Genistein, a Soy Phytoestrogen, Reverses Severe Pulmonary Hypertension and Prevents Right Heart Failure in Rats

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Abstract—Pretreatment with a phytoestrogen genistein has been shown to attenuate the development of pulmonary hypertension (PH). Because PH is not always diagnosed early, we examined whether genistein could also reverse preexisting established PH and prevent associated right heart failure (RHF). PH was induced in male rats by 60 mg/kg of monocrotaline. After 21 days, when PH was well established, rats received daily injection of genistein (1 mg/kg per day) for 10 days or were left untreated to develop RHF by day 30. Effects of genistein on human pulmonary artery smooth muscle cell and endothelial cell proliferation and neonatal rat ventricular myocyte hypertrophy were assessed in vitro. Severe PH was evident 21 days after monocrotaline, as peak systolic right ventricular pressure increased to 66.35±1.03 mm Hg and right ventricular ejection fraction reduced to 41.99±1.27%. PH progressed to RHF by day 30 (right ventricular pressure, 72.41±1.87 mm Hg; RV ejection fraction, 29.25±0.88%), and mortality was ~75% in RHF rats. Genistein therapy resulted in significant improvement in lung and heart function as right ventricular pressure was significantly reduced to 43.34±4.08 mm Hg and right ventricular ejection fraction was fully restored to 65.67±1.08% similar to control. Genistein reversed PH-induced pulmonary vascular remodeling in vivo and inhibited human pulmonary artery smooth muscle cell proliferation by ~50% in vitro likely through estrogen receptor-β. Genistein also reversed right ventricular hypertrophy (right ventricular hypertrophy index, 0.35±0.029 versus 0.70±0.080 in RHF), inhibited neonatal rat ventricular myocyte hypertrophy, and restored PH-induced loss of capillaries in the right ventricle. These improvements in cardiopulmonary function and structure resulted in 100% survival by day 30. Genistein restored PH-induced downregulation of estrogen receptor-β expression in the right ventricle and lung. In conclusion, genistein therapy not only rescues preexisting severe PH but also prevents the progression of severe PH to RHF. (Hypertension. 2012;60:00-00.) • Online Data Supplement

Key Words: pulmonary hypertension ■ right heart failure ■ genistein ■ angiogenesis ■ estrogen receptor-β

Pulmonary hypertension (PH) is a chronic, debilitating lung disorder associated with pulmonary vascular remodeling and progressive increase in pulmonary artery (PA) pressure leading to right ventricular (RV) hypertrophy, right heart failure (RHF), and death.1 Unfortunately all of the available drug therapies only alleviate the symptoms and slow down deterioration. However, their inefficacy in severe and more advanced cases often leads to invasive procedures, such as lung or heart-lung transplant.

The protective effects of estrogen on the cardiovascular system have been well documented in literature.2-3 However, we found that estrogen exerts most of its protective effects against PH via estrogen receptor-β (ERβ).4 However, the use of estrogen for therapy has been considered to be controversial because of its possible off-target effects.5 Genistein, a natural soybean-derived phytoestrogen, with much higher affinity for ERβ than estrogen receptor-α (ERα), has been shown to have vasodilator, cardioprotective, and anti-inflammatory effects.6,7 These properties make genistein a potential candidate for the treatment of PH. In fact, it has been shown that genistein pretreatment before the administration of monocrotaline (MCT) is able to slow down the progression of PH.9 Because PH is a chronic disease that is not always diagnosed early, this approach is not practical for patients who already have established PH. Here we tested the hypothesis that genistein therapy rescues preexisting severe PH and prevents RHF induced by MCT in rats.

Methods

Animals and Treatments

Male Sprague-Dawley rats (350–400 g) received a single SC injection of MCT (60 mg/kg; Sigma) at day 0 to induce severe PH by day 21. A group of randomly selected MCT-injected rats received genistein therapy (1 mg/kg per day, SC; Sigma; n=10) from day 21 to day 30. Another group of MCT-injected rats was left untreated to develop RHF by day 30 (RHF; n=10; Figure 1A). Saline-treated rats served as controls (CTRLs; n=10). Protocols received institutional review and committee approval.

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Cardiac and Pulmonary Hemodynamics
Sequential echocardiography was conducted using a VisualSonics Vevo770 echocardiogram device with a 30-MHz linear transducer to measure RV ejection fraction (RVEF) and to estimate RV pressure (RVP). RVP was also directly measured with a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Laboratory, ADInstruments) before euthanization.

