Moderate Pulmonary Arterial Hypertension in Male Mice Lacking the Vasoactive Intestinal Peptide Gene

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Background—Vasoactive intestinal peptide (VIP), a pulmonary vasodilator and inhibitor of vascular smooth muscle proliferation, has been reported absent in pulmonary arteries from patients with idiopathic pulmonary arterial hypertension (PAH). We have tested the hypothesis that targeted deletion of the VIP gene may lead to PAH with pulmonary vascular remodeling.

Methods and Results—We examined VIP knockout (VIP−/−) mice for evidence of PAH, right ventricular (RV) hypertrophy, and pulmonary vascular remodeling. Relative to wild-type control mice, VIP−/− mice showed moderate RV hypertrophy, RV hypertrophy confirmed by increased ratio of RV to left ventricle plus septum weight, and enlarged, thickened pulmonary artery and smaller branches with increased muscularization and narrowed lumen. Lung sections also showed perivascular inflammatory cell infiltrates. No systemic hypertension and no arterial hypoxemia existed to explain the PAH. The condition was associated with increased mortality. Both the vascular remodeling and RV remodeling were attenuated after a 4-week treatment with VIP.

Conclusions—Deletion of the VIP gene leads to spontaneous expression of moderately severe PAH in mice during air breathing. Although not an exact model of idiopathic PAH, the VIP−/− mouse should be useful for studying molecular mechanisms of PAH and evaluating potential therapeutic agents. VIP replacement therapy holds promise for the treatment of PAH, and mutations of the VIP gene may be a factor in the pathogenesis of idiopathic PAH. (Circulation. 2007;115:1260-1268.)

Key Words: cardiovascular diseases ▪ genetics ▪ hypertension, pulmonary ▪ pathology ▪ peptides ▪ remodeling ▪ vasculature

I diopathic (primary) pulmonary arterial hypertension (IPAH) is a relatively rare but highly fatal disease characterized by progressive PAH and increased thickening of smaller pulmonary arteries and arterioles, culminating in right ventricular (RV) failure.1–3 Considerable advances have been made in recent years in our knowledge of the pathophysiology, pathology, and genetic basis of the disease, and its treatment is now more successful.4–8 Much remains to be learned, however, about the pathogenetic mechanisms of the disease, particularly the interactions among multiple predisposing genes, and the influence of selected environmental factors.

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A variety of observations over the years have linked the neuropeptide vasoactive intestinal peptide (VIP) to the pulmonary and systemic circulation. With special reference to the pulmonary vascular bed and its alterations in IPAH, VIP relaxes pulmonary vascular smooth muscle from several mammalian species in vitro, neutralizes or attenuates the actions of endothelin and other vasoconstrictors; reduces hypoxic pulmonary vasoconstriction in cats, newborn lambs, Fawn-Hooded rats, and rabbits with monocrotaline-induced pulmonary hypertension; and inhibits the proliferation of pulmonary vascular smooth muscle from patients with IPAH. Furthermore, VIP is a cotransmitter of the physiological nonadrenergic, noncholinergic system of pulmonary vascular smooth muscle relaxation. Finally, VIP-containing nerves, normally plentiful in the pulmonary artery, were recently reported absent in pulmonary arteries from IPAH patients, and inhalation of the peptide had a beneficial therapeutic effect on those patients. Here we report that mice with targeted deletion of the VIP gene (VIP−/−) show hemodynamic, echocardiographic, anatomic, and histological changes in pulmonary hypertension that are not attributable to arterial hypoxemia or any significant cardiopulmonary disease.
Methods

Animals
VIP−/− mice, backcrossed to C57BL/6 mice, were prepared locally as described and genotyped to confirm the absence of the VIP gene.22,23 We mated homozygous (VIP−/−) males with homozygous (VIP−/−) females or, if necessary, with heterozygous (VIP+/−) females. For genotyping, we extracted DNA from 1-cm-long tail snips using a DNA isolation kit (Qiagen Inc, Valencia, Calif). DNA (100 ng) was subjected to polymerase chain reaction using primers to detect both VIP and the neomycin cassette. Control, wild-type (WT) C57BL/6 mice were from Taconic Labs (Germantown, NY). We examined animals ranging in age from 9 to 52 weeks. The entire study was approved by the institutional animal review committees.

