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Pulmonary Hypertension and Right Heart Failure in Pituitary Adenylate Cyclase–Activating Polypeptide Type I Receptor–Deficient Mice

Christiane Otto, MD, PhD; Lutz Hein, MD; Marc Brede, MD; Roland Jahns, MD; Stefan Engelhardt, MD, PhD; Hermann-Josef Gröne, MD; Günther Schütz, MD

Background—Pituitary adenylate cyclase–activating polypeptide (PACAP), acting via 3 different G protein–coupled receptors, has been implicated in the regulation of several homeostatic systems in the body, including cardiopulmonary control. To define the physiologic role of the PACAP-preferring type I receptor, PAC1, in cardiopulmonary function, we developed a mutant mouse strain lacking functional PAC1 receptors.

Methods and Results—When PAC1-deficient mice were crossed onto a C57BL/6 background, almost all mutants died during the second postnatal week. Whereas mutant mice were indistinguishable from their wild-type littermates at birth, they showed progressive weakness and died from rapidly developing heart failure. Right ventricles of PAC1 mutants were massively dilated and showed cardiac myocyte hypertrophy, whereas left ventricular structure was unaltered. On direct cardiac catheterization, right ventricular pressure was elevated by 45% in PAC1-deficient mice, indicating increased pulmonary artery pressure, as no malformations were detected in the valves or outflow tract of the right ventricle. Consistent with elevated pulmonary pressure, lung capillary density was decreased by 30% and small pulmonary arteries of mutant mice had significant vascular smooth muscle cell hypertrophy compared with wild-type mice.

Conclusions—Whereas PACAP induces vasodilation in isolated pulmonary vessels in wild-type mice, the absence of its specific receptor PAC1 causes pulmonary hypertension and right heart failure after birth. These in vivo findings demonstrate the crucial importance of PAC1-mediated signaling for the maintenance of normal pulmonary vascular tone during early postnatal life. (Circulation. 2004;110:3245-3251.)

Key Words: hypertension, pulmonary heart failure genetics

The highly conserved neuropeptide, pituitary adenylate cyclase–activating polypeptide (PACAP), binds to 2 classes of G protein–coupled receptors. PACAP type II receptors (VPAC1 and VPAC2) display equally high affinity for PACAP and its related peptide, vasoactive intestinal peptide (VIP), whereas the PACAP type I receptor (PAC1) exhibits 1000-fold higher affinity for PACAP than for VIP.1 PAC1-mediated signaling has been implicated in a variety of biologic processes, such as neurotrophic actions, immune and pituitary function, circadian rhythmicity, learning and memory, and catecholamine biosynthesis.2 In addition, PACAP and its receptors have been implicated in the regulation of cardiopulmonary function.3 Within the lung, PAC1 as well as VPAC1 and VPAC2 mRNA and protein has been detected by immunoblot analysis.4,5 VPAC1 and VPAC2 proteins are expressed in smooth muscle cells of blood vessels and bronchi.4 PACAP-positive nerve fibers have been found among smooth muscle bundles in tracheal and bronchial walls and around small blood vessels.6 PACAP as well as VIP exerts dilatory effects on pulmonary blood vessels7–10 and bronchi.9–11 In contrast to the effects of VIP, PACAP-induced dilation of pulmonary arteries depends on an intact endothelium and can be blocked by nitric oxide synthase inhibitors.8,12 Thus, different mechanisms underlying VIP’s and PACAP’s dilatory effects on pulmonary arteries as well protein expression data support the existence of both PACAP receptor classes within the lung.4,5

Recently, it has been suggested that VIP could serve as a new drug for treatment of primary pulmonary hypertension.13 Patients suffering from pulmonary hypertension exhibit reduced VIP levels within their sera and lung tissue, whereas the receptors VPAC1 and VPAC2 are upregulated. Expression of the PACAP-preferring receptor PAC1 was not analyzed.13 Taking into account that VPAC2-deficient mice do...
not develop pulmonary hypertension, there are 2 receptor candidates left, VPAC1 and PAC1, that possibly mediate VIP’s beneficial effects in patients with pulmonary hypertension. In this study, we provide genetic evidence that PAC1 is critically involved in cardiopulmonary regulation, because PAC1-deficient mice suffer from pulmonary hypertension leading to severe right heart failure and death.

