity was allowed to stabilize for 10 minutes. Enhanced pause (P_{enh}) was measured as an indirect assessment of airway resistance. Following equilibration, baseline P_{enh} was recorded for 5 minutes. Animals were then exposed to nebulized saline for 4 minutes, and P_{enh} was recorded for 5 minutes to establish a baseline for vehicle. Methacholine (MCh) was dissolved in normal saline to concentrations of 0.75–48 mg/ml. Animals were exposed to increasing doses of nebulized MCh for 4 minutes. P_{enh} was monitored continuously for 5 min following each dose of MCh. Sensitization was confirmed by a left shift in the dose response curve (airway resistance changes to methacholine) and further verified by increased number of eosinophils in the bronchoalveolar lavage (BAL) fluid. Animals used for the pulmonary function test were not used for the aortic ring studies, because of the possibility that methacholine nebulization might have effects on the systemic vasculature.

2.4 Brochoalveolar lavage (BAL) and cell counts

BAL fluid was collected 24 hours after the last intratracheal challenge. Animals were euthanized with sodium pentobarbital (120 mg/kg i.p.) and lungs were lavaged with 0.3 ml of phosphate buffered saline (PBS). Total cell counts were determined using a Coulter counter (Beckman Coulter Instruments; Miami, FL). Differential cell counts were performed on cytocentrifuge preparations stained with Diff-Quick (VWR Scientific; S. Plainfield, NJ). 200 cells were counted per slide, and 2 slides were evaluated per animal.

2.5 Isolated aortic rings

Twenty-four hours after the last intratracheal challenge, mice were sacrificed and thoracic aortas were isolated. Blood, surrounding connective tissue and fat were removed and 5 mm long rings were prepared. Rings were placed in cold physiological saline solution (PSS). The PSS had the following composition: NaCl 140.0 mM, KCl 5.0 mM, $CaCl_2$ 1.6 mM, $MgSO_4$ 1.2mM, 3-[N-morpholino]-propane sulfonic acid (MOPS) 1.2 mM, D-glucose 5.6mM, pH adjusted to 7.4 at 37 °C.

All tissue bath experiments were conducted in either endothelial intact or denuded rings. The endothelium was removed mechanically by insertion of a small stainless steel needle in to the lumen of the ring and lightly rolling the ring across a paper towel. The integrity of the endothelium was then tested pharmacological by application of 1 μ M acetylcholine to 1 M phenylephrine pre-stimulated ring following washout of a 109 mM K^+ -depolarization to test for tissue overall viability. Endothelial response was considered adequate when > 50% of the force generated by the phenylephrine stimulation was lost with acetylcholine administration. Endothelial denudation was verified by the lack of a relaxation response to acetylcholine (< 10% loss of force).

All ring force measurements were normalized to the rings cross-sectional area and reported as stress in mN/mm^2 . The area calculation was based on the formula of: Area = blot wet weight / length $\times$ 1.055 (density normalization). Stress values were further normalized to maximal responses of the agents of interest.

2.6 Measurement of isometric force generation

Endothelium-intact and -denuded isolated aortic rings were suspended between 2 mounting posts of a DMT 610 M 4 channel myograph system (DMT-USA International, Atlanta, GA) between an isometric force transducer and length positioning support post, and bathed in PSS warmed to 37 °C and gassed continuously with compressed air. A resting tension of 10 mN was imposed on all rings, and rings were allowed to equilibrate in PSS for 30 minutes prior to the start of pharmacological assessment. This tension was previously determined to reflect a passive force on the length-tension relationship for these rings that corresponded to a length for optimal force generation with K⁺-depolarization (data not shown).

2.7 Cumulative Dose Response Protocols

Following equilibration, rings were stimulated with 109 mM K⁺ PSS solution for 10 minutes to test viability. K⁺PSS solution was prepared by all equal molar substitution of Na⁺ for K⁺ to maintain osmotic conductions. Rings which did not achieve a steady-state stress generation greater than 1mN/mm² were not used for subsequent analysis. Rings were then rinsed with PSS until force returned to passive tension level. Cumulative dose response curves for the α -adrenergic agonist phenylephrine (PE, 0.01–30 μ M) were performed, and the tissues were washed for a minimum of 60 minutes before examining relaxation response of 1 μ M PE pre-constricted tissues to either acetylcholine (0.001–10 μ M) or adenosine (0.01–100 μ M) in intact aortas. In separate rings cumulative dose response profiles were constructed for forskolin (0.001–1 μ M) and sodium nitroprusside (SNP) (0.001–30 μ M) in denuded aortic rings.