Chemicals and Reagents
VIP was from the Karolinska Institute (Stockholm, Sweden). All other chemicals, unless otherwise noted, were from Sigma Chemical Co (St Louis, Mo).

Hemodynamic Measurements
Five VIP−/− mice and 5 WT mice were anesthetized with ketamine (100 mg/kg) and fentanyl (0.05 mg/kg IP). A 1.4F 3-cm Mikro-Tip catheter (Millar Instruments Inc, Houston, Tex) was inserted through the right jugular vein and advanced to the right ventricle for digital recording of RV pressure. We also monitored left ventricular pressure in both groups of mice by direct catheterization via the carotid artery.

Echocardiographic Examination
Five VIP−/− and 5 WT mice were lightly anesthetized with pentobarbital (100 mg/kg IP). Echocardiographic examination was performed with a Vivid 7 (GE Medical Systems, Milwaukee, Wis) for mice equipped with a miniaturized high-frequency 13-MHz transducer. Evaluations were made offline, following the recommendations of the American Society of Echocardiography.24 RV and left ventricular size and function and pulmonary artery size were assessed in all animals.

Anatomic Assessment of RV Hypertrophy
The heart was isolated and placed under a dissecting microscope. Attached vessels and both atria were dissected and removed. The RV wall was cut out, blotted, and weighed; then the left ventricular wall and septum (LV+septum) were treated the same way and weighed. The RV(LV+septum) ratio was calculated in 6 male VIP−/− mice and 5 male WT mice as an index of RV hypertrophy. To evaluate the differences in vascular pathology between male and female mice, we assessed RV mass as measured by the mean RV(LV+septum) weight ratio in 6 female VIP−/− mice and 6 female WT mice.

Arterial Blood Gas Analysis
To explore the possibility that the PAH in VIP−/− mice was secondary to arterial hypoxemia, we measured arterial blood PO2 in 6 female VIP−/− mice, also assessed RV mass as measured by the mean RV(LV+septum) weight ratio in 6 female VIP−/− mice and 5 male WT mice.

Histological Examination and Morphometric Analysis
For all histological procedures, the lungs were inflated to full capacity and fixed by intratracheal instillation of 1 mL 10% neutral buffered formalin, immersed in formalin overnight, and then embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin or Masson’s trichrome stain for general morphology and morphometric analysis. Pulmonary arteries from 6 WT and 6 VIP−/− mice were analyzed; measurements were taken of 4 separate vessels from each mouse and averaged to 1 set of values. Only arteries near smaller bronchi or terminal bronchioles, ∼50 μm in diameter, were selected for analysis. We used the Image J program, version 1.34r (http://rsb.info.nih.gov/ij/), for measurement of total vessel area (mm²), luminal area (μm²), and inner circumference (μm). Medial area (μm²) was calculated as the difference between total and luminal areas. Standard medial thickness (μm) was calculated as the ratio of medial area to inner circumference as described by Weibel.26 Average vessel diameter (μm) was derived from total area measurements.

For immunohistochemical detection of α-smooth muscle actin, paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 5 minutes. Immunostaining was performed with a mouse monoclonal antibody directed against α-smooth muscle actin (Sigma) in conjunction with an avidin/biotin-based kit designed to detect mouse primary antibodies in mouse tissue (Mouse-on-Mouse Peroxidase Kit, Vector Labs, Burlingame, Calif) used according to the manufacturer’s instructions. The primary antibody was at a final dilution of 1:1000. Color was developed by 3-minute incubation with diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Labs), after which sections were washed, counterstained for 30 seconds with Hematoxylin QS (Vector Labs), dehydrated, and then mounted.

Progression of Vascular Pathology and Survival Rates
Possible progression of the pathological lesions was evaluated by assessing the degree of RV hypertrophy in 9 male VIP−/− mice 5 to 18 weeks of age, 9 mice 30 to 38 weeks of age, and 13 mice 51 to 143 weeks of age. Mortality rates were compared in 38 male VIP−/− mice and 15 WT controls.

VIP Replacement Therapy
Nine male VIP−/− mice 4 to 12 weeks of age received VIP (15 μg IP in 0.2 mL phosphate-buffered saline) every other day for 4 weeks for a total of 14 injections, ending the day before examination. Another group of 9 male VIP−/− mice of a similar age received 0.2 mL phosphate-buffered saline, without VIP, in the same manner and for the same duration. Our choice of the dosage, duration, frequency, and mode of administration of VIP was guided by protocols for related studies by other investigators.27 At the end of this treatment period, we evaluated the degree of RV thickening and vascular remodeling in smaller pulmonary arteries from the 2 groups of mice.