**Methods**

**Animals**

The PAC1-deficient mouse strain has been described earlier. Mice used in this study were offspring of N3 heterozygous mice backcrossed onto a C57BL/6 background following the regulations for animal experimentation of the German Cancer Research Center. For analysis of postnatal lethality and weight gain, 25 mutant and 18 wild-type animals as well as heterozygous mice from 13 litters were examined daily from birth to postnatal day 22.

**Histologic Analysis**

For hematoxylin/eosin and Masson’s trichrome staining, hearts and livers of 8-day-old littermates were immersion-fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. For oil red O staining, tissues were embedded in Tissue-Tek (Sakura) and frozen on dry ice. For electron microscopy, tissues were fixed in Karnovsky’s fixative and embedded in araldite. For lung histology and morphometry, anesthetized mice were perfused via the right ventricle with 1% phosphate-buffered paraformaldehyde at 30 cm H2O, followed by tracheal perfusion at constant pressure of 20 cm H2O. For determination of myocyte cross-sectional area, individual cells per genotype from at least 3 different animals were analyzed morphometrically. Only nucleated cardiac myocytes from areas of transversely cut muscle fibers were included in the analysis.

**Corticosterone and Metabolic Measurements**

Blood samples were collected into heparinized capillary tubes after decapitation of 4- to 9-day-old mice at 10 AM. Corticosterone was measured by radioimmunoassay (ICN Pharmaceuticals). Determinations of triglyceride, free fatty acid, ketone body, and lactate concentrations were performed according to the manufacturer’s instructions (Sigma). Glucose measurements were performed with a glucometer (Accutrend).

**RNase Protection Analysis**

Total RNA from heart ventricles of 9-day-old mice was prepared and subjected to RNase protection analysis as described. The atrial natriuretic peptide (ANP) probe used spanned nucleotides 594 to 937 of the murine ANP gene.

**Echocardiography**

Transthraxic echo Doppler examinations were performed as described in lightly sedated (20 μL of 2.5% tribromoethanol IP), 10-day-old mice with an echocardiographic system (Vivid FiVe, GE Vingmed Ultrasound) equipped with a 8-MHz transducer. Measurements were performed offline by a reader blinded to the genotypes and following the recommendations of the American Society of Echocardiography.

![Figure 1](image1.png)

**Figure 1.** Postnatal arrest in body weight gain and death in PAC1-deficient mice. Within second postnatal week, majority of PAC1-deficient mice (−/−; filled squares, n=25) died while heterozygous and wild-type littermates (+/+; open triangles, n=18) survived (a). Typical pair of wild-type and mutant littermates is shown at postnatal day 8 (b). While PAC1-deficient mice were indistinguishable from their wild-type littermates at birth, there was arrest in body weight gain starting at postnatal day 6 (c). pp. indicates postpartum. All other abbreviations are as defined in text.

![Figure 2](image2.png)

**Figure 2.** Metabolic decompensation in PAC1-deficient mice shortly before death. From day 4 to postnatal day 7, there were no significant differences in corticosterone (mutants, black bars, n=5; wild types, white bars, n=5) (a) and triglyceride (mutants, n=12; wild types, n=14) (b) levels. On postnatal day 9, corticosterone levels in mutant mice (n=10) were almost 3-fold as high as in wild-type littermates (n=8), indicating that PAC1-deficient mice were severely stressed. In these later stages, triglyceride (b), free fatty acid (c), and lactate (d) levels were increased in mutants. In prefinal stages, mutant livers exhibited lipid accumulation around central veins (asterisks), as evident in oil red O–stained sections (f). Abbreviations are as defined in text. *P<0.05, ***P<0.005.
Twelve-day-old mice (7 mice per genotype) were anesthetized with tribromethanol (100 μL of 2.5% solution IP) and ventilated with a Harvard Minivent pump. After a thoracotomy was performed, a 1.4F microtip catheter (Millar Instruments) was inserted through the apex of the right ventricle, and right ventricular pressure traces were digitally recorded.17

### Hemodynamic Analysis

Twelve-day-old mice (7 mice per genotype) were anesthetized with tribromethanol (100 μL of 2.5% solution IP) and ventilated with a Harvard Minivent pump. After a thoracotomy was performed, a 1.4F microtip catheter (Millar Instruments) was inserted through the apex of the right ventricle, and right ventricular pressure traces were digitally recorded.17

### Statistical Analysis

Averaged data are presented as mean±SEM. Statistical analysis was carried out using the Prism software package (GraphPad). ANOVA followed by Bonferroni’s test was used for comparisons unless indicated otherwise. Differences were considered significant when $P<0.05$.

