

# Expression of Vasoactive Intestinal Peptide and Related Receptors in Overcirculation-Induced Pulmonary Hypertension in Piglets

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**ABSTRACT:** The pathobiology of pulmonary arterial hypertension (PAH) is not understood completely. Recent observations in patients with PAH and in knockout models have raised the idea that a defect in vasoactive intestinal peptide (VIP) may be involved in PAH pathophysiology. Therefore, we investigated the expressions of VIP, the related pituitary adenylate cyclase-activating polypeptide (PACAP), and their receptors (VPAC<sub>1</sub>, VPAC<sub>2</sub>, and PAC<sub>1</sub>) in piglets with overcirculation-induced pulmonary hypertension as an early-stage PAH model. Seventeen piglets were randomized to an anastomosis between the innominate and the main pulmonary artery, or to a sham operation. After 3 mo, a hemodynamic evaluation was performed and the lung tissue was sampled for biologic and histologic studies. The shunting increased pulmonary vascular resistance (PVR) by 100% and arteriolar medial thickness by 85%. VIP and VPAC<sub>1</sub> gene expressions were decreased and increased, respectively. VPAC<sub>1</sub> gene expression was positively correlated to PVR. VPAC<sub>2</sub> and PAC<sub>1</sub> immunoreactivity was seen in pulmonary arterial smooth muscle. VIP and PACAP immunostaining was observed in nerve fibers surrounding the pulmonary arterial smooth muscle. In conclusion, overcirculation-induced pulmonary hypertension is accompanied by a down-regulation of VIP signaling, without change in PACAP expression. These results are consistent with the notion that abnormal VIP signaling takes part in PAH pathogenesis. (*Pediatr Res* 66: 395–399, 2009)

The pathobiology of pulmonary arterial hypertension (PAH) is complex and is not understood completely (1). Several abnormal signaling pathways have been identified, involving the three layers of the pulmonary arterial walls, but their respective roles in initiating or perpetuating the disease remain undefined (1).

Recent observations have drawn attention to a possible implication of the vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) systems. VIP belongs to a family of structurally related hormones, including the PACAP. PACAP exists in two forms in mammals (PACAP<sub>27</sub> and PACAP<sub>38</sub>), derived from the same precursor. These ubiquitous and homologous neuropeptides

share pleiotropic functions and display highly preserved genes during evolution, suggesting their involvement in important physiologic mechanisms (2). Their actions are mediated through interaction with G-protein-coupled receptors (VPAC<sub>1</sub>, VPAC<sub>2</sub>, and PAC<sub>1</sub>): PAC<sub>1</sub> receptors have greater affinity for PACAP than for VIP, whereas VPAC receptors bind both the peptides with comparable affinity (2). VIP has been reported to promote the relaxation of vascular smooth muscle cells (3); to inhibit the proliferation of pulmonary arterial smooth muscle cells *in vitro* (4); to reduce pulmonary vasoconstriction induced by endothelin or hypoxia (5,6); and to present with antithrombotic effects (7). The cotransmitter PACAP also demonstrates actions similar to VIP on the pulmonary vasculature, such as vasodilation and inhibition of vascular remodeling (8).

Recent reports have focused on the assessment of the VIP/PACAP system in both patients with PAH and knockout murine models. On one hand, a VIP deficiency together with an overexpression of both VPAC receptors has been reported in a small series of patients with PAH (4). Chronic inhalation of VIP improved hemodynamics and exercise capacity in these patients, introducing the peptide as a possible new treatment for PAH (4). On the other hand, VIP knockout mice developed features of PAH in normoxia, with right ventricular hypertrophy and pulmonary vascular remodeling, both reduced after VIP replacement therapy (9). However, data regarding PACAP and its possible involvement in PAH are scarcer (10).