2.8 Statistics

Data are expressed as mean $\pm$ SEM. Statistical significance was determined at P values of less than 0.05. Paired t-test was used to compare individual cell types before and after treatment. Non-linear regression analysis for individual concentration response curves was performed using a Hill algorithm in Sigma Plot (ver 10, Systat Software Inc, Richmond, CA) allowing for an individual geometric 'EC₅₀' value to be calculated. Mean data were analyzed by one-way ANOVA. When the ANOVA manifested a statistical difference, the Student-Newman-Keuls test was applied for comparison to between groups (Sigma Stat, Ver 3.1.1).

3. Results

3.1 Airway inflammation and hyperresponsiveness to methacholine in ragweed sensitized and challenged mice

Twenty-four hours after the third intratracheal administration of ragweed or normal saline, airway reactivity was assessed by whole body plethysmography. Baseline Penh and the response to nebulized saline was similar in both groups, but the RW-challenged group showed significantly higher Penh to all MCh concentrations compared to the Control group (Figure 1A). The RW-challenged group also exhibited higher eosinophil counts in the BAL fluid (Figure 1B), thereby confirming that ragweed sensitization and challenge induced allergic pulmonary inflammation in these animals.

3.2 Aortic vasomotor responses

Endothelial intact aortic rings from both groups exhibited a dose-dependent contractile response to the α -adrenergic agonist phenylephrine (PE). There was no difference in the sensitivity or magnitude of contractile response to PE between the control and the RW-challenged group (Figure 2). In addition, the stress responses of the endothelial intact and denuded aortic rings to 1 μ M PE were not significantly different for each other. The response to maximal K⁺ (KPSS) depolarization was not different, but the denuded rings exhibited higher stresses (Figure 3). These results indicate that ragweed sensitization and challenge has no direct

effect on the adrenergic or depolarized contractile ability of the thoracic aorta, The data also demonstrate the presence of an underlying endothelial-associated relaxation mechanism in aortic rings from allergen challenged mice.

Aortic rings from RW-challenged mice were pre-contracted with 1 μ M PE and then exposed to acetylcholine (ACH) to evaluate relaxation responses. Aortic rings that were submaximally stimulated with 1 μ M PE showed depressed relaxations and lower sensitivity to ACH (Figure 4). Additionally, the relative stress from aortas maximally stimulated with 30 μ M PE and then relaxed with ACH was higher in rings from RW-challenged (0.49 ± 0.06 in control vs. 0.68 ± 0.05 in RW-challenged; $n = 8-10$; $p < 0.01$), indicating that ragweed sensitization leads to an impaired endothelial-dependent ACH-induced dilation response.

To determine if attenuated relaxation to ACH could be explained solely by endothelial dysfunction, we examined the vascular smooth muscle responses to the nitric oxide donor sodium nitroprusside (SNP) and forskolin (FSK), activators of cGMP and cAMP pathways, respectively. Denuded aortic rings from RW-challenged mice showed reduced relaxation to SNP (Figure 5). Maximal relaxation of control vs. RW-challenged aortas expressed as relative stress from 30 μ M PE were 0.02 ± 0.01 vs. 0.12 ± 0.04 ; $n = 8-10$; $p < 0.05$). This observation was consistent with impaired endothelial-independent vascular smooth muscle relaxation mediated by cGMP. RW-challenge was also associated with reduced sensitivity to FSK. Denuded rings from control mice exhibited lower EC_{50} values to FSK than rings from RW-challenged animals (Figure 6, calculated EC_{50} 0.05 ± 0.01 vs. 0.10 ± 0.02 , respectively; $n = 8-10$, $p < 0.001$). However, no difference in the maximal relaxation responses were observed

Since adenosine can play an important role in the regulation of systemic and pulmonary vascular tone, aortic responses to cumulative doses of adenosine were evaluated. Adenosine in the range of (0.001–100 μ M) exhibited a biphasic response in the isolated endothelial intact thoracic aortas. A contractile response was observed up to 1 μ M and then relaxation was observed at higher doses. There was no significant difference in the magnitude of maximal contraction to 1 μ M adenosine between the groups. However, the relaxation response appeared earlier in aortas from control mice, resulting in a maximal relaxation of greater than 80% at 100 μ M adenosine. The concentration of adenosine necessary for a relaxation effect was higher in aortas from RW-challenged animals, but those aortas did achieve 75% relaxation at 100 μ M adenosine. Thus the sensitivity to adenosine was impaired in the aortas from RW-sensitized animals, as evidenced by a significantly reduced relaxation response to 10 μ M adenosine (% relaxation from maximal adenosine contraction in control vs. RW-challenged aortas were 50 ± 4 and 27 ± 7 respectively; $n = 8$, $p < 0.05$). These results indicate that ragweed sensitization and challenge altered the thoracic aorta vasodilator response involving the endothelium, as well as cGMP and cAMP pathways in the smooth muscle.

