Pulmonary Hypertension

Resveratrol Prevents Monocrotaline-Induced Pulmonary Hypertension in Rats

Anna Csizsar, Nazar Labinskyy, Susan Olson, John T. Pinto, Sachin Gupte, Joseph M. Wu, Furong Hu, Praveen Ballabh, Andrej Podlutsky, Gyorgy Losonczy, Rafael de Cabo, Rajamma Mathew, Michael S. Wolin, Zoltan Ungvari

Abstract—Proliferation of pulmonary arterial smooth muscle cells, endothelial dysfunction, oxidative stress, and inflammation promote the development of pulmonary hypertension. Resveratrol is a polyphenolic compound that exerts antioxidant and anti-inflammatory protective effects in the systemic circulation, but its effects on pulmonary arteries remain poorly defined. The present study was undertaken to investigate the efficacy of resveratrol to prevent pulmonary hypertension. Rats injected with monocrotaline progressively developed pulmonary hypertension. Resveratrol treatment (25 mg/kg per day, PO, from day 1 postmonocrotaline) attenuated right ventricular systolic pressure and pulmonary arterial remodeling, decreased expression of inflammatory cytokines (tumor necrosis factor-α, interleukin 1β, interleukin 6, and platelet-derived growth factor-α/β), and limited leukocyte infiltration in the lung. Resveratrol also inhibited proliferation of pulmonary arterial smooth muscle cells. Treatment of rats with resveratrol increased expression of endothelial NO synthase, decreased oxidative stress, and improved endothelial function in small pulmonary arteries. Pulmonary hypertension was associated with an upregulation of NAD(P)H oxidase in small pulmonary arteries, which was significantly attenuated by resveratrol treatment. Our studies show that resveratrol exerts anti-inflammatory, antioxidant, and antiproliferative effects in the pulmonary arteries, which may contribute to the prevention of pulmonary hypertension. (Hypertension. 2009;54:668-675.)

Key Words: pulmonary hypertension • resveratrol • oxidative stress • endothelial dysfunction • inflammation

Pulmonary hypertension is a syndrome that encompasses several diseases, all of which have in common increased pulmonary artery pressures. Idiopathic (“primary”) pulmonary hypertension is a rare disease caused by genetic defects in the bone morphogenetic protein signaling pathways. Common causes of “secondary” forms of pulmonary hypertension include the following: (1) pulmonary hypertension associated with chronic obstructive pulmonary disease; (2) pulmonary embolism; and (3) pressure/volume overload–related pulmonary hypertension. In addition, pulmonary hypertension often develops in patients with autoimmune diseases or as a severe adverse effect of anorectic drug treatment. Despite the diverse etiologic differences, many similarities in the pathological alterations in pulmonary arteries occur among the various forms of pulmonary hypertension, ie, vascular remodeling, including cellular proliferation in both the intima and media; endothelial dysfunction/increased vasoconstriction; and activation of inflammatory processes (eg, inflammatory cytokine expression and monocyte infiltration).

Current therapies for chronic pulmonary hypertension are designed to reduce pulmonary arterial resistance by inducing vasodilation (eg, NO inhalation, stimulation of cGMP production by phosphodiesterase inhibitors, endothelin receptor antagonists, and prostacyclin analogs). These therapeutic approaches mainly provide symptomatic relief. Novel treatments are required to prevent progression of pulmonary hypertension by interfering with the pathomechanisms of the disease at multiple levels. For example, in a preclinical setting, experimental therapeutics that exert antimitotic effects on proliferation of pulmonary arterial smooth muscle cell (PASMC), in addition to promoting vasodilatation, show promise in enhancing overall prognosis.

Resveratrol (3,5,4′-trihydroxystilbene) is a dietary polyphenolic compound that exerts significant antioxidant, anti-inflammatory, and endothelial protective effects in the systemic circulation. Importantly, our previous study shows that resveratrol inhibits proliferation of cultured aortic smooth muscle cells. Thus, we hypothesize that resveratrol...
exerts antiproliferative, antioxidant, anti-inflammatory, and endothelial protective effects in the pulmonary circulation and prevents the progression of pulmonary hypertension.