These findings provide a new aspect of PAH pathobiology that deserves to be explored in early stages of the disease. To our knowledge, VIP, PACAP, and their related receptors have never been explored together in a validated and satisfactory model of PAH. Therefore, we investigated the global VIP/PACAP system in overcirculation-induced pulmonary hypertension in growing piglets. This experimental model reproduces, in a 3-mo period, significant clinical pulmonary hypertension and small arteriole medial hypertrophy corre-

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**Abbreviations:** HR, heart rate; PACAP, pituitary adenylate cyclase-activating polypeptide; PAH, pulmonary arterial hypertension; PAP, mean pulmonary artery pressure; PAPO, occluded PAP; PVR, pulmonary vascular resistance; Q, cardiac output; SAP, systemic arterial pressure; VIP, vasoactive intestinal peptide

sponding to early stages of the disease (11). In the current study, we hypothesized that the VIP/PACAP signaling might be down-regulated in early PAH.

## MATERIALS AND METHODS

**Animals and surgical procedure.** These experiments were conducted in agreement with the guide for the care and use of laboratory animals of the U.S. National Institutes of Health. Seventeen 3-wk-old piglets were included in this study, with approval by the Committee on the Care and Use of Animals in Research of the Faculty of Medicine of the Free University of Brussels. The left subclavian artery was dissected and anastomosed to the pulmonary arterial trunk, according to the adapted Blalock-Taussig procedure (11). After randomization, the anastomosis was ligated in eight animals (sham-operated controls).

**Hemodynamic evaluation and tissue preparation.** After a 3-mo observation period, the animals were anesthetized, ventilated, and equipped with catheters and an ultrasonic flow probe on the pulmonary arterial trunk for measurements of mean pulmonary artery pressure (PAP), occluded PAP (PAPO), systemic arterial pressure (SAP), cardiac output (Q), pulmonary arterial flow, heart rate (HR), and blood gases. Pulmonary vascular resistance (PVR) was defined by multipoint (PAP-PAPO)/Q plots obtained by rapid inflation of the inferior vena cava balloon (11,12). Hemodynamic and blood gas measurements were obtained after ensuring steady-state conditions (stable HR, SAP, and PAP) for 60 min after shunt closure (11). After measurements, the animals were killed with an overdose of anesthesia. Pulmonary tissue samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ , or after overnight fixation in 10% formalin, embedded in paraffin for real-time quantitative PCR and histologic procedures, respectively.

**Morphometry.** Pulmonary arterial morphometry was performed on sections stained immunohistochemically with anti- $\alpha$ -smooth muscle actin antibody, as reported previously (11). Only arteries with an external diameter (ED)  $<500\ \mu\text{m}$  and a complete muscular coat were measured and assigned to five groups according to the ED: 0–75, 76–150, 151–225, 226–300, and 300–500  $\mu\text{m}$ . Medial thickness (MT) was related to arterial size using the following formula:  $\%MT = (2 \times MT)/(ED \times 100)$ .

**RNA isolation and reverse transcription.** Total RNA was extracted from whole lung tissue samples (100 mg), using TRIzol reagent (Invitrogen, <http://www.invitrogen.com>), according to the manufacturer's protocol. The extracted RNA was quantified by absorbance at 260 nm (BioPhotometer, Eppendorf, <http://www.eppendorf.de>), and its concentration was adjusted at  $0.25\ \mu\text{g}/\mu\text{L}$ . Reverse transcription was performed with the GeneAmp PCR system 2400 (Applied Biosystems, <http://www.appliedbiosystems.com>), using  $1\ \mu\text{g}$  of total RNA in a total reaction volume of  $20\ \mu\text{L}$ , containing random hexamers, a mix of deoxyribonucleotides, reverse transcription buffer, DTT, ribonuclease inhibitor, and Superscript II reverse transcriptase (Applied Biosystems), according to the manufacturer's instructions. The cDNA synthesis was conducted using the following cycle procedure:  $22^{\circ}\text{C}$  for 40 min,  $42^{\circ}\text{C}$  for 1 h, and  $99^{\circ}\text{C}$  for 5 min.