4. Discussion

In this study, a murine model of pulmonary allergic response was found to have altered thoracic aorta relaxation responses. The pulmonary allergic response was confirmed by increased airway reactivity to aerosolized methacholine and the presence of eosinophilic airway inflammation following ragweed sensitization and challenge. Ragweed sensitization and challenge did not alter the constrictor response of the thoracic aorta to α -adrenergic or depolarization, suggesting that sensitization and challenge with allergen did not affect the elements of excitation-contraction coupling. In contrast, maximal relaxation and sensitivity to both endothelial-dependent and-independent relaxation agents were reduced in aortas from animals sensitized and challenged with ragweed. Together, these findings indicate that airway allergen exposure leading to pulmonary inflammation attenuates systemic vascular relaxation responses and may contribute to increased risk for adverse cardiovascular events.

In contrast to the acute murine model used in this study, human asthma is a chronic, multifactorial condition involving genetic and environmental factors. The relevance of the vascular response observed in this acute study to the complex chronic human disease is as of yet not fully investigated. However, frequent cardiovascular complications are reported in patients with asthma. Since the medications used to treat asthma have effects on the cardiovascular system, most complications are attributed to the side effects of these drugs. Emerging evidence indicates that inflammation plays a vital role in the initiation and progression of cardiovascular diseases (Hansson, G.K., 2005; Willerson, J.T. et al, 2004; Zebrack, J.S. et al, 2002). Recent evidence also indicates the presence of systemic inflammation in asthma (Jousilahti, P. et al, 2002; Ohkawara, Y. et al, 1997; Togias, A., 2004). So, it is possible that systemic inflammation present in asthma affects the cardiovascular system by altering systemic parameters, including vascular reactivity. Inflammatory cells produce several pro-inflammatory cytokines, which in turn may have an effect on systemic vasculature. Although extensive evidence of altered smooth muscle reactivity exists in the pulmonary vasculature (Chetta, A. et al, 2007; Horvath, G. et al, 2007; Tazzeo, T. et al, 2003; Theodorou, A. et al, 1997; Wanner, A. et al, 2004; Wilson, J.W. et al, 2003; Witzentrath, M. et al, 2006), little is known about the impact on the systemic vasculature.

Information on systemic vascular response in asthma is limited to a single report from the clinical setting demonstrating that endothelial dependent brachial artery flow was impaired in asthmatic patients (Yildiz, P. et al, 2004) and further supported by epidemiological reports linking cardiovascular disease to asthma (Dogra, S. et al, 2007; Schanen, J.G. et al, 2005). While the conclusion drawn in that report would suggest that the systemic vascular responsiveness was altered because of asthma, additional studies are warranted to establish the relative importance of intrinsic changes in vascular reactivity compared to side effects of therapeutic agents.

The murine model of allergic asthma used in our study exhibits features characteristic of human asthma, including pulmonary inflammation with increased presence of eosinophils in the bronchoalveolar lavage, airway hyperresponsiveness in vivo, and enhanced sensitivity and responsiveness of tracheal smooth muscle to methacholine (Blyth, D.I. et al, 1996; Justice, J.P. et al, 2001). While there are publications documenting the effect of allergen sensitization and challenge in the pulmonary vasculature, little is known about the extra-pulmonary vascular changes. However, there are epidemiological studies that have confirmed the link between inhaled material and non-respiratory effects (Yeates, D.B. et al, 2001). The use of this mouse model has demonstrated that pulmonary allergic reactions are associated with increased myocardial infarction following ischemia and reperfusion, providing a direct evidence for an extra-pulmonary cardiovascular effect of allergen sensitization and challenge (Hazarika, S. et al, 2007). This study provides the first and direct evidence that pulmonary allergic sensitization affects a systemic vasculature tissue and establishes the utility of the murine model for investigating the mechanisms linking pulmonary inflammation to extra-pulmonary events and in particular to an altered systemic vascular reactivity. However, as with all isolated vascular tissue studies, care must be made in interpreting the response of the tissue to overall in situ vascular reactivity.

The aortic responses observed in this study are in agreement with observations of other investigators examining systemic vascular reactivity changes following pulmonary exposure to various kinds of particulate matter. The absence of an altered phenylephrine response is similar to observations with isolated rat small mesenteric arteries in response to pulmonary particulate matter exposure (Bagate, K. et al, 2004) and to a mouse model of ultrafine particulate matter instillation (Cozzi, E. et al, 2006). Similarly, the response to adenosine are consistent with observations by Talukdar et al. (Talukder, M.A. et al, 2002). Adenosine plays a significant role in modulating coronary and systemic vascular tone in different animal models

(Freilich, A. et al, 1992; Stella, L. et al, 1993). Observations by Talukder and collaborators indicated that adenosine in the micromolar range induces relaxation of mouse aorta, whereas vasodilatation of coronary vessels is observed at nanomolar concentrations (Talukder, M.A. et al, 2002). This is in agreement with the biphasic response to adenosine that we observed in our murine model. At lower concentrations of adenosine (0.001–1 μM), there was aortic ring contraction while at concentrations above 1 μM there was aortic relaxation. In addition, findings from this study are consistent with clinical observation that flow mediated dilatation of the brachial artery was impaired in patients with asthma (Yildiz, P. et al, 2004).