Gene Microarray Analysis
RNA was isolated from lung samples from male VIP−/− and WT mice and subjected to Affymetrix gene profiling (Expression Analysis, Durham, NC). The objective was to search for significant differences between the 2 groups in the expression of genes relevant to the pulmonary circulation. Genes of compounds that influence vasomotor tone, vascular smooth muscle proliferation, and collagen deposition were in special focus.

Statistical Analysis
All summary data for continuous variables were expressed as mean±SEM. For continuous variables, 2-group comparisons were performed with both the parametric 2-sample t test and nonparametric Mann-Whitney test. When the statistical results were similar between the 2 approaches, probability values from parametric t tests were reported. When the results were different from each another, probability values from both parametric and nonparametric tests were reported. For mortality data, Kaplan-Meier curves were generated and compared through the use of the log-rank test. All analyses were performed with SPSS software (Stata Corp, Inc, College Station, Tex), and a 2-sided value of P<0.05 was regarded as statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Hemodynamic Evidence of RV Hypertension in VIP Knockout Mice
Mean RV pressure in VIP\(^{-/-}\) mice (n=5) was significantly elevated relative to that in WT mice (n=5) (29.5±1.1 versus 16.3±2.2 mm Hg; \(P=0.001\)). Systolic left ventricular pressure, however, was normal (96.1±6.0 mm Hg; n=7) and was not significantly different from that in WT mice (96.2±4.5 mm Hg; n=6; \(P=0.76\); Tables 1 and 2).

Echocardiographic Confirmation of Pulmonary Arterial Thickening and RV Dilatation
Echocardiographic analysis showed the wall of the main pulmonary artery in VIP\(^{-/-}\) mice (n=5) to be 0.24±0.02 mm thick versus 0.16±0.02 mm in WT mice (n=5; \(P=0.013\)) and the pulmonary artery diameter to be wider in VIP\(^{-/-}\) mice (1.57±0.02 versus 1.24±0.14 mm; \(P=0.046\) for 2-sample t test, \(P=0.16\) for Mann-Whitney test). The area of the RV, a correlate of RV size, was greater in VIP\(^{-/-}\) mice than in WT mice: 8.47±1.08 mm\(^2\) during diastole and 5.99±1.01 mm\(^2\) during systole compared with 4.07±0.85 and 2.58±0.67 mm\(^2\), respectively, in WT mice (\(P=0.013\) and \(P=0.022\), respectively).

Anatomic Confirmation of RV Hypertrophy
The RV/(LV+septum) weight ratio, used here as a measure of RV hypertrophy, was 0.34±0.01 in male VIP\(^{-/-}\) mice (n=6), significantly higher than in male control WT mice (0.21±0.01; n=5; \(P<0.001\)). The same weight ratio in 6 female VIP\(^{-/-}\) mice was 0.24±0.01, not significantly different from the corresponding value in 6 female WT mice (0.26±0.02), suggesting no significant RV hypertrophy in female VIP\(^{-/-}\) mice.

Histological and Morphometric Evidence of Thickened, Remodeled Pulmonary Arteries
Comparing pulmonary arteries of similar diameter (45 to 50 μm), the medial wall was significantly thicker and the lumen was significantly narrower in VIP\(^{-/-}\) mice (n=6) than in control WT mice (n=6; Figure 1) (medial thickness, 14.45±2.86 versus 5.88±0.53 μm, \(P=0.030\); ratio of medial area to total area, 0.68±0.04 versus 0.43±0.04, \(P<0.001\)). The most striking abnormality was a marked increase in the ratio of medial area to luminal area, which averaged 2.78±0.45 versus 0.83±0.13 (\(P=0.006\)). Numerous vessels were so severely narrowed they appeared almost totally occluded.

In addition to the hematoxylin and eosin stain, which formed the primary basis for morphometric analysis (Figure 2), Masson’s trichrome stain demonstrated pronounced proliferation of medial smooth muscle and collagen (Figure 3), which was corroborated by α-smooth muscle actin immuno-
staining (Figure 4). Little or no endothelial cell proliferation was observed.