Gross Histological Evaluation
The lung, RV wall, left ventricular (LV) wall, and the interventricular septum (IVS) were used to measure the lung weight and RV hypertrophy index (RV/(LV + IVS)).

Western Blot Analysis, Immunohistochemistry, and Imaging
Lung and RV lysates were used for standard Western immunoblotting. Whole lungs and hearts were fixed, and tissue sections were used for immunofluorescence, hematoxylin-eosin, and Masson trichrome stainings. Images were captured using a light microscope or a laser-scanning confocal microscope.

Cell Proliferation and Apoptosis Assays
Cryopreserved human PA smooth muscle cells (Invitrogen) and human PA endothelial cells (Invitrogen) were cultured. Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell proliferation assay (American Type Culture Collection), measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell proliferation assay (American Type Culture Collection). Cell proliferation and apoptosis were assessed using a confocal microscope.

In Vitro Cardiomyocyte Hypertrophy Studies
Neonatal rat ventricular myocytes were isolated as described previously. Cells were cultured overnight and treated with phenylephrine (10 μmol/L) in the presence or absence of genistein (1 μmol/L), selective ERα agonist PPT (4,4’,4”-[propyl-[(1H)-pyrazole-1,3,5-triy]l]trisphenol; 10 μmol/L), selective ERβ agonist DPN [2,3-bis (4-hydroxyphenyl) propionitrile; 10 μmol/L], or ER antagonist ICI 182780 (ICI; 10 μmol/L) + genistein (1 μmol/L) for 48 hours. Cells were fixed and stained with anti–α-actinin. Images were taken with a confocal microscope.

Reagents
For the list of primary and secondary antibodies used for Western blots and immunostainings please see the online-only Data Supplement.

Statistical Analysis
One-way ANOVA was performed to compare between groups using SPSS13.0. Post hoc tests (Bonferroni) were carried out to compare individual mean values when significant differences were found. P values <0.05 were considered as statistically significant. Values are represented as mean±SD.

Results
Genistein Rescues Severe PH by Restoring Cardiopulmonary Structure and Function
Severe PH was clearly evident at day 21, because the RVP increased by 2-fold from 30.85±0.52 mm Hg in the CTRL group to 66.35±1.03 mm Hg in the PH group (Figure 1B and 1D), and RVEF decreased by 40% to 41.99±1.27% compared with 67.26±1.16% in the CTRL group (Figure 1C and 1E). There was also a significant increase in lung weight (2.32±0.082 g versus 1.38±0.075 g in the CTRL group; Figure 1F, available in the online-only Data Supplement), RV hypertrophy index (RV/(LV+IVS) = 0.63±0.06 versus 0.25±0.02 in the CTRL group; Figure 1F), and in medial thickness of pulmonary arterioles (Figure 2A and Table S1). Untreated rats in the RHF group developed even higher RVP (72.41±1.87 versus 66.35±0.88% versus 41.99±1.27% in the PH group) by day 30. Surprisingly, genistein therapy of MCT-injected rats not only prevented the transition from PH to RHF but also managed to gradually improve RVP (43.34±4.08 mm Hg), RVEF (65.67±1.08%), RV hypertrophy index (0.35±0.02 in genistein versus 0.63±0.06 in PH), and RV dilatation within a short
period of 10 days (Figures 1B through 1F and S1). Genistein therapy also resulted in the disappearance of midsystolic notching on PA Doppler echocardiography (Figure S1) and restored PH-induced RV systolic dysfunction (Table S2). Genistein therapy also reversed the adverse changes in the lung structure by restoring the lung weight (1.81 \pm 0.08 g in genistein versus 2.32 \pm 0.08 g in PH; Figure S2) and pulmonary microvascular remodeling (Figure 2A and Table S1).

Genistein therapy not only prevented the increase in pulmonary fibrosis, as observed in RHF, but it also substantially reversed preexisting fibrosis in the PH group (Figure 2B). These improvements in cardiopulmonary function and structure (Figures 1C and 1E and 2A through 2C) associated with genistein therapy resulted in 100% survival of genistein-treated MCT rats by day 30. In MCT-injected untreated rats, mortality began as early as day 24 and reached 75% by day 30.