To test this hypothesis we investigated the chronic efficacy of oral resveratrol treatment in monocrotaline-treated rats. Rats develop severe pulmonary hypertension after a single injection of monocrotaline (MCT), and this model mimics several key aspects of both primary and secondary forms of human pulmonary hypertension, including vascular remodeling, proliferation of PASMCs, oxidative stress, endothelial dysfunction, and upregulation of inflammatory cytokines and leukocyte infiltration. We particularly addressed the question of whether resveratrol exerts beneficial effects on proliferation of PASMCs, pulmonary arterial endothelial function, and inflammation.

Methods
A detailed Methods section can be found in the online Data Supplement (please see http://hyper.ahajournals.org).

Animal Models and Experimental Design
Animal use protocols were approved by the New York Medical College Institutional Animal Care and Use Committee. To assess preventive effects of resveratrol on MCT-induced pulmonary hypertension, adult male Sprague-Dawley rats (300 g) were randomly assigned to the following groups (n=6 animals in each group) to receive either vehicle or resveratrol in the drinking water: (1) control animals for 14 and 21 days; (2) MCT (60 mg/kg SC)-treated animals for 14 and 21 days; or (3) MCT-treated animals receiving resveratrol (25 mg/kg per day, PO, in the drinking water) from day 1 to day 14 or day 21 after MCT injection.

Assessment of Right Ventricular Hemodynamics
Two or 3 weeks after MCT administration, rats were anesthetized with ketamine (60 mg/kg IM) and xylazine (3 mg/kg IM). A microtip Millar pressure catheter was introduced into the right ventricle through the jugular vein to measure right ventricular (RV) pressure. After hemodynamic measurements, the thorax was opened, and pieces of the left lung and isolated segments of small intrapulmonary arteries were processed for histological evaluation or frozen in liquid nitrogen for subsequent biochemical measurements.

Measurement of RV Hypertrophy
The heart was dissected, and the ratio of the RV free wall weight divided by the length of the tibia was calculated as an index of RV hypertrophy (which is unaffected by changes in body weight or left ventricular mass).

Cell Proliferation and Apoptosis Assays
Immunofluorescent labeling for α-smooth muscle actin was performed to visualize the medial layer of the vessels. Index of smooth muscle hypertrophy of small pulmonary arteries (diameter: 50 to 150 µm) was calculated. Cell proliferation was also assessed in the walls of distal pulmonary vessels by use of a monoclonal antibody against proliferating cell nuclear antigen (PCNA). The percentage of PCNA-positive cells was calculated in 10 randomly chosen fields. To quantify the rate of endothelial cell apoptosis, TUNEL assay was performed, as described. To assess the antiproliferative effects of resveratrol in vitro, primary human PASMCs were stimulated in vitro with platelet-derived growth factor (PDGF; 10 ng/mL) in the presence or absence of resveratrol. Cultured cells were stained with the membrane-permeable nucleic acid stain 4′,6′-diamidino-2-phenylindole, and cell number was determined using a hemocytometer. The inhibitory effect of resveratrol on cell proliferation was also tested using a colony formation assay. In other experiments, PASMCs grown in a 96-well plate were stimulated in vitro with transforming growth factor (TGF)-β (10 ng/mL), interleukin (IL) 1β (10 ng/mL), or IL-6 (10 ng/mL) in the presence or absence of resveratrol. Cell proliferation was assessed by measuring the fluorescence of the DNA-binding CyQUANT dye (Invitrogen) in cell lysates with a fluorescence microplate reader. The effects of resveratrol on apoptotic cell death (annexin V and TUNEL staining) in PASMCs and pulmonary arterial endothelial cells were quantified by flow cytometry (Guava EasyCyte). Resveratrol-induced inhibition of the cell cycle in PASMCs was assessed by the Guava Cell Cycle Assay by flow cytometry.

Functional Studies
Acetylcholine-induced relaxation was assessed in segments of small intrapulmonary arteries precontracted by phenylephrine. In separate experiments, the vasorelaxant effect of in vitro administration of resveratrol (10⁻⁷ to 3×10⁻⁴ mol/L) was also assessed.

Measurement of Tissue H₂O₂ and Superoxide Production
Production of superoxide in isolated intrapulmonary arteries was assessed using the dihydroethidine staining method, as described previously. The cell-permeant oxidative fluorescent indicator dye 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (C-

Western Blotting
Western blotting was performed to assess protein expression of Nox-1, gp91phox, and endothelial NO synthase (eNOS) in small intrapulmonary arteries.