**Real-time quantitative PCR.** Amplification of reverse-transcribed cDNA sequence for VIP, PACAP, PAC<sub>1</sub>, VPAC<sub>1</sub>, and VPAC<sub>2</sub> was obtained by SYBR Green real-time quantitative PCR (Applied Biosystems). The primer sequences (Table 1) were designed on Primer Express software (Applied Biosystems), knowing the already reported porcine sequence for VPAC<sub>1</sub> (GenBank NM214036) and the human sequences for VIP (GenBank

NM003381), PACAP (GenBank NM001117), VPAC<sub>2</sub> (GenBank NM003382), and PAC<sub>1</sub> (GenBank NM001118). Gene expressions were normalized using hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene product as an endogenous reference (11). The primers were produced on an automated synthesizer (Applied Biosystems), according to the manufacturer's protocol (Eurogentec, <http://www.eurogentec.com>). SYBR Green real-time quantitative PCR analysis was performed with the GeneAmp PCR system 7700 (Applied Biosystems). For each gene, the procedure was realized in duplicate with  $25\ \mu\text{L}$  reaction volume containing 10 ng of cDNA,  $5\ \text{pmol}/\mu\text{L}$  of each primer, and  $12.5\ \mu\text{L}$  of SYBR Green PCR Master Mix (Applied Biosystems). Measurements were made in duplicate. Amplification was conducted as follows: initial denaturation ( $95^{\circ}\text{C}$  for 10 min), then a two-step cycle program (15 s at  $95^{\circ}\text{C}$  followed by 1 min at  $60^{\circ}\text{C}$ ) repeated 40 times. To ensure the quality of measurements, we systematically included both negative (no template control) and positive controls in duplicate in each plate. The statistical analysis of PCR results was completed with the comparison between the  $C_t$  values of the gene of interest and HPRT ( $\Delta C_t$ ). Relative gene expression was obtained by  $\Delta\Delta C_t$  methods ( $\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator}$ ), with the sham group as a calibrator, to compare every unknown-sample gene expression levels. The conversion between  $\Delta\Delta C_t$  and relative gene expression levels is given by fold induction =  $2^{-\Delta\Delta C_t}$  (12).

**Immunohistochemistry.** The immunohistochemistry analysis was performed using the avidin–biotin complex technique (13). After routine deparaffinization and rehydration, microwave heat pretreatment in citrate buffer was performed before immunolabeling with primary antibodies against VIP, PACAP, and PAC<sub>1</sub>. Whole lung sections were washed in Tris-buffered saline for 30 min, treated with normal goat serum (1/10 dilution) for 1 h, and incubated overnight at  $4^{\circ}\text{C}$  with a commercial primary antibody (rabbit polyclonal IgG; Table 2). After rinsing, the tissue sections were incubated for 30 min with a goat biotinylated anti-rabbit IgG at 1/300 dilution (BA-1000, Vector Laboratories, <http://www.vectorlabs.com>), followed by incubation with the ABC reagent (Vectastain Elite ABC Kit Standard, PK-6100, Vector Laboratories). After 30 min, the slides were rinsed again before developing in 5% diaminobenzidine tetrahydrochloride chromogenic solution (Dakocytomation, <http://www.dako.com>). Finally, the specimens were routinely dehydrated, mounted in DPX agent, and coverslipped. Human tissue samples were used as positive controls (liver for VPAC receptors, placenta for PACAP and PAC<sub>1</sub>, and colon for VIP). Negative controls were generated by omitting the primary antibody.

**Data analysis.** The values are reported as mean  $\pm$  SEM. Tests of statistical significance were performed using the SigmaStat version 3.0 software (Systat Software, <http://www.sigmaplot.com>). The effects of the shunt on hemodynamic parameters were analyzed by a repeated-measures ANOVA. For relative amounts of mRNA, statistical significance between groups was determined by a one-tailed unpaired *t* test, after ensuring normality and equality of variance (14). Correlations between PAP, PVR, or MT and mRNA contents were calculated by means of a linear regression analysis. A *p* value of less than 0.05 was considered as significant.