Genistein Therapy Restores PH-Induced Loss of Capillaries in the Right Ventricle

RV ischemia has been described in PH patients in the absence of coronary artery disease,\textsuperscript{10} which is usually caused by loss of RV microvessels.\textsuperscript{11,12} RHF was associated with a 42% reduction in RV capillary density (0.54 \pm 0.01 versus 0.94 \pm 0.03 microvessels per cardiomyocyte in the CTRL group), and genistein therapy fully restored the loss of capillaries (0.97 \pm 0.02 microvessels per cardiomyocyte; Figure 3A and 3B). Genistein also partially restored 4-fold downregulation of vascular endothelial growth factor expression induced by RHF in the RV (0.64 \pm 0.02 versus 0.23 \pm 0.08 in RHF, normalized to the CTRL group; Figure 3C).

![Figure 2](image-url)

**Figure 2.** Genistein (GEN) therapy reverses lung remodeling induced by pulmonary hypertension (PH). A, Immunofluorescence colabeling of pulmonary arterioles with anti-\(\alpha\)-smooth muscle actin antibody (red) together with anti-von Willebrand factor antibody (green). B, Masson trichrome staining of lung sections; fibrosis indicated by blue color; black arrows indicate areas of fibrosis. C, Hematoxylin and eosin staining of heart cross-sections. (control [CTRL], \(n=3–5\); PH, \(n=3–4\); right heart failure [RHF], \(n=3–5\); GEN, \(n=3–5\)).

![Figure 3](image-url)

**Figure 3.** Restoration of right ventricular (RV) capillary density, as well as vascular endothelial growth factor (VEGF) expression by genistein (GEN) therapy. A, Single confocal images of RV sections immunostained for endothelial marker CD31 (green, top panel), overlay of CD31 (green) and wheat germ agglutinin (red, middle panel), and at higher display magnification (bottom panel). B, Quantification of capillary density (microvessels per cardiomyocyte). C, Representative Western immunoblots (top panel) of RV lysates labeled with anti-VEGF and vinculin antibodies; Western blot analysis (bottom panel) of anti-VEGF normalized to vinculin in the right ventricle. *\(P<0.01\) vs control [CTRL]; **\(P<0.05\) vs right heart failure [RHF]; \(P<0.01\) vs RHF (\(n=4–5\) animals per group).
Genistein Inhibits the Proliferation of Human PA Smooth Muscle Cells In Vitro Through an ER-β-Mediated Mechanism

PH is associated with excessive proliferation of PA smooth muscle cells and endothelial cells. Genistein (1 μmol/L) inhibited the proliferation of human PA smooth muscle cells by ≈50% (from 1.00±0.05 to 0.50±0.06; P<0.05; Figure 5A). These inhibitory effects of genistein were completely absent in the presence of ER antagonist ICI (1.1±0.05 normalized to CTRL). An ERβ agonist, DPN, was as effective as genistein in inhibiting proliferation (0.65±0.05), whereas treatment with the ERα agonist PPT had no significant effect on the proliferation rate of human PA smooth muscle cells (1.068±0.026; Figure 5A). ICI alone also had no effect. Regarding the human PA endothelial cells, genistein at 1 μmol/L (relative proliferation, 0.94±0.06 versus 1.00±0.07 in the CTRL group; Figure 5B) or even at 10 μmol/L had no effect on proliferation rate of human PA endothelial cells.

Genistein Inhibits Neonatal Rat Ventricular Myocyte Hypertrophy In Vitro

Next we examined whether genistein can directly inhibit cardiomyocyte hypertrophy. Genistein inhibited phenylephrine-induced neonatal rat ventricular myocyte hypertrophy (cell

**Figure 4.** Restoration of estrogen receptor (ER)-β protein levels by genistein (GEN) therapy. Representative immunoblots of right ventricle (RV) and lung lysates labeled with anti-ERα (A and B) or anti-ERβ (C and D) together with their normalized Western blot analysis (bottom panel) to their corresponding loading controls (GAPDH or vinculin). *P<0.05 vs CTRL; **P<0.05 vs right heart failure (RHF; n=4–5 animals per group).