Quantitative Real-Time PCR
Real time RT-PCR technique was used to analyze mRNA expression, as reported previously.

Assessment of Leukocyte Infiltration
Immunolabeling on lung sections was performed for the rat macrophage marker ED-1, the number of ED-1±cells was determined in 10 randomly chosen fields.

Data Analysis
Data were normalized to the respective control mean values and are expressed as mean±SD or SEM. Statistical analyses of data were performed by Student’s t test or by 2-way ANOVA followed by a Tukey’s posthoc test, as appropriate. P<0.05 was considered statistically significant.

Results
Resveratrol Prevents the Development of Pulmonary Hypertension
Rats challenged with MCT consistently developed significant pulmonary hypertension within 14 days, which progressively increased until day 21. Consequently, RV systolic pressure was increased significantly as compared with the saline-challenged group (Figure 1A). Resveratrol treatment from day 1 normalized RV systolic pressure in MCT-injected rats at both the 2- and 3-week periods (Figure 1A). Mean systemic arterial pressure was comparable in each group (data not shown). In the MCT groups, a significant RV hypertrophy (Figure 1B) associated with RV oxidative stress (Figure S1) developed as a consequence of increased pulmonary pres-
Resveratrol treatment prevented MCT-induced RV hypertrophy (Figure 1B) and oxidative stress.

**Resveratrol Inhibits Proliferation of PASMCs**

Smooth muscle cell mass in the small pulmonary arteries was markedly increased in the MCT groups, both at day 14 and day 21 (Figure 1C and 1D), as compared with control animals. Resveratrol treatment normalized medial wall thickness, preventing the increase in PASMC mass in vessels of MCT-treated rats (Figure 1C and 1D). Medial hypertrophy of pulmonary resistance vessels was associated with an increased number of PCNA-positive proliferating vascular cells in MCT-induced pulmonary hypertension (Figure 1E and Figure S2). In parallel to normalization of vessel morphology, the number of PCNA-positive cells was considerably reduced in animals treated with resveratrol (Figure 1E and Figure S2). Neither MCT pulmonary hypertension nor resveratrol treatment elicited apoptotic cell death in small pulmonary arteries (Figure 1F and Figure S2).

PDGF (10 ng/mL for 48 hours) stimulated proliferation in cultured PASMCs, which was prevented by resveratrol (Figure 2A). The inhibitory effect of resveratrol on PASMC proliferation was also tested using a colony formation assay (Figure 2B). We confirmed that resveratrol potently inhibited PASMC colony formation and proliferation in a physiologically relevant concentration range (Figure 2B and 2C). Resveratrol also inhibited proliferation of PASMCs stimulated by IL-1β, TGF-β, or IL-6 (Figure 2D). In the concentration range, in which the antiproliferative effects were evident, resveratrol did not significantly increase apoptotic cell death either in PASMCs or pulmonary arterial endothelial cells (Figure 2E and 2F). Using flow cytometry, we demonstrated that the inhibitory effect of resveratrol on cell proliferation could be attributed to the cell cycle arrest in S phase (Figure 2G through 2I). Resveratrol significantly inhibited cytokine-induced nuclear factor κB (NF-κB) activation in PASMCs (Figure S3).

Development of pulmonary hypertension was associated with dysregulation of the expression of components of the bone morphogenetic protein (BMP)-4 signaling pathway. Resveratrol treatment normalized alterations in BMP receptors (ACVR1 and ACVRL1), BMP antagonists (chordin), and SMAD signaling molecules (SMAD1/4) in the lung (Figure S4).

**Resveratrol Attenuates Inflammatory Gene Expression and Monocyte Infiltration**

Development of pulmonary hypertension was associated with upregulation of mRNA expression of IL-6, IL-1, tumor necrosis factor-α, PDGF-α, PDGF-β, TGF-β, MCP-1, inducible NO synthase, and intercellular adhesion molecule 1 in the lung of MCT-treated rats (Figure 3). Resveratrol treatment significantly reduced the expression of these genes.