## RESULTS

**Effect of shunting on hemodynamic and morphometric measurements.** After a follow-up of 3 mo, the piglets gained 40 kg weight, with no difference between the two study groups. Blood gases measurements were normal, with no difference between shunted and sham animals. The chronic

**Table 1.** Primers used for RTQ-PCR for the genes of interest and HPRT gene

Genes	Primers sequences
HPRT	S: 5'-TCAGGCAGTATAATCAAAGATGGT-3' AS: 5'-AGTCTGGCTTATATCCAACACTTCG-3'
VIP	S: 5'-CAGCCAACCGTTAGCCATTG-3' AS: 5'-TGGTGTCCATTTCTGTGCCCTC-3'
PACAP	S: 5'-GATGACTCGGAGCCCCTCTC-3' AS: 5'-CGGCCGCAAGTATTTCTTA-3'
PAC <sub>1</sub>	S: 5'-TACCTGCACACCCTACTTGCC-3' AS: 5'-ACCATGATGAAGGTGCTGGG-3'
VPAC <sub>1</sub>	S: 5'-GAGACCCCTGAATCCCCAAA-3' AS: 5'-GTAGATCCAACCGCCTCACC-3'
VPAC <sub>2</sub>	S: 5'-AAGCCATGTGCTGGAGAAG-3' AS: 5'-CCAACACGTGATGTTGTC-3'

S, sense primer; AS, antisense primer.

**Table 2.** Primary antibodies used for immunohistochemistry

Commercial primary antibodies	Cross-reaction	Dilution
Anti-VIP (T-4464, Bachem, <a href="https://www.bachem.com">https://www.bachem.com</a> )	0% PACAP	1/500
Anti-PACAP <sub>38</sub> (T-4472, Bachem)	0% PACAP <sub>27</sub> – 0% VIP	1/500
Anti-PACAP <sub>27</sub> (T-4245, Bachem)	1% PACAP <sub>38</sub> – $<0.1\%$ VIP	1/250
Anti-VPAC <sub>1</sub> (ab13286, Abcam, <a href="https://www.abcam.com">https://www.abcam.com</a> )	Not reported	1/100
Anti-VPAC <sub>2</sub> (ab13145, Abcam)	Not reported	1/100
Anti-PAC <sub>1</sub> (ab13374, Abcam)	Not reported	1/100

left-to-right shunting increased PAP (from  $20.1 \pm 1.8$  to  $33.2 \pm 1.4$  mm Hg,  $p < 0.01$ ), PVR (from  $3.7 \pm 0.4$  to  $7.5 \pm 0.5$  mm Hg/L/min/m<sup>2</sup>,  $p < 0.001$ ), and PAPO (from  $7 \pm 1$  to  $11 \pm 1$  mm Hg,  $p < 0.05$ ), with no change in Q (from  $3.3 \pm 0.1$  to  $3.1 \pm 0.1$  L/min), HR (from  $112 \pm 8$  to  $120 \pm 6$  beats/min), and SAP (from  $121 \pm 8$  to  $130 \pm 8$  mm Hg). Arterial MT of the smallest pulmonary arterioles ( $<75 \mu\text{m}$ ) increased by 85% in the shunt group in comparison to the sham group (from  $13.6 \pm 1.8$  to  $25.2 \pm 0.2\%$ ,  $p = 0.005$ ; Table 3). The MT was positively correlated to PAP ( $p < 0.05$ ; Fig. 1).