**Figure 5.** Genistein (GEN) inhibits the proliferation of human pulmonary artery smooth muscle cells (hPASMCs) and phenylephrine (PE)-induced hypertrophy of neonatal rat ventricular myocytes (NRVMs). The proliferation rate of hPASMCs (A) and human pulmonary artery endothelial cells (hPAECs; B) incubated with GEN (1 μmol/L), GEN+ICI 182780 (ICI; 10 μmol/L), ICI alone (10 μmol/L), DPN (estrogen receptor [ER]-β agonist; 1 μmol/L), or PPT (ERα agonist; 1 μmol/L) normalized to control (CTRL). Cells incubated with only growth medium served as negative CTRL. *P<0.01 vs CTRL; #P<0.05, ##P<0.01 vs GEN; $$P<0.01$$ vs GEN. (Experiments were repeated with ≥8 replicates and 3–5 independent times.). C, Single confocal images of NRVM labeled with anti-α-smooth muscle actin (red) in CTRL, PE (10 μmol/L), PE+GEN (1 μmol/L), PE+GEN+ICI (10 μmol/L), PE+DPN (ERβ agonist; 10 μmol/L), PE+PPT (ERα agonist; 10 μmol/L). D, The cardiomyocyte size normalized to CTRL. *P<0.05, **P<0.01 vs CTRL; #P<0.05, ##P<0.01 vs GEN; $$P<0.01$$ vs PE (All experiments were performed in duplicate and ≥3 times; n=100 cells per group).
surface area: phenylephrine, 3.16±0.60 versus genistein, 0.80±0.18, normalized to the CTRL group; Figure 5C and 5D). Interestingly, in the presence of the ER antagonist ICI, genistein was unable to inhibit hypertrophy (2.42±0.21; Figure 5C and 5D). Treatment with ERβ agonist DPN resulted in antihypertrophic effect similar to genistein (1.01±0.03; Figure 5C and 5D), whereas the ERα agonist PPT was not able to inhibit phenylephrine-induced hypertrophy of neonatal rat ventricular myocytes (2.85±0.53; Figure 5C and 5D).

Discussion
Although pretreatment with genistein has been shown to attenuate PH,9 our study demonstrates for the first time that genistein therapy is able to rescue preexisting advanced PH. Because PH is a chronic disease that is not always diagnosed early, our approach of starting genistein treatment after the establishment of fatal PH is more practical and clinically relevant. Our therapy also requires shorter duration and relatively lower dose of genistein. Here we show that genistein treatment restores PH-induced severe abnormalities in cardiopulmonary function and structure resulting in 100% survival, whereas in untreated rats, the mortality was ~75% by day 30. The rescue action of genistein seems to be mediated via ERβ. Restoration of cardiac capillary density by genistein might be another key factor in nurturing and healing the failing RV.

Genistein Therapy Reverses PH-Induced Lung Fibrosis and Pulmonary Vascular Remodeling
Lung fibrosis13 and pulmonary vascular remodeling1 are associated with severe PH in patients. Our data strongly demonstrate that genistein reverses interstitial and perivascular lung fibrosis, as well as arteriolar wall thickness. We also find that both genistein and the ERβ agonist DPN inhibit human PA smooth muscle cell proliferation in vitro mainly through ERβ-dependent mechanisms. However, the inhibition is more pronounced in genistein-treated cells than DPN, possibly as a result of the slight increase in apoptosis (Figure S3), although the contribution of other antiproliferative mechanisms cannot be ruled out.

Restoration of Cardiac Structure and Function by Genistein Therapy
It is generally believed that the improved RV function by any given therapy in PH is merely a result of improved lung function. Although genistein improves pulmonary hemodynamics, it is much more effective in fully restoring RV contractility and function. RVFs of PH rats treated with genistein varied largely from 28 to 67 mm Hg, whereas the RVEFs of these animals were all >60% (Figures 1E and S4). Genistein was also very efficient in suppressing cardiac hypertrophy both in vivo and in vitro (Figures 1F, 5C and 5D).

Loss of blood vessels in RV has been reported in experimental models of advanced PH, as well as in patients.11,14,15 Down-regulation of RV vascular endothelial growth factor has been reported in advanced PH.12 Increased production of vascular endothelial growth factor has been shown to be cardioprotective by improving myocardial functional recovery after ischemia/reperfusion injury.16 Both decreased RV capillary density and downregulation of vascular endothelial growth factor induced by PH were restored by genistein therapy (Figure 3). Although genistein did not increase the proliferation of human PA endothelial cells in vitro, it resulted in the restoration of capillary density in the RV. This dichotomy could be explained by the fact that endothelial cells originating from different tissues, as well as from different organisms, may elicit distinct responses. We speculate that, in addition to the effects of genistein on the lung, it also acts on the RV to reverse RV hypertrophy, replenish microvessels, and restore RV contractility and function.