Perivascular Inflammation
Clusters of inflammatory cell, predominantly mononuclear, infiltrates were observed around smaller pulmonary vessels and airways (Figure 5).

No Hypoxemia to Explain the Pulmonary Hypertension
Mean arterial oxygen tension (PaO₂), measured in samples collected from the carotid artery from 7 VIP⁻⁻⁻⁻ mice during air breathing, was 93.9±9.8 mm Hg compared with 93.1±9.1 mm Hg for WT mice (n=7; P=0.96). Confirming this normal finding, hemoglobin O₂ saturation in VIP⁻⁻⁻⁻ mice measured by direct oximetry was 96%.

No Systemic Vascular Pathology
The renal arteries, examined as representative of systemic vascular beds, showed no evidence of vascular thickening such as that observed in the pulmonary arteries.

VIP Treatment Reduces RV Hypertrophy and Pulmonary Vascular Remodeling
Nine male VIP⁻⁻⁻⁻ mice that had been treated with VIP for 4 weeks showed considerably less RV hypertrophy than 9 control mice that had merely received buffer. The RV/(LV+septum) ratio was 0.25±0.01 in the VIP-treated group (n=9), significantly lower than that in the buffer-treated group (0.34±0.01; n=9; P<0.001; Table 3) but not as low as in WT mice (0.21±0.01; n=5; P=0.002). In the same 2 groups of mice, the walls of smaller pulmonary arteries were proportionately less thickened in the VIP-treated than in the buffer-treated controls. Thus, the mean ratio of medial area to total area in smaller vessels from the VIP⁻⁻⁻⁻ mice (n=9) was 0.59±0.06 compared with 0.74±0.03 in the buffer-treated mice (n=9; P=0.045 for 2-sample t test and P=0.065 for Mann-Whitney test; Table 3).

Progression of Pathological Lesions and Decreased Survival in Knockout Mice
Despite the generally moderate severity of PAH and the lack of intimal proliferation, the pulmonary vascular pathology in the VIP⁻⁻⁻⁻ mice showed evidence of being progressive; mice >30 weeks of age had an RV/(LV+septum) ratio of
0.34±0.01 compared with 0.38±0.01 in mice <30 weeks of age (P=0.047 based on 2-sample t test; P=0.026 based on nonparametric Mann-Whitney test). In addition, the VIP−/− mice had a higher mortality rate relative to WT controls (P<0.001 for log-rank test; Figure 6).

Gene Microarray Analysis
Lungs from VIP−/− mice showed significant alterations in the expression of several genes pertinent to pulmonary vascular tone and vascular remodeling. Genes for platelet-derived growth factor receptor β polypeptide and platelet-derived growth factor-β polypeptide, procollagen type I, α1, endothelin receptor A, and angiopoietin 2 were upregulated by 1.8-, 1.3-, 1.3-, 1.4-, and 1.6-fold, respectively. On the other hand, the gene for adrenomedullin, a pulmonary vasodilator and antiproliferative peptide, was downregulated by 50%.

Discussion
Our results demonstrate that male VIP−/− mice exhibit moderately severe PAH, with remodeled, muscularized pulmonary arterioles and smaller arteries, RV hypertension, and RV hypertrophy. Despite the presence of peribronchial cellular infiltrates and airway hyperresponsiveness in VIP−/− mice, as recently reported,23 no arterial hypoxemia or other signs of significant pulmonary or cardiac disease was present.

The pulmonary vascular alterations in this experimental model closely resemble those in patients with moderately severe IPAH.2 Morphometric analysis showed that, for pulmonary arteries of comparable external diameter, vessels from VIP−/− mice typically had markedly thickened medial layer and narrowed lumen, with a mean medial area/luminal area ratio 3.35 times that in WT mice. Complementary immunohistochemical studies revealed the medial thickening to result from accumulation of multiple layers of smooth muscle and collagen. The degree of medial thickening varied somewhat within the VIP−/− mice, reflecting some degree of phenotypic heterogeneity. Endothelial cell proliferation, typically seen in advanced forms of the disease,28 was not observed in these mice, possibly reflecting the moderate severity of the pathological process or its relatively short duration. Alternatively, deletion of the VIP gene may result in partial expression of the pathological features of the human disease, other genetic defects being required for the missing features such as endothelial cell proliferation and the fuller expression of the disease process.
Our observations were largely limited to male mice, both 
VIP/H11002/H11002 and WT. Preliminary comparisons of lung sections from male and female mice confirmed the impression that medial thickening was less pronounced in female VIP/H11002/H11002 mice than in their male counterparts. Similar gender differences have been reported in other models of PAH. In a study of mice with deletion of the endothelial nitric oxide synthase gene, structural evidence of pulmonary hypertension was evident in male but not in female adult mice. Further investigation of gender differences in the expression of the PAH phenotype in VIP−/− mice and of the possible role of estrogen in such differences is clearly in order. A predominant expression of the pulmonary vascular abnormalities in male VIP−/− mice, if confirmed, would be clearly different from human IPAH, which is more prevalent in women.