**Effect of shunting on whole lung mRNA levels.** As illustrated in Figure 2, the shunting decreased VIP gene expression (from  $1.00 \pm 0.57$  to  $0.42 \pm 0.14$  units of relative gene expression;  $p = 0.05$ ), with an overexpression of VPAC<sub>1</sub> (from  $1.00 \pm 0.32$  to  $3.05 \pm 1.57$  units of relative gene expression;  $p < 0.05$ ). PAC<sub>1</sub> expression was also increased by  $2.28 \pm 1.68$  times, but this change did not reach statistical significance. There was no modification in mRNA contents for PACAP. No result was available for VPAC<sub>2</sub> gene expression because we failed in our attempt to design specific primers able to specifically amplify porcine cDNA. VPAC<sub>1</sub> mRNA content was correlated to PVR (Fig. 3), with no correlation to

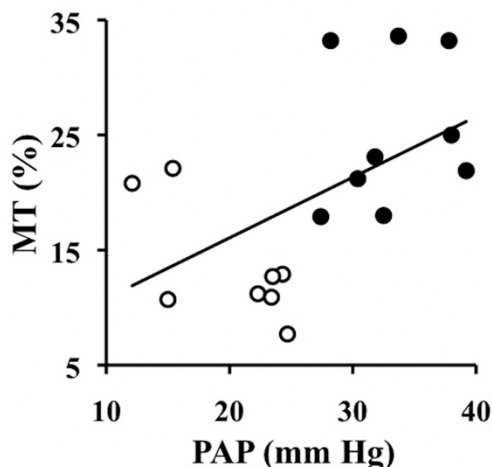
**Table 3.** Hemodynamic and morphometric effects of chronic left-to-right shunt in piglets

	Sham (n = 8)	Shunt (n = 9)
PAP (mm Hg)	$20.1 \pm 1.8$	$33.2 \pm 1.4^*$
PVR (mm Hg/L/min/m <sup>2</sup> )	$3.7 \pm 0.4$	$7.5 \pm 0.5^*$
HR (bpm)	$117 \pm 4$	$121 \pm 6$
Q (L/min/m <sup>2</sup> )	$3.4 \pm 0.1$	$3.1 \pm 0.1$
SAP (mm Hg)	$131 \pm 3$	$132 \pm 6$
PAPO (mm Hg)	$8 \pm 1$	$10 \pm 1$
$<75 \mu\text{m}$ pulmonary artery medial thickness (%)	$13.6 \pm 1.8$	$25.2 \pm 1.2^\dagger$

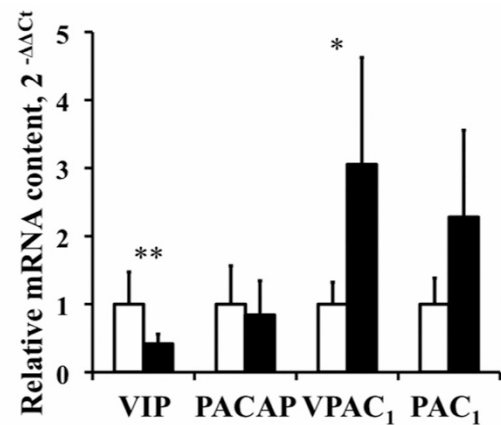
Data are expressed as mean  $\pm$  SEM.

\*  $p < 0.001$  sham group vs shunt group.

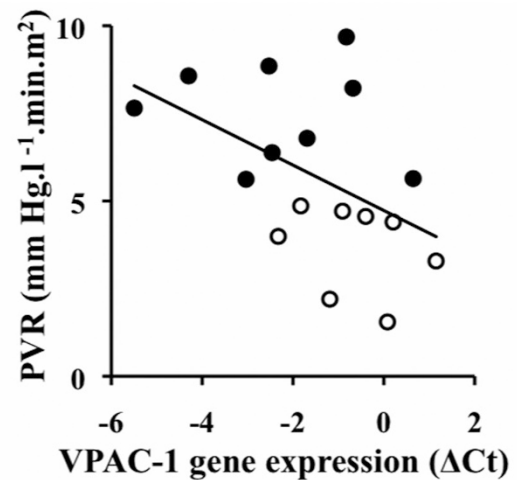
†  $p < 0.05$  sham group vs shunt group.