Potential Mechanisms of Action of Genistein in Rescuing PH
Estrogen or estrogen metabolites have been shown to prevent and attenuate the development of PH.2,17 Recently, we discovered that estrogen can even rescue PH and exerts most of its protective effects against PH via ERβ.5 The vital implications of ERβ on the lung have been demonstrated previously, because the ablation of ERβ resulted in severe structural abnormalities in the lungs of ERβ-deficient mice.19 ERβ also plays an important role in cardioprotection, because estrogen therapy was able to reduce cardiac hypertrophy in ERα-deficient mice but was ineffective in ERβ-deficient mice.5 Here we found that genistein, a natural soybean-derived phytoestrogen, with much higher affinity for ERβ than ERα, efficiently reverses PH-induced cardiopulmonary structure and function possibly through an ERβ-mediated mechanism.

Genistein may also act through a host of other potential pathways to rescue PH. Genistein is a well-known tyrosine kinase inhibitor. Tyrosine kinase inhibitors play an important role in pulmonary vasodilation.20 Currently, tyrosine kinase inhibitors, such as imatinib, are used in clinical trials for the treatment of PH.21 Genistein also increases endothelial NO synthase levels9 and restores NO-mediated PA relaxation.22 Thus, it is possible that tyrosine kinase inhibitory effects, as well as endothelial NO synthase–induced vasodilatory effects, of genistein also participate in rescuing preexisting PH.

Limitations
The plexiform lesions that are found in the lungs of PAH patients, as well as in angioproliferative models of PH, are not usually seen in the MCT model.20 Still, the MCT model of PH shares several main characteristics with both primary and secondary PH in humans, such as vascular remodeling and proliferation of pulmonary artery smooth muscle cells, as well as RV and endothelial dysfunction.24 As genistein restores most of these parameters, we propose that genistein could potentially serve as a treatment option for both forms of PH in humans.

Perspectives
PAH in patients is a chronic, debilitating disease that is refractory to most of the available pharmacological therapies, which only alleviate the symptoms and slow down the deterioration. Some clinical forms of PAH are more prevalent in females than in males, whereas various animal models of PH have established a protection in females, exacerbation of the disease with ovariectomy, and a therapeutic role of estrogen. Recently, we discovered that estrogen can rescue PH and exerts most of its protective effects against PH via ERβ. However, the use of estrogen for therapy has been considered to be controversial because of its possible off-target effects.6 Genistein, a natural soybean derived
phytoestrogen, with much higher affinity for ERβ than ERα, has been shown previously to attenuate PH in rats. Here we show for the first time that genistein therapy is able to rescue preexisting advanced PH by restoring PH-induced severe abnormalities in cardiopulmonary function and structure resulting in 100% survival. A selective ERβ ligand, such as genistein, which is a phytoestrogen with additional tyrosine kinase inhibition properties, shows promise as a rescue agent for experimental PH. Further preclinical and clinical studies are warranted to establish genistein as a novel and safe therapeutic agent for treating patients with PAH and RHF.

Sources of Funding

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Disclosures

None.

References


Novelty and Significance

What Is New?

- Genistein, a soy-derived phytoestrogen, has never been used to rescue preexisting severe PH.
- We investigated therapeutic effects of genistein on the lung and heart, as well as the direct effects of genistein on human pulmonary artery smooth muscle cells, endothelial cells, and neonatal rat cardiomyocytes.

What Is Relevant?

- PH is a type of hypertension that involves increased pressure in the pulmonary circulation leading to cardiac failure and death.

Summary

Genistein effectively rescues preexisting PH and raises the prospect of expanding the application of genistein for the treatment of chronic PH.
Genistein, a soy phytoestrogen, reverses severe pulmonary hypertension and prevents right heart failure in rats

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Animals and treatments

Male Sprague-Dawley rats (350-400 g) were used. MCT was dissolved in 1N HCl, adjusted to a pH of 7.4, and diluted with PBS. Rats were given a single subcutaneous injection of MCT (60 mg/kg, Sigma) at day-0 to induce PH and were randomly assigned to i) PH group, rats were sacrificed at day-21 after MCT to confirm the establishment of severe PH at this time point; ii) RHF group, MCT-injected rats kept untreated until day-30 to develop severe PH-induced right heart failure and iii) GEN group, rats received a daily subcutaneous injection of genistein (1 mg/kg/day; Sigma) from day-21 until day-30. In the control group (CTRL), rats were injected with saline and monitored until day-30. All rats were sacrificed at day-30 except the PH group in which rats were scarified at day 21. Protocols received institutional review and committee approval. The investigation conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Cardiac and pulmonary hemodynamics