### Results

#### PAC1-Deficient Mice Die Within the Second Postnatal Week

PAC1-deficient mice were born at the expected mendelian ratio independent of the genetic background analyzed. In previous studies, we and others had already observed that on a mixed genetic background, 20% of mutant animals were lost until weaning for unknown reasons.15,22 On backcrossing of the mutant mouse strain onto a C57BL/6 background, we realized that almost all mutant mice were lost within the second postnatal week (Figure 1a). To facilitate analysis of the mechanisms leading to death in the PAC1 mutants, in this study we focused our analysis on mice backcrossed onto the C57BL/6 background. At birth, mutant mice were completely indistinguishable from their wild-type littermates. At approximately postnatal days 5 and 6, homozygous mutant mice started to suffer from progressive weakness and rapid fatigue. From postnatal day 6 onward, there was a significant difference in body weight between wild-type and mutant mice (Figure 1b and 1c).

Because the neuropeptide PACAP has been implicated in metabolic regulation, we analyzed several metabolic parameters on postnatal day 4 when the mutants still appeared healthy and on postnatal day 9 when most of the PAC1-deficient mice started to die (Figure 2). These measurements revealed metabolic decompensation in PAC1 mutants shortly before death. From postnatal day 4 up to postnatal day 7, corticosterone and triglyceride levels were normal in mutant mice. Only at prefinal stages were corticosterone, triglyceride, free fatty acid (Figure 2a–2c), ketone body (wild type, n=6, 6±0.9 mg/dL; mutants, n=5, 10±1.2 mg/dL; $P<0.05$) and lactate levels (Figure 2d) elevated in the sera of mutant mice.

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Figure 3. Enlarged hearts in PAC1-deficient mice. Compared with wild-type hearts (a), mutant hearts (b) were enlarged. Especially right ventricles (RV) of mutant mice (d) were dilated in comparison with wild-type littermates (c). Left ventricles (LV) appeared normal. Right ventricular cardiomyocytes of mutant animals (f) were hypertrophied. Scale bar=1.5 mm, a–d; 100 μm, e and f. Electron microscopy revealed disturbance of myofibrillar architecture (arrows) in septa of 8-day-old mutant mouse (h) compared with wild-type mice (g); mitochondria were swollen and had lost their cristae (arrowheads). There was increase of large lipid droplets in hearts from mutant mice (asterisks) (h). Magnification ×10 400. All other abbreviations are as defined in text.

Figure 4. Early postnatal onset of heart failure in PAC1 mutants. On postnatal day 4, first significant increase in heart weight–body weight index (b) was noted in PAC1-deficient mice (n=12, black bars), whereas body weight was not different from that of wild-type littermates (n=14, white bars) (a). On postnatal day 9, heart weight–body weight index was almost doubled in PAC1-deficient mice (b), and ANP expression was strongly induced in mutant heart ventricles (c). Morphometric analysis demonstrated hypertrophy of right ventricular but not left ventricular cardiac myocytes in PAC1 mutants (black bars) (d). GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase. All other abbreviations are as defined in text. *$P<0.05$, **$P<0.005$. 

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animals. Catecholamine levels in sera, hearts, and brains from PAC1-deficient mice and wild-type littermates were indistinguishable (data not shown). At prefinal stages, PAC1-deficient mice but not wild-type mice started to display fatty livers, showing lipid deposits restricted to the centrilobular areas surrounding the hepatic central veins (Figure 2e and 2f).

Blood gas analysis carried out in 8-day-old wild-type mice and mutants that did not show fatty livers at the time of analysis revealed hypoxemia in mutant animals (PO2 mutants, 43.4±1.8 mm Hg; PO2 wild types, 52.5±3.3 mm Hg; P<0.05).

Cardiac Hypertrophy in PAC1-Deficient Mice
Hearts of 9-day-old PAC1-deficient mice were severely enlarged (Figure 3a and 3b). Within the hearts, no malformations of valves or septal walls were detected, and there was no persistent ductus arteriosus. Right ventricles of mutant animals showed prominent dilatation (Figure 3c and 3d), whereas left ventricles did not appear conspicuously altered. There were no signs of inflammatory infiltration, fibrosis (Figure 3e and 3f), or enhanced apoptosis in mutant hearts (data not shown). Right ventricular cardiomyocytes of mutant animals (Figure 3f) were larger than in wild-type littermates (Figure 3e). At the ultrastructural level, hearts from 8-day-old PAC1-deficient mice displayed clear disruption of the myofibrillar architecture. Many lipid droplets (the lipid character was confirmed by oil red O staining; data not shown) were visible, and the mitochondria were swollen and had almost lost their typical cristae structure (Figure 3g and 3h).