**Figure 1.** A, Pulmonary hypertension developed progressively in MCT-injected rats, as shown by increases in the RV systolic pressure at 14 and 21 days post-MCT injection. Resveratrol treatment (25 mg/kg per day from day 1) prevented development of pulmonary hypertension. B, Adaptive RV hypertrophy was demonstrated by RV weight:tibia length ratio in MCT-treated rats. Resveratrol treatment prevented RV hypertrophy. Data are mean±SEM. *P<0.05 vs control, #P<0.05 vs no resveratrol. C, Immunofluorescent labeling (red) for α-smooth muscle actin for identifying vascular smooth muscle cells (blue fluorescence: nuclei). Medial wall thickness of small pulmonary arteries was significantly increased in MCT-treated rats. Resveratrol treatment prevented increases in vascular smooth muscle mass. Scale bar: 20 μm. D, Bar graphs are summary data for medial hypertrophy index in control, MCT-, and MCT plus resveratrol–treated rats. E and F, Relative ratio of PCNA-positive nuclei (E) or TUNEL-positive nuclei (F) in the wall of small pulmonary arteries of MCT- and MCT plus resveratrol–treated rats (n=6 animals for each group). Data are expressed as mean±SEM. *P<0.05 vs control, #P<0.05 vs no resveratrol.
significantly attenuated expression of each inflammatory marker (Figure 3).

In the lungs of MCT-treated rats, the number of ED-1–positive leukocytes was significantly increased (Figure 4). Resveratrol treatment prevented infiltration of ED-1–positive cells (Figure 4). Thus, signaling for leukocyte invasion into lung tissue is markedly attenuated by resveratrol treatment.

**Resveratrol Upregulates eNOS, Attenuates Oxidative Stress, and Improves Endothelial Function in Small Pulmonary Arteries**

Acetylcholine-induced relaxation was impaired in small pulmonary arteries of MCT-treated rats, and relaxation was restored after resveratrol treatment (Figure 5A). Vascular relaxations to the NO donor S-Nitroso-N-Acetyl-d, l-Penicillamine (SNAP) were unaffected in MCT rats (data not shown). In small pulmonary arteries of MCT-treated rats, reactive oxygen species generation was elevated, whereas resveratrol treatment significantly attenuated oxidative stress (detected with dihydroethidine [Figure 5B] and C-H$_2$DCFDA fluorescence [data not shown] methods). Resveratrol in vitro did not elicit significant vasorelaxation in the physiological relevant concentration range ($\leq$10 $\mu$mol/L; Figure 5C). In small pulmonary arteries of MCT-treated rats, expression of NAD(P)H oxidase subunits was upregulated (Figures 5D, 5E, and S4). Attenuation of vascular oxidative stress by resveratrol (Figure 5B) was associated with downregulation of NOX-1 and gp91$^{phox}$, as well as improved eNOS expression (Figures 5D through 5F and S5).

**Discussion**

Resveratrol belongs to a new class of drugs$^{12}$ that exhibits cytoprotective, antioxidative, and anti-inflammatory vasoprotective properties in the systemic circulation.$^4$ Our studies show that resveratrol in the pulmonary arteries of MCT-treated rats improved endothelial function, attenuated oxida-
Resveratrol treatment also inhibited vascular remodeling, thus preventing development of pulmonary hypertension. In addition, treatment of cultured PASMCs with resveratrol inhibited cell proliferation, recapitulating in vitro the effects observed in vivo on MCT-treated animals.

Previous studies focused on the effects of resveratrol on the systemic circulation but provided little information on its pulmonary effects. In our study, we found a prominent prevention of progression of pulmonary hypertension in response to resveratrol therapy (Figure 1A). Accordingly, reducing right heart load also prevented adaptive hypertrophy.
(Figure 1B) and strain-induced oxidative stress (Figure S1) in the right ventricle of MCT-treated rats. Additional studies are needed to determine whether resveratrol treatment can reverse/delay the progression of the disease once pulmonary hypertension is established, which is the ultimate clinical relevance of a new therapeutic paradigm.

Structural changes observed in MCT-induced pulmonary hypertension resemble characteristics of human pulmonary hypertension in terms of marked medial wall thickening (Figure 1C and 1D), resulting in a dramatic increase in pulmonary arterial resistance.13 The current study is in agreement with previous reports2 that smooth muscle cell proliferation markedly increases in pulmonary resistance vessels of MCT-challenged animals (Figure 1C through 1E). Resveratrol treatment resulted in near normal vessel morphology and reduced pulmonary arterial smooth muscle proliferation (Figure 1C through 1E) without increasing the rate of apoptosis (Figure 1F). The lack of increased apoptotic cell death may explain the lack of adverse effects and good tolerability of resveratrol treatment in clinical studies.