Sequential echocardiography was conducted to measure cardiac and pulmonary hemodynamic parameters and RV architecture to properly assess the progression of the disease. A VisualSonics Vevo 770 echocardiogram device with a 30-MHz linear transducer was used to perform the B-Mode, M-mode and pulmonary pulsed wave Doppler echocardiography. RV ejection fraction was measured using the M-mode echocardiography. The peak systolic RV pressure was measured directly by inserting a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, ADInstruments) into the RV at the end of experiment prior to sacrifice. The recording speed for Millar-catheter based measurements was 1k/sec. The RV pressure was also estimated non-invasively at different time points in the same animal using serial pulsed-wave Doppler echocardiography to monitor the progression of the disease. The RV pressure were calculated from echocardiographic data using Mahan’s regression equation: MPAP = 79−(0.45×PAAT)\(^1\) in which MPAP is mean pulmonary artery pressure and PAAT is the pulmonary artery acceleration time, that is measured from the pulsed-wave Doppler echocardiogram. The values of RV pressure measured by both methods (echocardiography and direct RV catheterisation) were, on average, similar.

Western blot analysis

Western Blot analysis was done using lung and RV lysates. Lung and RV tissue was homogenized at 4°C (mM): 150 NaCl, 50 Tris-HCl, 1 EGTA, 1 EDTA, 1 NaF, 1 PMSF, 1 Na\(_3\)VO\(_4\), 1% NP-40, 0.1% SDS and 0.5% Sodium Deoxycholate (pH 7.4) combined with Protease and Phosphatase Inhibitor cocktails (Roche). All samples were centrifuged for 10 minutes at 12,000 RPM and the supernatant were gathered. Protein concentrations were determined and 100 µg of total protein was loaded on a 4-20% gradient Tris/HCl SDS polyacrylamide gel, electrotransferred to nitrocellulose paper, blocked with 5% non-fat dry milk in 20 mM of TBS with 0.1% Tween and incubated with primary antibodies. Vinculin or GAPDH served as a marker for equal loading of protein in each lane. The blots were labeled with infrared fluorophore conjugated anti-mouse and anti-rabbit
secondary antibodies for 1 h. Later on, the blots were observed using Odyssey™ Imaging System (Li-Cor). In the immunoblots in Fig 3 and 4, all samples from CTRL, RHF and GEN were run on the same gel or on two gels at the same time due to the lack of space. The blots were incubated together with the primary and secondary antibodies and were scanned at the same time with the same laser intensity. Since we are only showing one representative lane from a total of 4-5 samples per group, some of the intervening lanes were not shown and are separated by a dotted line if the samples were run on the same gel or a continuous line if they were from different blots. The Western blot images were captured with the Odyssey imaging system and the signal intensity was measured using Image-J software in each lane for the loading CTRL as well as the protein of interest. The signal intensity of the protein of interest in each lane was divided by the signal intensity of its corresponding loading control. The average of normalized intensities (protein of interest/loading control) in CTRL group was then calculated. All the individual normalized protein of interest/loading control in each group even in the CTRL group were divided by the average normalized protein of interest/loading control value in CTRL to set the CTRL to 1. The SEMs were calculated using the individual normalized protein of interest/loading control in each group.

**Tissue fixation and stainings**

Lungs were fixed in situ via the trachea cannula with buffered formaldehyde (4% paraformaldehyde in PBS, pH 7.4) at a pressure of 25 cmH2O for 5 min as described in our previous studies. Whole heart and lungs were fixed in 4% paraformaldehyde (PFA) in 0.1M Na2HPO4 and 23 mM NaH2PO4 (pH 7.4) for 4 h on ice. The tissue was then immersed in ice-cold 20% sucrose overnight to cryoprotect the tissue, was mounted using OCT, and transversal 6-7 μm sections were obtained with a cryostat.

**Immunohistochemistry** - Tissue sections were stained with immunofluorescence, standard hematoxylin-eosin and Masson trichrome staining. The images were acquired using a light microscope (Axiovert 135, Zeiss Germany) or with a laser scanning confocal microscope (Olympus).

**Immunofluorescence staining** - Heart and lung sections (4-5μ) were placed in acetone at −20°C for 15 min. Then, the sections were washed with PBS + 0.1% Triton three times for three minutes each. Next, the sections were incubated with PBS + 0.1% Triton and 10% normal goat serum (NGS) for 30 min in order to block the background. Afterwards, the sections were incubated with primary antibodies mixed in PBS + 0.1% Triton and 1% NGS at 4°C overnight. The following day, the sections were again washed using PBS + 0.1% Triton three times and incubated with corresponding secondary antibodies mixed in PBS + 0.1% Triton and 1% NGS at room temperature for 1 hr. Heart sections were washed with PBS + 0.1% Triton three times while the lung sections were washed with only PBS three times. After the washing step, only the heart sections were incubated with wheat germ agglutinin (WGA, 1:200 dilution) in a PBS + 0.1% Triton and 1% NGS mixture at room temperature for 1 hr for the purpose of quantifying capillary density. During this time, the lung sections were mounted using Prolong gold (Molecular Probes).
After the 1 hr incubation with WGA, the heart sections were washed with PBS three times and mounted using Prolong gold for confocal microscopy.