**Figure 1.** Correlation between mean PAP and the percentage of MT for smallest size pulmonary arterioles (diameter inferior to  $75 \mu\text{m}$ ) in sham-operated controls (○) and shunted (●) piglets ( $r = 0.52$ ;  $p < 0.05$ ).



**Figure 2.** Bar graph showing relative lung tissue mRNA contents for VIP, PACAP, VPAC<sub>1</sub> receptor, and PAC<sub>1</sub> receptor of sham-operated (□) and shunted (■) groups. Values are expressed as mean  $\pm$  SEM. \* $p < 0.05$  shunt group vs sham group; \*\* $p = 0.05$  shunt group vs sham group.

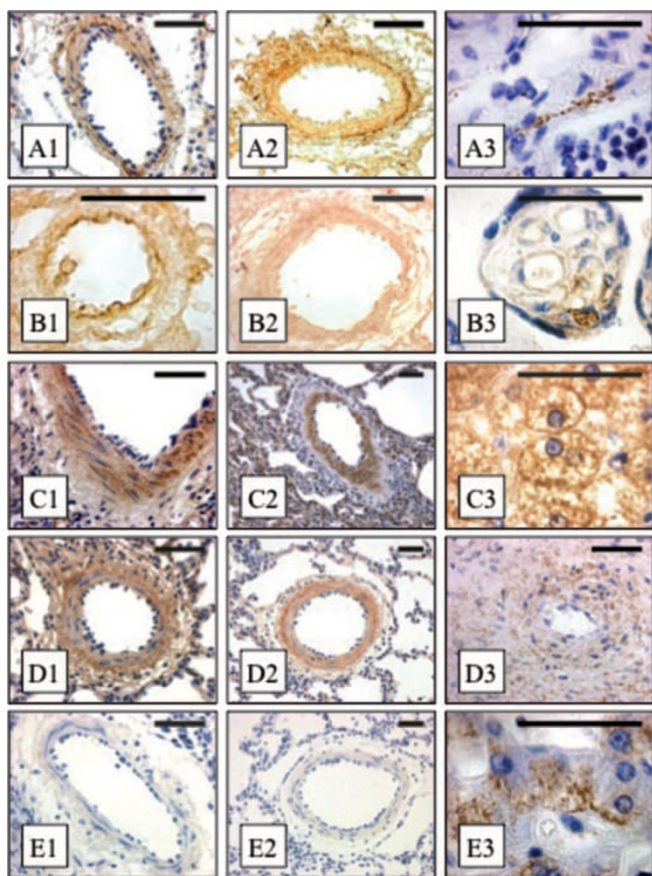


**Figure 3.** Correlation between relative lung tissue mRNA content for VPAC<sub>1</sub> and PVR in sham-operated controls (○) and shunted (●) piglets. Values are expressed as mean  $\pm$  SEM ( $r = 0.48$ ;  $p = 0.05$ ).

PAP or MT. No other significant correlation was seen between the other gene expressions and hemodynamic or morphometric parameters.

**Immunohistochemical localization and distribution of peptides and related receptors in whole lung paraffin-embedded sections.** The distribution of VIP, PACAP, VPAC<sub>2</sub>, and PAC<sub>1</sub> immunoreactivity was similar in both sham and shunt piglets (Fig. 4). VIP and PACAP immunoreactivity was localized in small nerve fibers surrounding the respiratory epithelium and bronchial submucosal glands (data not shown). Immunoreactivity for VIP and PACAP was also observed in nerve fibers around vascular and bronchial smooth muscle cells (panels A and B). Vascular smooth muscle cells appeared as immunopositive cells for VPAC<sub>2</sub> (panel C). Strong immunoreactivity for PAC<sub>1</sub> was shown in arterial smooth muscle cells (panel D), as well as in chondrocytes, bronchial smooth muscle cells, and respiratory epithelial cells (data not shown). There was no reactivity for VPAC<sub>2</sub> and PAC<sub>1</sub> in the extracellular matrix. Despite the strong VPAC<sub>1</sub> staining shown on positive control specimen (human liver), no immunoreactivity





