Pulmonary blood pressure, not flow, is associated with net endothelin-1 production in the lungs of patients with congenital heart disease and normal pulmonary vascular resistance

Sohrab Fratz, MD^a
 Ralf Geiger, MD^b
 Hans Kresse, PhD^a
 Gabriele Roemer, BS^a
 Michael Hennig, PhD^c
 Walter Sebening, MD^a
 John Hess, MD, PhD, FESC^a

Objective: Endothelin-1 concentrations are increased in patients with increased mean pulmonary arterial pressure, pulmonary blood flow, and pulmonary vascular resistance. However, endothelin-1 concentrations have not been well characterized in patients with congenital heart disease and normal pulmonary vascular resistance. In particular, it is unclear whether pressure or flow is the key regulator of endothelin-1 in this setting. We tested the hypothesis that pulmonary blood pressure and not flow is associated with net endothelin-1 production in patients with congenital heart disease and normal pulmonary vascular resistance.

Methods: With a commercially available immunoassay, we measured endothelin-1 concentrations in pulmonary arterial and pulmonary venous plasma of 56 consecutive patients with congenital heart disease and pulmonary vascular resistance less than $2 \text{ U} \cdot \text{m}^2$ undergoing cardiac catheterization. We used multiple linear regression to analyze the effect of demographic and hemodynamic variables on pulmonary arterial and venous endothelin-1 concentrations and on the change of endothelin-1 concentration over the pulmonary vascular bed.

Results: Multiple linear regression revealed that of all the hemodynamic variables tested, mean pulmonary arterial pressure had the greatest effect on increasing the change of endothelin-1 concentration over the pulmonary vascular bed ($P < .0001$). Pulmonary blood flow did not have any effect on endothelin-1 concentrations or on the change of endothelin-1 concentration over the pulmonary vascular bed.

Conclusions: This study shows that pulmonary blood pressure and not flow is associated with net endothelin-1 production in patients with congenital heart disease and normal pulmonary vascular resistance.

From the Department of Pediatric Cardiology and Congenital Heart Disease, Deutsches Herzzentrum München, Klinik an der Technischen Universität München,^a Munich, Germany, the Department of Pediatric Cardiology at the University Hospital Innsbruck,^b Innsbruck, Austria, and the Institute for Medical Statistics and Epidemiology, Technischen Universität München,^c Munich, Germany.

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Address for reprints: Sohrab Fratz, MD, Department of Pediatric Cardiology and Congenital Heart Disease, Deutsches Herzzentrum München, Klinik an der Technischen Universität München, Lazarettstrasse 36, 80636 Munich, Germany (E-mail: fratz@dhm.mhn.de).

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Pulmonary hypertension and increased pulmonary vascular resistance (PVR) may develop in patients with congenital heart disease (CHD) and increased pulmonary blood pressure and flow.¹ Ultimately, pulmonary hypertension and increased PVR lead to advanced pulmonary vascular disease. Although the morphologic features of pulmonary hypertension have been well described,²⁻⁴ the mechanisms leading to advanced pulmonary vascular disease are still poorly understood.⁵⁻⁸

Increasing evidence emphasizes the role of endothelin-1 (ET-1) in patients with various forms of pulmonary hypertension, increased PVR, and advanced pulmonary

vascular disease.^{6,9} In these patients there is a net production of ET-1 by the pulmonary vascular bed, caused either by decreased clearance, true increased production, or a combination of two.^{6,9} In light of these findings, the first ET-1 receptor antagonist (bosentan) has recently been approved for treatment of patients with pulmonary hypertension.¹⁰

However, little is known about the alterations of ET-1 concentrations in patients with CHD and normal PVR. In particular, it is uncertain whether pressure or flow is the major trigger for net ET-1 production in these patients.¹¹⁻¹⁶ The reason for this uncertainty is that research has focused on the effects of flow, not pressure.^{11,17,18}

Recently, a study in bovine pulmonary cell cultures indicated that pressure and not flow is the major trigger for ET-1 production by the pulmonary endothelium.¹⁹ We therefore tested the hypothesis that pulmonary blood pressure and not flow is associated with net ET-1 production in patients with CHD and normal PVR.