**Pulmonary morphometry**

Pulmonary morphometry was performed using ImageJ software. Various parameters of vascular remodelling were measured as shown in Table S1. Medial thickness is the thickness of the smooth muscle cell layer of the arterioles. Intimal thickness is the thickness of the endothelial cell layer of the arterioles. Endoluminal diameter is the inner diameter of the lumen of the arterioles; and the mediointimal thickness is the combined thickness of media and intima.

**Cell Proliferation Assays**

Cryopreserved human pulmonary artery smooth muscle cells (hPASMC, Invitrogen) and human pulmonary artery endothelial cells (hPAEC, Invitrogen) were cultured in Medium 231 supplemented with smooth muscle growth supplement (SMGS, Invitrogen) or Medium 200 supplemented with low serum growth supplement (LSGS, Invitrogen) respectively. Cells were plated into a 96 well plate at a density of 1000 cells/well. Cells were serum starved overnight and proliferation was induced by replenishment with serum, phenol red free medium and SMGS (for hPASMCs) or LSGS (for hPAEC) in the presence or absence of genistein. Cell proliferation was measured by the MTT Cell Proliferation Assay (ATCC) according to the manual. Proliferation rate without serum replenishment served as a negative control. All experiments were repeated with at least 8 replicates and 3-5 independent times.

**Detection of cellular apoptosis**

hPASMCs were plated on poly-D-lysine coated coverslips, starved overnight, and replenished with serum and phenol red free medium in the presence of genistein (1μM), DPN (1μM), or left untreated. After 24 hours, cells were fixed in 4% paraformaldehyde and stained with Roche In Situ Cell Death Detection Kit TMR red (Catalog #12156792910), exactly as described in the company protocol. Cells were costained with DAPI and the ratio of apoptotic cells to total cells was calculated. All experiments were performed in triplicate at least 3 independent times.

**In vitro cardiomyocyte hypertrophy studies**

Neonatal rat ventricular myocytes (NRVM) were isolated as previously described. Cells were cultured in phenol red free DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 100μM 5-bromo-2-deoxyuridine (Sigma) to inhibit the growth of cardiac fibroblasts. Cells were starved overnight and treated with Phenylephrine (10μM) in the presence or absence of genistein (1μM), selective ERα-agonist PPT (4,4',4''-(propyl-[ (1)H]-pyrazole-1,3,5-triy] trisphenol, 10μM), selective ERβ-agonist DPN [2,3-bis-(4-hydroxyphenyl) propionitrile, 10μM], or ER-antagonist ICI 182,780 (ICI, 10μM)+genistein (1μM) for 48 hours. Cells were fixed with acetone at -20°C for 15 min and stained with anti-α-actinin (1:500, Sigma). Images were taken with high-resolution confocal microscope and cell surface area was quantified with ImageJ.
software and normalized to that of control cells. All experiments were performed in duplicate and at least 3 times (n≥100 cells per group).

**Reagents**

The list of primary antibodies used: anti-smooth muscle actin (Thermo Scientific, 1:200), anti-PECAM (CD31, Millipore, 1:200), anti-VEGF (Santa Cruz Biotechnology Inc., 1:200), anti-ER alpha (Santa Cruz Biotechnology Inc., 1:200), anti-ER beta (Thermo Scientific, 1:200), anti-von Willebrand factor (Abcam, 1:500), anti-Vinculin (V 9131, Sigma, 1:10,000) anti-α-actinin (Sigma, 1:500) and anti-GAPDH (Novus Biologicals, 1:1000). The list of secondary antibodies used: goat anti rabbit alexa 488 (1:1000) for immunofluorescence, goat anti- rabbit alexa 680 (1: 100,000, Invitrogen) and goat anti-mouse IR Dye 800 CW (1: 100,000, Odyssey, LI-COR) for Western immunoblotting.
Reference List


Table S1. Genistein reverses pulmonary vascular remodeling.