**Figure 4.** Immunostaining of porcine pulmonary arteries on paraffin-embedded sections. Immunoreactivity for VIP (panel A) and for PACAP (panel B) showing small nerve fibers penetrating pulmonary arterial smooth muscle of sham-operated (A1, original magnification  $\times 40$ ; B1, magnification  $\times 100$ ) and shunted piglets (A2 and B2, magnification  $\times 40$ ); VIP immunoreactivity in human colon as a positive control (A3, magnification  $\times 100$ ); PACAP immunoreactivity in chorionic villi of human placenta as a positive control (B3, magnification  $\times 100$ ). Immunoreactivity for VPAC<sub>2</sub> receptor (panel C) and for PAC<sub>1</sub> receptor (panel D) showing granular immunoreaction of arterial smooth muscle cells of sham-operated (C1 and D1, magnification  $\times 40$ ) and shunted piglets (C2 and D2, magnification  $\times 20$ ); VPAC<sub>2</sub> immunostaining in human liver as a positive control (C3, magnification  $\times 100$ ); PAC<sub>1</sub> immunoreactivity in chorionic villi of human placenta as a positive control (D3, magnification  $\times 40$ ). Negative immunoreaction for VPAC<sub>1</sub> receptor in pulmonary tissue of sham-operated (E1, magnification  $\times 40$ ) and shunted piglets (E2, magnification  $\times 20$ ); VPAC<sub>1</sub> immunostaining in human liver as a positive control (E3, magnification  $\times 100$ ). Bars = 50  $\mu$ m.

could be seen when the porcine lung sections were immunostained for VPAC<sub>1</sub> (panel E). None of the slides used as negative controls showed immunopositive reaction.

## DISCUSSION

Our results demonstrate that growing piglets with overcirculation-induced pulmonary hypertension exhibit a down-regulation of VIP together with an up-regulation of VPAC<sub>1</sub>, without modification of the PACAP axis.

The pathobiology of PAH is characterized by an imbalance of vascular effectors, with vasoconstriction, thrombosis, smooth muscle, and endothelium proliferation. One way to investigate the initial biologic stages of the disease is to develop satisfactory animal models. We previously reported

that chronic aorta-pulmonary shunting in growing piglets induces severe pulmonary hypertension, with a PAP between 30 and 40 mm Hg at a normal cardiac output measured after shunt closure, and pulmonary arteriolar medial hypertrophy, compatible with reversible PAH related to congenital heart disease with left-to-right shunts. We showed, in this model, an up-regulation of endothelin-1 and phosphodiesterase-5, together with altered angiopoietin-1/BMP2 signaling (11,15). Pretreatment with phosphodiesterase-5 inhibitor (15), nonselective endothelin-1 receptor antagonist (11), or selective endothelin type A receptor blocker (16) partially prevented the changes in signaling molecules, the increase in PVR, and the thickening of the pulmonary arterial media.

The pulmonary alterations of VIP signaling in this experimental model closely resemble those in patients suffering from severe PAH. In patients with PAH, VIP serum concentrations were decreased, and VIP immunoreactive nerve fibers were nearly absent from the median layer of pulmonary arterial vessels (4). In the left-to-right shunting PAH model, the pulmonary expression of VIP gene was significantly decreased. However, VIP was still detectable by immunostaining in shunted animals and was analogous to that of normal human lung in nerve fibers penetrating arterial and bronchial smooth muscle layers and surrounding the mucous and serous glands (17). Because immunohistology is not sensitive enough for protein quantification, a moderate reduction in nerve fiber VIP protein might have been missed. Furthermore, the implication of VIP in early PAH physiopathology has been emphasized by another experimental model of moderate pulmonary hypertension, namely the VIP knockout model (9). Mice knockout for the VIP gene present with pulmonary hypertension and right ventricular hypertrophy that are reversible by the administration of VIP. In the piglet with shunt-induced PAH, decreased expression of VIP could have been the consequence as well as a contributing factor to increased PVR.