For this study we assessed the effects of pressure, flow, and several other hemodynamic and demographic variables on pulmonary arterial and venous ET-1 concentrations and on the change of ET-1 concentration over the pulmonary vascular bed in patients with CHD and normal PVR. We used the change of ET-1 concentration over the pulmonary vascular bed as an indicator of net ET-1 clearance or production by the pulmonary vascular bed.

Methods

Patient Population

During the 10-month study period, 65 consecutive patients in our hospital (Deutsches Herzzentrum München, Munich, Germany) who fulfilled the following criteria were enrolled in the study: routine diagnostic cardiac catheterization, routine catheter access to pulmonary veins, PVR less than $2 \text{ U} \cdot \text{m}^2$, and CHD without cavopulmonary connections. Patients with cavopulmonary connections were excluded because accurate determination of pulmonary blood flow is difficult in these patients. Because of a coefficient of variation of at least 0.15 between the dual measurements of ET-1 concentration, 9 original patients were later excluded. Thus the final study population consisted of 56 patients (29 of them female). The mean age of the patients was 10.8 ± 15.7 years. The underlying diagnoses were secundum type atrial septal defect ($n = 23$), sinus venosus defect ($n = 1$), ventricular septal defect ($n = 3$), atrioventricular septal defect ($n = 2$), congenitally corrected transposition of the great arteries with ventricular septal defect ($n = 2$), double-outlet right ventricle ($n = 3$), Ebstein anomaly ($n = 1$), persistent foramen ovale ($n = 2$), tetralogy of Fallot ($n = 4$), transposition of the great arteries with Mustard procedure ($n = 2$), uncorrected transposition of the great arteries ($n = 1$), pulmonary valve stenosis ($n = 1$), pulmonary sling ($n = 1$), distal pulmonary stenosis ($n = 1$), dilatation of the ascending aorta ($n = 1$), coarctation of the aorta ($n = 1$), and others ($n = 7$). None of the patients had large aortopulmonary collaterals. The study was approved by the institutional review board, and informed consent was obtained from the patient or from the parents.

Study Design

ET-1 concentrations were measured in the pulmonary artery and corresponding vein of all patients enrolled in the study. The effect of hemodynamic and demographic variables on pulmonary arterial and venous ET-1 concentrations and on the change of ET-1 over the pulmonary vascular bed were assessed by multiple linear regression analysis.

Measurement of Hemodynamic Variables

Hemodynamic variables were measured during cardiac catheterization with patients under general anesthesia or sedation. Oxygen consumption was measured directly with a flow-through system²⁰ in 57% of the patients. If this was not possible, oxygen consumption was estimated from age, sex, and heart rate by the method of LaFarge and Miettinen.²¹ Mean pulmonary arterial pressure (mPAP), mean pulmonary venous pressure, and mean ascending aortic pressure were measured with fluid-filled catheters connected to pressure transducers. Pulmonary and systemic blood flows were calculated by the standard Fick method and indexed for body surface area (BSA). The ratio of pulmonary to systemic blood flow, PVR, and systemic vascular resistance were calculated according to standard formulas and indexed for BSA.

Blood Sampling

During cardiac catheterization before angiography, 1.3-mL blood samples were taken from the left or right pulmonary artery and a corresponding pulmonary vein. The samples were collected in prechilled commercially available blood sample tubes containing ethylenediaminetetraacetic acid. After centrifugation at 4°C , the plasma samples were stored at -70°C until the ET-1 assay. The time lapse between blood sampling and freezing of the plasma samples was recorded.