It is likely that inhibition of pulmonary arterial smooth muscle proliferation and vascular remodeling is predominantly a direct effect of resveratrol, because resveratrol also effectively inhibited proliferation of cultured PASMCs, arresting the cell cycle in the S phase (Figure 2). These findings are in agreement with our previous studies that demonstrate that the antiproliferative properties of resveratrol are attributed to induction of p53 and heat shock protein HSP27 in human aortic smooth muscle cells.8,14

Current literature supports contributory effects of inflammation on the development and progression of pulmonary hypertension.15 In animal models of pulmonary hypertension, an increased presence and activity of inflammatory cells (including macrophages, polymorphonuclear neutrophils, lymphocytes, and mast cells) are routinely observed.15 Indeed, our study documents a substantial increase in ED-1-positive cells in the lungs of MCT-treated rats (Figure 4), which is accompanied by a significant upregulation of inflammatory cytokines16,17 and growth factors (tumor necrosis factor-α, IL-1β, IL-6, PDGF-α, PDGF-β, and TGF-β), as well as adhesion molecules (Figure 3). There is increasing appreciation of inflammation in various forms of clinical pulmonary hypertension on the basis of evidence ranging from increased plasma levels of inflammatory cytokines to pulmonary infiltration of inflammatory cells.15 In humans, a variety of diverse inflammatory conditions (eg, viral infections and autoimmune diseases) culminate in severe pulmonary hypertension. It has been generally accepted that inflammatory cytokines and growth factors can cause pulmonary vasoconstriction and promote proliferation of vascular cells. Recent studies suggest that disruption of PDGF signaling pathways prevents development and progression of MCT-induced pulmonary hypertension in rats.2 Importantly, we found that resveratrol treatment in MCT-treated rats normalized expression of inflammatory cytokines and growth factors (including PDGF-α/β; Figure 3) and significantly decreased the number of infiltrating ED-1–positive cells in the lung (Figure 4). The mechanisms by which resveratrol interferes with inflammatory processes in the lung are not well understood. Recently we showed that resveratrol effectively blocks NF-κB activation in endothelial cells, thus limiting expression of inflammatory cytokines and adhesion molecules and attenuating monocyte adhesiveness.8 Resveratrol also effectively inhibits cytokine-induced NF-κB activation in PASMCs (Figure S3). NF-κB activation is known to regulate PASMC proliferation in vitro.18,19 Because development of pulmonary hypertension in MCT-treated rats is associated with NF-κB activation,20 and administration of an inhibitor of
NF-κB prevents pulmonary hypertension in MCT-treated rodents, we speculate that inhibition of NF-κB by resveratrol may contribute to its therapeutic potential in pulmonary hypertension.

Mutations in BMP receptors have been identified in patients with primary pulmonary arterial hypertension, implicating BMP signaling in pulmonary hypertension. Importantly, recent studies have demonstrated that the expression and function of the BMP/Smad signaling axis are perturbed in MCT-treated animals as well. Because BMP signals antagonize PASMC proliferation induced by inflammatory cytokines, altered BMP/Smad signaling may play a role in the pathogenesis of pulmonary hypertension. Resveratrol normalized the expression of many components of the BMP/Smad signaling pathway (Figure S4). Additional studies are needed to elucidate whether these effects contribute to the therapeutic benefits of resveratrol treatment.

There is solid evidence in MCT-treated rats that oxidative injury to the pulmonary vascular endothelium precedes PASMC proliferation and medial hypertrophy in the distal pulmonary vascular bed and the rise in pulmonary artery pressure. We found that pulmonary hypertension in small pulmonary arteries is associated with endothelial dysfunction (Figure 5A), oxidative stress (Figure 5B), and upregulation of NAD(P)H oxidases (Figure 5D and 5E). By contrast, resveratrol treatment significantly improved pulmonary arterial endothelial function (Figure 5A) and attenuated oxidative stress and NADPH oxidase expression (Figures 5D, 5E, and Figure S5). These findings are significant because recent studies on p91^phox knockout mice suggest that NAD(P)H oxidases contribute to the development of pulmonary hypertension. The activity and expression of vascular NAD(P)H oxidases are known to be regulated by inflammatory cytokines (eg, tumor necrosis factor-α and TGFβ), the expression of which is upregulated in lungs of rats with pulmonary hypertension (Figure 2). Thus, we suggest that the observed resveratrol-induced downregulation of NAD(P)H oxidase (Figure 5) is attributed to the resveratrol-induced attenuation of inflammatory cytokine production (Figure 3).