The pivotal role of VIP/PACAP receptors in PAH has been pointed out in patients with severe disease and in animal models. Overexpression of both VPAC receptors at mRNA and protein levels has been reported in pulmonary arterial smooth muscle cells from patients with PAH, in which VPAC<sub>1</sub> and VPAC<sub>2</sub> were strongly detected by immunohistochemical methods (4). In our porcine model, VPAC<sub>1</sub> gene was also up-regulated, which may reflect a response due to the lack of agonist (4). Data regarding VPAC<sub>2</sub> gene expression are lacking in this study. Nevertheless, we demonstrated the localization of the correspondent protein in pulmonary arterial smooth muscle cells in accordance with the findings in normal human lung (18). We cautiously refrained to perform semi-quantitative estimation only on the basis of VPAC<sub>2</sub> immunostaining intensity because the results might not be reproducible with this technique (19). Considering that VPAC<sub>2</sub> knockout mice do not develop pulmonary hypertension (20), VPAC<sub>1</sub> might be an essential receptor that mediates disease-limiting actions of VIP in PAH. To date, no transgenic mice have yet been generated with altered gene expression for VPAC<sub>1</sub>.

The pulmonary gene expression of PACAP and its preferential PAC<sub>1</sub> receptor remained unchanged in the current ex-

periments. PAC<sub>1</sub> receptor had a similar distribution to VPAC<sub>2</sub> in the porcine pulmonary tissue. PAC<sub>1</sub> has not yet been detected by immunohistochemistry in the human lung (18). These results do not favor a role for this pathway in early overcirculation-induced PAH. Our observations contradict previous findings in the PAC<sub>1</sub> knockout mice model, in which PAC<sub>1</sub> mutants display severe right heart failure and pulmonary arterial muscularization causing death within the first postnatal weeks (10). This might be due to the fact that PAC<sub>1</sub> knockout mice developed alveolar hypoxia, leading to secondary pulmonary hypertension. The chronically shunted piglets did not suffer from hypoxia.

Further interactions between VIP signaling and other pathways potentially involved in pulmonary arterial homeostasis will have to be investigated to elucidate the mechanisms by which VIP deficiency takes part in PAH pathophysiology. Apart from a direct effect on vascular smooth muscle cells, VIP vasorelaxant effects may be mediated following an endothelium-dependent manner with activation of endothelial NOS, releasing of NO, and subsequent elevation of cGMP content in arterial smooth muscle cells (21). Endothelin receptors are closely related to vascular endothelium because they participate in NO modulation of endothelin synthesis (22) and release of NO and prostacyclin (23). Taking into account that VIP inhibits the contractile effects of endothelin on pulmonary arteries (5), studies of interactions between endothelin and VIP pathways could take advantage of the existence of endothelin antagonists. Recently, the transforming growth factor- $\beta$  was described as an inducer of VIP gene transcription *via* the Smad signaling pathway (24). The Smad pathway is involved in angiotensin-1/BMP2 signaling, which plays a crucial role in PAH pathogenesis. Overexpression of angiotensin-1 or mutation of BMP2 gene down-regulates Smad signaling and enhances proliferative effects on vascular smooth muscle (25). Moreover, VIP deletion in mice is accompanied by an imbalance between vasodilator antiremodeling genes (down-regulation of BMP2, Smad1, endothelial NOS, and prostacyclin synthase) and vasoconstrictor proremodeling ones (up-regulation of angiotensin 1) (26). Additional knowledge of the relationship between the VIP/PACAP system and PAH pathobiology could be achieved, thanks to investigations with specific therapies (phosphodiesterase-5 inhibitor or endothelin blockers).

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