In addition to the pulmonary vascular abnormalities in male VIP−/− mice described here, lungs of the same mice showed perivascular and peribronchiolar inflammatory cell infiltrates (Figure 5). Coexistence of the 2 sets of findings is unlikely to be a mere coincidence; at least 2 groups of investigators have focused on the importance of inflammation as a factor in the pathogenesis of PAH.

The VIP-related pituitary adenylate cyclase–activating peptide has VIP-like actions on the pulmonary circulation, such as vasodilation and inhibition of vascular remodeling. Mutant mice lacking the principal receptor for pituitary adenylate cyclase–activating peptide, the PAC1 receptor, present with severe pulmonary hypertension and RV failure, causing their death within the first postnatal weeks. Thus, deletion of either VIP or pituitary adenylate cyclase–activating peptide (or its main receptor) results in an experimental model of PAH. Although the 2 peptides are closely related both structurally and functionally and their actions are mediated by common receptors (of which PAC1 binds with pituitary adenylate cyclase–activating peptide with considerably greater affinity than with VIP), it is clear that neither peptide compensates for the absence of the other. It appears likely therefore that both peptides and their signaling pathways are probably required for the maintenance of normal hemodynamics of the pulmonary circulation.

The mechanisms and pathways by which the absence of the VIP gene leads to expression of pathophysiological features of PAH are under investigation. The sequence of events probably begins with pulmonary vasoconstriction, which

<table>
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<th>Mouse</th>
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LVSP indicates left ventricular systolic pressure.

*P=0.76.
causes increased pulmonary vascular resistance, leading to pulmonary and RV hypertension. Vascular remodeling would follow as an adaptive response to give the pulmonary vessels sufficient support to withstand the elevated pressure, and the process would culminate in RV hypertrophy and failure.

Strong evidence has linked the pathogenesis of IPAH to mutations in the bone morphogenetic protein receptor-2 gene. Dysfunctional Smad signaling of transforming growth factor- probably accounts, at least in part, for the excessive smooth muscle cell proliferation in IPAH. Interactions between VIP and transforming growth factor- have already been reported. Thus, transforming growth factor-β, together with ciliary neurotrophic factor, synergistically induces VIP gene expression through the cooperation of Smad and other pathways. A variety of additional mechanisms may contribute to the smooth muscle cell proliferation and migration, a prominent feature in our experimental model. Preliminary data from gene microarray analysis suggest the involvement of other pathways with an established role in the pathogenesis of that disease. These include upregulation of endothelin and platelet-derived growth factor signaling, the angiopeptin/Tie 2 pathway, and collagen deposition. On the other hand, the gene for adrenomedullin, a pulmonary vasodilator and antiproliferative peptide, was significantly downregulated. VIP also has been demonstrated to induce the biosynthesis of tetrahydrobiopterin, a critical cofactor in endothelial nitric oxide synthase function. Thus, the lack of the VIP gene may be expected to lead to decreased endothelial nitric oxide production.

Because bone morphogenetic protein receptor-2 heterozygous mice show only mild pulmonary hypertension, many believe that “multiple genetic hits” are needed for the full expression of the disease. Our results suggest that a single hit may suffice for the expression of at least 1 experimental model of the disease; deficiency of the VIP gene alone resulted in the expression of a moderate PAH phenotype, although other gene alterations, secondary to the loss of the VIP gene as outlined above, may have contributed. Our findings also suggest the need for investigating the possibility that mutations in the VIP gene may be a factor in the pathogenesis of IPAH in humans.