We confirmed that eNOS is downregulated (Figure 5E) in rats with pulmonary hypertension. Importantly, this attenuation was corrected by resveratrol (Figure 5E). Previous studies showed that resveratrol directly regulates eNOS expression at the level of transcription in cultured endothelial cells. Because some studies suggest that treatment with NO donors or overexpression of eNOS itself may attenuate pulmonary hypertension, it is possible that upregulation of eNOS contributes to the therapeutic action of resveratrol. We cannot exclude the possibility that resveratrol, in addition to upregulating eNOS, may also affect BH4 levels and/or caveolin and, thus, directly influence eNOS activity. Previously, resveratrol was shown to upregulate the expression of Nrf2-regulated antioxidant gene battery, including heme oxygenase 1 in systemic arteries. In the present study, resveratrol treatment also induced heme oxygenase 1 in the pulmonary arteries of MCT-treated rats (Figure S6), which is known to confer antiproliferative and vasoprotective effects.

**Perspectives**

We have demonstrated that treatment with resveratrol prevents the development of pulmonary hypertension in a recognized animal model of the disease. The demonstrated antiproliferative action of resveratrol on PASMCs may be contributory to its beneficial effects. Furthermore, we have provided evidence for anti-inflammatory, antioxidative, and endothelial protective effects in the beneficial actions of resveratrol in pulmonary arteries. Because phase I clinical trials are currently underway for anticancer efficacy of oral resveratrol treatment in humans, the use of resveratrol as a new therapy for pulmonary hypertension might, therefore, be plausible. Future studies should determine the following: (1) whether resveratrol treatment can reverse or delay the progression of established pulmonary hypertension; (2) whether resveratrol may potentiate the effects of current therapies; and (3) whether resveratrol can exert direct cardioprotective effects preventing RV failure in patients with pulmonary hypertension.

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**Disclosures**

None.

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Extended Materials and Methods

Animal Models and Experimental Design

Animal use protocols were approved by the Institutional Animal Care and Use Committee of the New York Medical College, Valhalla, NY. To assess the preventive effects of resveratrol on MCT-induced pulmonary hypertension, adult male Sprague-Dawley rats (300 g, purchased from Charles River Co, Austin, TX) were randomized to the following groups (n=6 animals in each group) to receive either vehicle or resveratrol in the drinking water: control animals for 14 and 21 days; MCT (60 mg/kg s.c.) -treated animals for 14 and 21 days; MCT-treated animals receiving resveratrol (25 mg/kg/day, p.o., in the drinking water) from day 1 to day 14 or day 21 after MCT injection. This dose of resveratrol was shown to be vasoprotective in the systemic circulation1,2.

Assessment of right ventricular hemodynamics

Three or 5 weeks after MCT administration, rats were anesthetized with ketamine (60 mg/kg i.m.) and xylazine (3 mg/kg i.m.). A microtip Millar pressure catheter was introduced into the right ventricle through the jugular vein to measure right ventricular pressure. After hemodynamic measurements the thorax was opened and pieces of the left lung and isolated segments of small intrapulmonary arteries were processed for histological evaluation or frozen in liquid nitrogen for subsequent biochemical measurements.

Measurement of right ventricular hypertrophy

The heart was dissected, and the ratio of the right ventricular free wall weight divided by the length of the tibia was calculated as an index of right ventricular hypertrophy (which is unaffected by changes in body weight).

Cell proliferation and apoptosis assays

Immunofluorescent labeling for α-smooth muscle actin was performed to visualize the medial layer of the vessels. Index of smooth muscle hypertrophy of small pulmonary arteries (d: 50-150 μm) was calculated as follows: \((a+b+c+d)x(A+B)\)\(^{-1}\), where a,b,c,d and A,B are measurements of medial wall thickness (at 3, 6, 9 and 12 o’clock direction, respectively) and external diameter (vertically and diagonally, respectively). Cell proliferation was also assessed in the walls of distal pulmonary vessels by use of a monoclonal antibody against proliferating cell nuclear antigen (PCNA). The percentage of PCNA–positive cells was calculated in 10 randomly chosen fields. To assess the rate of endothelial cell apoptosis, TUNEL assay was performed as described1.