The onset of the observed cardiac phenotype was first evident on postnatal day 4, when mutant mice exhibiting the same body weight as their wild-type littermates (Figure 4a) showed a slight increase in their heart weight–body weight index (Figure 4b). On postnatal day 9, the heart weight–body weight index was almost doubled in homozygous mutant mice in comparison with wild-type littermates (Figure 4b). At this time, ANP expression was strongly induced in ventricles from PAC1-deficient mice (Figure 4c), an observation characteristic of failing hearts. These data in concert with the clinical performance of the animals demonstrated that PAC1 mutants suffered from heart failure.

PAC1-Deficient Mice Display Signs of Right Heart Failure
As evidenced by morphometric analysis, cardiac myocytes of the right ventricle were significantly enlarged in 9-day-old PAC1-deficient mice (mutants, n=53, 150.2±4.4% of left ventricular wild-type cardiomyocyte size; wild types, n=49, 116.3±3.1%; P<0.0001). In contrast, cardiac myocytes of the left ventricle did not show any significant size differences between genotypes (Figure 4d). In PAC1-deficient mice,
electrocardiography showed clear dilatation of the right ventri-
cle in vivo (Figure 5a–5c). No differences were detected in
wall thickness, end-systolic or end-diastolic left ventricular
chamber diameters (Figure 5a, 5b, 5d), or left ventricular
fractional shortening between genotypes (Table 1). Together
with the results obtained from morphometric analysis, electro-
cardiography led to the conclusion that PAC1-deficient mice
suffered from selective right heart failure.

Pulmonary Hypertension in PAC1-Deficient Mice

Ten days after birth, maximal systolic pressure in the right
ventricle was significantly higher in mice lacking PAC1
(20.6±2.9 mm Hg, n=7, P<0.0005) than in wild-type litter-
mates (14.2±1.8 mm Hg, n=7; Figure 5e). Right ventricular
end-diastolic pressure and maximal rates of contraction (dP/
dt max) and relaxation (dP/dt relax) (Table 1) as well as heart rate
(Figure 5f) did not differ between genotypes.

The elevated systolic right ventricular pressure in PAC1-
deficient mice indicated an increased resistance in the right
ventricular outflow tract. Lungs from PAC1-deficient mice
did not exhibit any gross anatomic abnormalities, and there
was no evidence of lung edema (lung weight–body weight
index and rapid fatigue in mutant mice, these studies were performed in 1- to 2-week-
old mice weighing 5 to 6 g. At this small size, echocardiogra-
phy and invasive catheterization techniques are at their
limits. The first signs of heart failure, ie, increase in heart
weight–body weight index and rapid fatigue in mutant mice,

65±11% increase in media-lumen ratio in small pulmonary
arteries in PAC1-deficient mice compared with wild-type
littermates. The media hypertrophy of small pulmonary
vessels in concert with the elevated right ventricular systolic
pressure points to the existence of pulmonary hypertension in
PAC1 mutants.

Discussion

The present in vivo study elucidates a pivotal role for
PAC1-mediated signaling in cardiopulmonary function. Ge-
etic disruption of PAC1 in mice led to pulmonary hyperten-
sion, with subsequent right heart failure causing death within
the second postnatal week. Because of the early lethality of
mutant mice, these studies were performed in 1- to 2-week-
old mouse weighing 5 to 6 g. At this small size, echocardio-
yography and invasive catheterization techniques are at their
limits. The first signs of heart failure, ie, increase in heart
weight–body weight index and rapid fatigue in mutant mice,