<table>
<thead>
<tr>
<th>Remodeling parameters</th>
<th>CTRL</th>
<th>PH</th>
<th>RHF</th>
<th>GEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial thickness (μm)</td>
<td>5.1±0.37</td>
<td>15.9±1.26**</td>
<td>17.7±1.27**</td>
<td>5.1±0.48††‡‡</td>
</tr>
<tr>
<td>Intimal thickness (μm)</td>
<td>3.1±0.20</td>
<td>4.9±0.41**</td>
<td>5.3±0.42**</td>
<td>4.1±0.33*‡</td>
</tr>
<tr>
<td>Endoluminal diameter (μm)</td>
<td>70.9±3.78</td>
<td>41.4±3.67**</td>
<td>35.2±2.50**</td>
<td>63.9±3.08††‡‡</td>
</tr>
<tr>
<td>Endoluminal diameter/</td>
<td>8.6±0.61</td>
<td>1.9±0.12**</td>
<td>1.5±0.13**</td>
<td>6.9±0.59††‡‡</td>
</tr>
<tr>
<td>Mediointimal thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Medial thickness (the thickness of the smooth muscle cell layer of the arterioles), intimal thickness (the thickness of the endothelial cell layer of the arterioles), endoluminal diameter (the inner diameter of the lumen of the arterioles) and the ratio of endoluminal diameter to mediointimal thickness (the combined thickness of media and intima) in Control (CTRL), pulmonary hypertension (PH), right heart failure (RHF) and genistein treated group (GEN). Values are mean±SEM. *p<0.05 vs. CTRL, ‡p<0.05 vs. RHF, **p<0.01 vs. CTRL; ††p<0.01 vs. PH; †††p<0.01 vs. RHF.
Table S2. Genistein therapy restores RV systolic dysfunction associated with PH-induced RHF.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CTRL (n=7)</th>
<th>RHF (n=10)</th>
<th>GEN (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (BPM)</td>
<td>281.1±5.0</td>
<td>259.7±10.8</td>
<td>295.7±14.6</td>
</tr>
<tr>
<td>RV dp/dt\text{Max} (mmHg/s)</td>
<td>2115.1±119.9</td>
<td>1752.8±106.8*</td>
<td>2529.1±143.5†</td>
</tr>
<tr>
<td>RV dp/dt\text{Min} (mmHg/s)</td>
<td>-2024.2±216.2</td>
<td>-1618.4±118.1</td>
<td>-1935.5±152.3</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>104.3±2.3</td>
<td>105.1±4.2</td>
<td>100.8±2.9</td>
</tr>
<tr>
<td>LV dp/dt\text{Max} (mmHg/s)</td>
<td>5967.6±188.0</td>
<td>5914.6±260.9</td>
<td>5426.8±326.5</td>
</tr>
<tr>
<td>LV dp/dt\text{Min} (mmHg/s)</td>
<td>-4371.1±143.9</td>
<td>-3808.8±213.6</td>
<td>-3980.9±312.8</td>
</tr>
</tbody>
</table>

Cardiac hemodynamic parameters measured by direct cardiac catheterization in CTRL, RHF and GEN groups. (dp/dt)\text{Max}, maximum rate of pressure rise and (dp/dt)\text{Min}, maximum rate of pressure decline in RV or LV. Values are Mean±SEM. *p<0.05 vs. CTRL, †p<0.05 vs. RHF.
Figure S1. Genistein therapy reverses cardiac hemodynamic and structural changes induced by pulmonary hypertension. Echocardiographic images of M-mode (A) showing RV, RV end diastolic diameter (EDD), LV and Pulsed-wave Doppler of pulmonary artery flow (B). Red arrows show mid-systolic notch present in PH and RHF only.
Figure S2. Genistein therapy reverses increase in lung weight associated with PH. Bar graphs showing lung weight (g) in CTRL, PH, RHF and GEN groups. *p<0.05 vs. CTRL, **p<0.01 vs. CTRL; ††p<0.01 vs. PH; ^^p<0.01 vs. RHF.
Fig. S3. Genistein Therapy results in a marginal increase in cell death.
A. TUNEL (red) and DAPI (blue) staining of HPASMCs treated with DPN (1µM), genistein (1µM) or not treated (CTRL). B. The apoptosis% (the number of TUNEL positive stained cells divided by the total cell number in CTRL (black), DPN (blue) or Genistein (pink). *p<0.05 vs. CTRL.
Figure S4. RVP (mmHg) and RVEF (%) of individual rats treated with genistein (open circles for RVP and open squares for RVEF) together with their mean (closed circles for RVP and closed squares for RVEF).