To assess the anti-proliferative effects of resveratrol in vitro, primary human pulmonary arterial smooth muscle cells (PASMC; cultured in 0.2% FBS-DMEM as described3) were stimulated in vitro with PDGF (10 ng/mL) in the presence or absence of resveratrol. Cultured cells were stained with the membrane-permeable nucleic acid stain 4',6'-diamidino-2-phenylindole (5 μM; Molecular Probes) and cell number was determined using a hemocytometer. The inhibitory effect of resveratrol on cell proliferation was also tested using a colony formation assay as described by Smith et al.4 with few modifications. In brief, cells were grown in 60-mm dishes for 14 days in the presence or absence of various concentrations of resveratrol. Three dishes were seeded for each treatment, at a concentration of 200 cells per dish. Cells were then fixed and stained and total colony number and cell numbers per colony scored. In other experiments PASMC grown in a 96 well plate were stimulated in vitro with TGFβ (10 ng/mL), IL-1β (10 ng/mL) or IL-6 (10 ng/mL) in the presence or absence of resveratrol (1 to 30 μmol/L). Cell proliferation was assessed by measuring the fluorescence of the DNA-binding CyQUANT dye (Invitrogen) in cell lysates with a fluorescence microplate reader. The effect of resveratrol on apoptotic cell death (annexin V and TUNEL staining) in PASMC and pulmonary arterial endothelial cells (PAEC) were quantified by flow cytometry (Guava Easycyte). Resveratrol-induced inhibition of the cell cycle in PASMC was assessed by the Guava Cell Cycle Assay by flow cytometry.
Transient transfection and luciferase assays

Effect of PDGF (10 ng/mL, for 24 h) and resveratrol (10 µmol/L, 24 h pre-treatment) on NF-κB activity in PASMCs was tested by a reporter gene assay as described5, 6. We used a NF-κB reporter comprised of an NF-κB response element upstream of firefly luciferase (NF-κB-Luc, Stratagene) and a renilla luciferase plasmid under the control of the CMV promoter (as an internal control). Transfections in PASMCs were performed using the Amava Nucleofector technology (Amava, Gaithersburg, MD), as we have previously reported5, 6. Firefly and renilla luciferase activities were assessed after 24 h using the Dual Luciferase Reporter Assay Kit (Promega) and a luminometer.

Functional studies

Acetylcholine-induced relaxation was assessed in segments of small intrapulmonary arteries pre-contracted by phenylephrine (1 µmol/L), as described7. Relaxation of arterial segments to the NO donor S-nitrosopenicillamine (SNAP, from 10⁻⁹ to 3x10⁻⁵ mol/L mol/L) was also evaluated. In separate experiments the vasorelaxant effect of in vitro administration of resveratrol (10⁻⁷ to 3x10⁻⁴ mol/L) was also assessed.

Measurement of tissue H₂O₂ and O₂⁻ production

Production of O₂⁻ in isolated intrapulmonary arteries, RV, and coronary arteries isolated from the RV was assessed using the dihydroethidine staining method, as described8. Untreated tissues were used for determining levels of autofluorescence. The cell-permeant oxidative fluorescent indicator dye C-H₂DCFDA (5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad CA) was used to assess peroxide levels in isolated intrapulmonary arteries, as we reported8. In addition, pressure overload-induced O₂⁻ generation in pieces of the right and left ventricles was measured by the lucigenin (10 µmol/L) chemiluminescence (CL) method, as reported8.

Quantitative real-time PCR

Total RNA was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript III RT (Invitrogen) as described previously5, 7, 10. Real time RT-PCR technique was used to analyze mRNA expression using the Strategen MX3000, as reported2, 10, 11. Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. Quantification was performed using the efficiency-corrected ΔΔCT method. The housekeeping genes GAPDH and β-actin were used for internal normalization. Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Western blotting

Western blotting was performed as described8, 10 to assess protein expression of Nox-1, gp91phox and eNOS in small intrapulmonary arteries.