ET-1 Assay

Plasma ET-1 concentrations were measured with a commercially available immunoassay kit (R&D Systems, Minneapolis, Minn). Cross-reactivities with ET-2, ET-3, and big ET-1 were 27%, 8%, and less than 1%, respectively. This assay used a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ET-1 was precoated onto a microplate. Standards and samples were pipetted into the wells, and the immobilized antibody bound any ET-1 present. After any unbound substance was washed away, an enzyme-linked monoclonal antibody specific for ET-1 was added to the wells. Any unbound antibody-enzyme reagent was then washed away. An enhanced luminol-peroxide substrate solution was then added to the wells, and light was produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer was used to measure the intensity of the light emitted. All samples were pipetted into two wells; thus each sample was measured twice. The arithmetic mean of the two measurements was used for the statistical analysis. A coefficient of variation of at least 0.15 between the two measurements led to a second dual measurement or exclusion of the patient if not enough plasma was available.

Statistical Analysis

Three multiple linear regressions with a backward selection algorithm analyzed the effects of several independent variables on

TABLE 1. Hemodynamic and demographic variables that significantly affected ET-1 concentrations after multiple linear regression analysis (n = 56)

	ET-1 concentration (standardized effect,* regression coefficient [95% CI])		
	In PA	In PV	Change over pulm vasc bed†
Mean pulmonary artery pressure		3.89†, 0.04 (0.02 to 0.07)	5.78†, 1.96 (1.28 to 2.64)
Body surface area	−4.28†, −0.58 (−0.85 to −0.31)	−4.24†, −0.62 (−0.91 to −0.33)	−2.22†, −8.79 (−16.72 to −0.85)
Pulmonary vascular resistance	2.98‡, 0.49 (0.16 to 0.82)		

*Regression coefficient/standard error of regression coefficient. PA, Pulmonary artery; PV, pulmonary vein; Change over pulm vasc bed, ET-1 concentration in the pulmonary vein minus the ET-1 concentration in the pulmonary artery divided by the ET-1 concentration in the pulmonary artery.

† $P < .0001$.

‡ $P = .0044$.

three dependent variables. The dependent variables were the ET-1 concentrations in the pulmonary artery and vein and the change in ET-1 concentration over the pulmonary vascular bed. The change in ET-1 concentration was defined as the ET-1 concentration in the pulmonary vein minus the ET-1 concentration in the pulmonary artery divided by the ET-1 concentration in the pulmonary artery. A positive change therefore indicates an increase of ET-1 concentration over the pulmonary vascular bed; a negative change indicates a decrease. The independent variables of the multiple regression analysis were age, sex, BSA, hemoglobin concentration, time between blood sampling and freezing, diagnosis group, and several hemodynamic variables: mPAP, mean pulmonary venous pressure, mean ascending aortic pressure, pulmonary blood flow, ratio of systemic to pulmonary flow, PVR, and systemic vascular resistance. Results are presented as mean $\pm$ SD. SPSS for Windows version 11.0 (SPSS Inc, Chicago, Ill) was used for statistical analysis.

Results

Our main finding from multiple linear regression analysis was that of all the hemodynamic variables tested, mPAP had the greatest effect on ET-1 concentrations in patients with CHD and normal PVR (Table 1). Specifically, increased mPAP increased the change of ET-1 concentration over the pulmonary vascular bed (Figure 1). The linear regression in Figure 1 seemed to be predominantly driven by the patient in the upper right corner. After exclusion of this patient, however, the linear regression did not change considerably. More importantly, when we repeated the multiple linear regression analysis without this patient (data not shown), the highly significant results remained as depicted in Table 1.

The other two variables that had a significant effect on ET-1 concentrations were BSA and PVR (Table 1). To ensure that these three variables were not dependent on each other, we carried out a correlation analysis for these three variables. None of the variable pairs had a coefficient of correlation greater than 0.5 (data not shown), thus ensuring the independence of these three variables. None of the other variables studied (pulmonary blood flow, ratio of pulmonary to systemic flow, mean pulmonary venous pressure, mean ascending aortic pressure, systemic vascular resistance,

sex, age, diagnosis, hemoglobin level, and time between blood sampling and freezing) had any significant effect on ET-1 concentrations.