Endothelial cells are major targets of cytokine signals. It has been shown that serum levels of several proinflammatory cytokines, including TNF- α are elevated in an ovalbumin sensitized murine model of asthma (Ohkawara, Y. et al, 1997). It is therefore possible that prolonged exposure to elevated levels of proinflammatory cytokines modulates vascular responses by altering endothelial function and resultant vascular smooth muscle properties. As observed by Browner and coworkers, cytokines such as Interleukin-1 β and TNF- α downregulate cGMP-dependent protein kinase expression in vascular smooth muscle cells (Browner, N.C. et al, 2004). In addition, proinflammatory cytokines also downregulate endothelial nitric oxide production by activating Rho (Kofler, S. et al, 2005). These observations provide a possible link between the inflammation and reduced vasorelaxation responses observed in this study. These potential signaling mechanisms along with the known influence of the cyclooxygenase pathway are yet to be explored.

In conclusion, epidemiological studies reveal an association of several cardiac and vascular complications with asthma, suggests that underlying inflammation in asthma may impair systemic vascular responses. Results from this study indicate that airway allergen exposure results in impaired vasodilatory response of the aorta. These results provide evidence for a potential mechanism by which systemic inflammatory responses are linked to adverse cardiovascular effects in individuals with asthma.

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Figure 1.

Figure 1A. Airway reactivity assessed by whole body plethysmography 24 hours after intratracheal administration of ragweed (8 μ g) or normal saline. Mice were exposed to nebulized Methacholine (MCh) in the range of 0.75–48 mg/ml. Ragweed sensitized and challenged mice showed an enhanced sensitivity and airway reactivity to methacholine challenge compared to controls. N = 5; *P < 0.05.

Figure 1B. Differential leukocyte counts in the bronchoalveolar lavage (BAL) fluid of RW-challenged and control mice. Lavage was done 24 hours after the last airway challenge. N = 6 in each group; *p < 0.05. L: Lymphocytes; E: Eosinophils; N: Neutrophils; M: Monocytes.

Figure 2.

Cumulative dose response of endothelial intact thoracic aorta rings from control and RW-challenged mice to phenylephrine (PE). Curves represent Hill fit to the mean data. Data points represent mean $\pm$ SEM, n=8, control and 10, RW-challenged, respectively. Closed circles: Controls, open circles: RW-challenged animals.

Figure 3.

Active stress generation of thoracic aorta rings, normalized to cross-sectional area, to phenylephrine and KPSS stimulation from control endothelial intact (black bars) or denuded (dark gray) and RW-challenged endothelial intact (white bars) and denuded (light gray). Bars represent the mean $\pm$ SEM, n = 8, control and 10, RW-challenged, respectively. * Indicates statistical significance at P < 0.05 from the endothelium intact condition.

Figure 4. Cumulative dose response of endothelial intact thoracic aorta rings from control and RW-challenged mice to acetylcholine (ACH). Curves represent Hill fit to the mean data. Data points represent mean $\pm$ SEM, $n = 8-10$ animals. * Indicates statistical significance at $P < 0.05$.

Figure 5. Cumulative relaxation dose response of endothelial denuded thoracic aorta rings of control and RW-challenged mice to sodium nitroprusside. Curves represent Hill fit to the mean data. Data points represent mean $\pm$ SEM, $n = 6$ animals. * Indicates statistical significance at $P < 0.05$.

Figure 6.

Cumulative relaxation dose response of endothelial denuded thoracic aorta rings from control and RW-challenged mice to forskolin. Curves represent Hill fit to the mean data. Data points represent mean $\pm$ SEM, $n = 8-10$ animals. * Indicates statistical significance at $P < 0.05$.

Figure 7.

Cumulative adenosine concentration response curve of endothelial intact thoracic aorta rings from control and RW-challenged mice. Adenosine exhibited a contractile response at lower concentrations, up to 1 μM. At concentrations above 1 μM, adenosine exhibited a relaxation response. Maximal constrictive response to 1 μM adenosine and maximal relaxation response to 100 μM adenosine were not different between the groups. However, RW-challenged aorta exhibited a reduced sensitivity to adenosine mediated relaxation as evident by reduced relaxation response to 10 μM adenosine. Data points represent mean ± SEM, N = 7 in each group, *Indicates statistical significance at $p < 0.05$.