Assessment of leukocyte infiltration

Immunolabeling on lung sections was performed for the rat monocyte/macrophage marker ED1. The number of ED1-positive cells was determined in 10 randomly chosen fields. Histological analysis was performed in a blinded fashion by two observers.

Data analysis
Data were normalized to the respective control mean values and are expressed as means ± S.D. or S.E.M. Statistical analyses of data were performed by Student’s t-test or by two-way ANOVA followed by a Tukey's post hoc test, as appropriate. *p*<0.05 was considered statistically significant.

References for Online Supplement

Figure S1, A: Representative fluorescent images showing increased nuclear dihydroethidine staining in the right ventricle (RV) of MCT treated rats (middle; 21 days post-MCT), as compared to controls (left). Resveratrol treatment normalized O\textsubscript{2}^- production in the RV. B: RV hypertrophy in MCT-treated rats is associated with increased O\textsubscript{2}^- production (assessed by the lucigenin chemiluminescence (CL) method) in the RV, but not in the left ventricle (LV). Resveratrol treatment prevented oxidative stress in the RV. Data are mean ± S.E.M. *P<0.05 vs. control, #P<0.05 vs. no resveratrol.; (n=6 for each group). C: Relaxation of intramural coronary arteries from the RV of control rats and MCT-injected rats in response to acetylcholine. The effect of chronic resveratrol treatment on vascular relaxations is also shown. Data are mean ± S.E.M. *P<0.05 vs. control, #P<0.05 vs. no resveratrol; (n=6 for each group). D: Representative fluorescent images showing increased nuclear dihydroethidine staining in the coronary arteries isolated from the RV of MCT treated rats (middle), as compared to controls (left), which was prevented by resveratrol treatment (right). E: endothelium, M: media, Ad: adventitia.
Figure S2, panel A: Medial hypertrophy (red fluorescence: α-smooth muscle actin) of small pulmonary arteries (d: 50-150 μm) was associated with an increased number of proliferating vascular cells in MCT-induced pulmonary hypertension. Immunolabeling for PCNA (green nuclei are PCNA-positive cells; arrows) revealed increased proliferation in MCT-challenged animals (as compared with controls). Prevention of medial hypertrophy induced by resveratrol was attributed to reduced SMC proliferation. B: TUNEL assay results showing that neither MCT-PAH nor resveratrol increases apoptotic cell death in small pulmonary arteries (left: arrows point to non-vascular TUNEL positive cell nuclei (green); middle: overlay with nuclear staining (blue); right: overlay with α-smooth muscle actin (red)). Scale bars: 25 μm
Figure S3: To determine the effect of resveratrol on PDGF-induced NF-κB activation, we transiently transfected PASMCs with a NF-κB-driven reporter gene construct and then pre-treated the cells with resveratrol (10 µmol/L, for 24 h) followed by stimulation with PDGF (10 ng/mL, for 24 h). A significant increase in luciferase activity over the vector control was noted upon stimulation with PDGF in the absence of resveratrol. Pre-treatment of PASMCs with resveratrol prevented PDGF-induced NF-κB activation. Data are mean ± S.E.M. *P<0.05 vs. control, #P<0.05 vs. no resveratrol.
Figure S4. mRNA expression of BMP-4, BMP receptors (BMPR1a, BMPR1b, BMPR2, ACVR2a, ACVR2b, ACVR1, ACVRL1), BMP antagonists (chordin, noggin) and SMAD signaling molecules (SMAD1/4/5/6/7) in the lung of control, MCT-treated and MCT plus resveratrol treated rats. Analysis of mRNA expression was performed by real-time QRT-PCR. Data are mean ± S.E.M. *P<0.05 vs. control, #P<0.05 vs. no resveratrol.
Figure S5. mRNA expression of Nox-1 (A), gp91\(^{phox}\) (B) and Nox-4 (C) in small intrapulmonary arteries from control, MCT-treated and MCT plus resveratrol treated rats. Analysis of mRNA expression was performed by real-time QRT-PCR. Data are presented as mean ± S.E.M. *P<0.05 vs. control, #P<0.05 vs. no resveratrol.
**Figure S6.** Expression of HO-1 mRNA in small intrapulmonary arteries from control, MCT-treated and MCT plus resveratrol treated rats. β-actin was used for normalization.