![Figure 6. Pulmonary hypertension in PAC1-deficient mice. Morphometric analysis (a and b) showed increased muscularization of small pulmonary vessels in PAC1 mutants. Scale bar=200 µm. Abbreviations are as defined in text.](https://circ.ahajournals.org)
were observed on postnatal day 4. There was prominent dilatation of right ventricles and selective hypertrophy of right ventricular cardiomyocytes. The end-diastolic right ventricular diameter was enlarged, and right ventricular systolic pressure was significantly increased in mutant mice, whereas left ventricular function and structure remained normal. Typically, right heart failure was accompanied by hypoxia, provoking the accumulation of neutral lipids, first around the hepatic central veins where oxygen pressure is already low under physiologic conditions. A direct causal relation between impaired PAC1-mediated signaling and the development of fatty livers seemed unlikely, because PAC1 expression was barely detectable in mouse livers.18 In the end stages of heart failure, mutants suffered from severe weakness and metabolic decompensation, revealing signs of enhanced catabolism, a situation typical of cardiac cachexia.24 We conclude that the metabolic decompensation shortly before death is a consequence of severe right heart failure in mice lacking PAC1 and therefore, does not account for a specific primary role of PAC1 in lipid metabolism, as has been proposed for its ligand, in PACAP-deficient mice.25

Mainly 3 signs, rarefaction of lung capillaries, increased muscularization of small pulmonary vessels, and elevated right ventricular end-systolic pressure, directly pointed out that PAC1-deficient mice suffered from pulmonary hypertension. It is interesting to note that the observed alterations in media thickness (plus 65% in mutant mice) and right ventricular end-systolic pressure (plus 45% in mutant mice) in PAC1 mutants are identical to those seen in other murine models of pulmonary hypertension.26–28 Because of technical limitations, we were unable to directly measure pulmonary artery pressure in newborn mice. Nevertheless, it is generally accepted that elevations in right ventricular end-systolic pressure parallel elevations in pulmonary artery pressure.29 In contrast to our open-chest measurements, right ventricular systolic pressure is expected to be even higher, by 10 to 20 mm Hg, under intact-chest conditions.27,29,30

It remains an open question whether PAC1-deficient mice suffer from primary pulmonary hypertension (due to primary vasoconstriction of small pulmonary vessels) or whether pulmonary hypertension is secondary to primary alveolar hypoxia (due to bronchoconstriction followed by vasoconstriction). Recently, it has been suggested that PACAP-deficient mice suffer from reduced ventilation and a blunted response to hypoxia.31 Taking into account these findings may lead to the assumption that bronchoconstriction promotes secondary pulmonary hypertension in PAC1-deficient mice. However, the observation that hypoxia in our PAC1-mutant mice occurred in rather late stages of the disease may account for the opposite, ie, the existence of primary pulmonary hypertension in PAC1-deficient mice. Technically, this problem is difficult to solve in our conventional knockout animal model because the mice are too small at the potential time of analysis. Future studies with tissue-specific inactivation of PAC1 are needed to elucidate the mechanisms involved in the development of pulmonary hypertension in PAC1-deficient mice and will help clarify whether these mice suffer from primary or secondary pulmonary hypertension. The observation that PAC1-mediated signaling seems to play a pivotal role in the prevention of early postnatal pulmonary hypertension is in accordance with previous findings describing PACAP as a potent dilator of bronchi7–11 and pulmonary blood vessels.7–10 Our own preliminary experiments confirmed vasodilatory effects of PACAP on murine pulmonary arteries (L. Hein, unpublished data). Although PAC1 mRNA is expressed in lungs,5,18 it was not possible to unravel the cellular localization of PAC1 protein in lungs (C. Otto, unpublished data). Obviously, PACAP type II receptors, which are strongly expressed in lung,4,5 were not able to compensate for the lack of PAC1 and thus, do not seem responsible for mediating the dilatory effects of PACAP on vascular smooth muscle cells in vivo. This finding is quite exciting, taking into account a recent publication describing the PACAP-related peptide VIP as a potential new drug for the treatment of pulmonary hypertension in humans.13 The authors suggested that VIP exerts its beneficial effects via PACAP type II receptors, which were found to be upregulated in patients suffering from pulmonary hypertension, whereas VIP was downregulated. Unfortunately, expression of PAC1, which can also bind VIP, was not studied in those patients.13 Taking into account our results demonstrating that the absence of PAC1 leads to postnatal pulmonary hypertension followed by deleterious right heart failure and the fact that VPAC2-deficient mice do not suffer from pulmonary hypertension,14 we would like to suggest that PAC1 may also play a key role in human pathology of pulmonary hypertension. We anticipate that these findings will stimulate future research with PAC1-specific agonists in patients suffering from pulmonary hypertension.

Acknowledgments

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