Unlike most other models of PAH, the VIP mouse expresses spontaneous PAH, including remodeled pulmonary vessels and RV hypertrophy, during normoxic breathing. In most other models, including those based on deletion of the endothelial nitric oxide synthase gene, vascular endothelial growth factor receptor-2 blockade, and platelet-derived growth factor pathway activation, frank expression of PAH and vascular remodeling requires the additional stimulus of hypoxia.

Finally, the marked and highly significant attenuation of RV hypertrophy and of medial thickening after treatment

<table>
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<th>Mouse</th>
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<th>RV Weight, mg</th>
<th>LV+Septum Weight, mg</th>
<th>RV/(LV+Septum)</th>
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| Buffer treated |
| 10    | 23.1      | 16      | 29.3         | 76.2                | 0.38           | 0.81                                          |
| 11    | 21.6      | 6       | 24.9         | 71.7                | 0.35           | 0.67                                          |
| 12    | 22.2      | 6       | 27.5         | 80.6                | 0.34           | 0.63                                          |
| 13    | 24.0      | 13      | 28.2         | 80.0                | 0.35           | 0.86                                          |
| 14    | 24.3      | 13      | 22.2         | 75.0                | 0.30           | 0.59                                          |
| 15    | 25.5      | 16      | 23.8         | 80.7                | 0.29           | 0.76                                          |
| 16    | 24.9      | 13      | 27.2         | 78.7                | 0.35           | 0.83                                          |
| 17    | 23.5      | 14      | 25.7         | 75.0                | 0.34           | 0.76                                          |
| 18    | 24.5      | 14      | 32.0         | 87.8                | 0.36           | 0.73                                          |
| Mean  | 23.7      | 12.3    | 26.8         | 78.4                | 0.34†          | 0.74†                                         |
| SEM   | 0.4       | 1.3     | 1.0          | 1.6                 | 0.01           | 0.03                                          |

*P < 0.001; †P = 0.045 (t test); ‡P = 0.065 (Mann-Whitney test).
with VIP suggests that the peptide may be capable of preventing or at least slowing the progression of the key pathological changes in PAH, thus reinforcing its potential therapeutic value in patients with IPAH.18

Acknowledgments
We thank Maria Rienzi for help with the preparation of the manuscript and Tarek Abdel-Razek and Mathew Chin for assistance with the research.

Sources of Funding
This work was supported by NIH grants HL–70212, HL–68188 (to Dr Said), K08 HL71263 (to Dr Szema), and DK62722 (to Dr Lin), by an AHA grant (to Dr Lin), and by VA research funds.

Disclosures
Dr Said is a consultant or on the advisory board at MondoBiotec. The other authors report no conflicts.

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**CLINICAL PERSPECTIVE**

Despite significant advances in our understanding of its pathogenesis and improvements in its prognosis, idiopathic pulmonary arterial hypertension remains an incompletely understood, incurable disease. In the present study, male mice lacking the gene for the vasoactive intestinal peptide, a vasodilator of pulmonary and systemic vessels and an inhibitor of vascular smooth muscle proliferation, showed features of moderately severe idiopathic pulmonary arterial hypertension. In addition to pulmonary hypertension, smaller pulmonary arteries were markedly thickened with medial accumulation of smooth muscle, and the right ventricle was hypertrophied. Confirming the cause-and-effect relationship between the vasoactive intestinal peptide gene deletion and the pulmonary vascular pathology, administration of vasoactive intestinal peptide (15 μg IP every other day for 4 weeks) attenuated the vascular remodeling and right ventricular hypertrophy. This experimental model, in which lesions resembling those of clinical idiopathic pulmonary arterial hypertension are expressed secondary to the loss of a single gene, should prove useful in exploring pathogenetic mechanisms of the disease, especially the interactions between genetic pathways, and in testing the efficacy of new investigational drugs. The ability of vasoactive intestinal peptide to ameliorate the pulmonary arterial hypertension pathology in these mice provides a solid rationale for its therapeutic potential in the human disease, as proposed in a recent clinical trial.
Moderate Pulmonary Arterial Hypertension in Male Mice Lacking the Vasoactive Intestinal Peptide Gene


*Circulation.* 2007;115:1260-1268; originally published online February 19, 2007;
doi: 10.1161/CIRCULATIONAHA.106.681718

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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