For the patient group as a whole, the following hemodynamic variables were determined: mPAP 12.3 ± 6.6 mm Hg, mean pulmonary venous pressure 5.9 ± 2.2 mm Hg, mean ascending aortic pressure 61.4 ± 11.1 mm Hg, pulmonary blood flow 7.0 ± 4.9 L/(min $\cdot$ m²), ratio of pulmonary to systemic flow 1.9 ± 1.1 , PVR 1.0 ± 0.5 U $\cdot$ m², and systemic vascular resistance 17.2 ± 9.1 U $\cdot$ m². Hemoglobin concentration was 12.0 ± 2.0 g/dL. The mean ET-1 concentration dropped from 1.3 ± 0.7 pg/mL in the pulmonary artery to 1.2 ± 0.8 pg/mL in the pulmonary vein. However, the change of ET-1 concentration over the pulmonary vascular bed ranged from a 40.7% decrease to a 71.0% increase (mean $9.5\% \pm 21.8\%$ decrease; Figure 1).

Discussion

This study demonstrates that pulmonary blood pressure and not flow is associated with net ET-1 production in the lungs of patients with CHD and normal PVR. Multiple linear regression analysis showed that of all the variables studied, mPAP had the greatest effect on ET-1 concentrations in patients with CHD and normal PVR (Table 1). Specifically, as mPAP increased, the change of ET-1 concentration over the pulmonary vascular bed increased (Figure 1). Similar results were found recently by studying bovine pulmonary cell cultures.¹⁹ Moreover, neither pulmonary blood flow nor the ratio of pulmonary to systemic flow had a significant effect on ET-1 concentrations. Greater BSA was associated with decreased ET-1 concentrations. Because BSA increases with age, this finding is consistent with previous observations that increasing age from infancy to adolescence is correlated with decreasing ET-1 concentrations.²²

A review of studies of the effects of pulmonary arterial pressure and flow on ET-1 concentrations in patients with CHD reveals conflicting results.¹¹⁻¹⁶ The explanation may be that all studies focused on the effects of flow, thus neglecting the effects of pressure. Further, none of the studies used multiple regression analysis, thus ignoring the

Figure 1. Linear regression of mPAP (variable with greatest effect as assessed by multiple linear regression) and change of ET-1 concentration over pulmonary vascular bed of 56 patients with CHD and normal PVR. Positive change indicates increase of ET-1 concentration over pulmonary vascular bed; negative change indicates decrease. Change of ET-1 concentration was defined as ET-1 concentration in pulmonary vein minus ET-1 concentration in pulmonary artery divided by ET-1 concentration in pulmonary artery. Linear regression results ($y = 2.1, x = 35.5, r^2 = 0.409, P < .0001$) seemed to be predominantly driven by patient in *upper right corner*. However, linear regression did not change considerably after exclusion of this patient. More importantly, when we repeated multiple linear regression analysis without this patient, highly significant results remained, as depicted in Table 1. Note that pulmonary vascular bed seems to be net ET-1 clearance site when mPAP is low (change is negative) and seems to shift to net ET-1 production site when mPAP is high (change is positive).

interdependence of pressure and flow. Our results clarify the reason for these conflicting results by showing with multiple regression analysis of a large number of patients that pressure, not flow, is the major trigger for ET-1 production in patients with CHD and normal PVR.

A detailed look at our results for patients with CHD and normal PVR suggests that the pulmonary vascular bed seems to be a net ET-1 clearance site when mPAP is low (decrease of ET-1 over the pulmonary vascular bed, change is negative; Figure 1). Further, it seems to shift to a net ET-1 production site when mPAP is high (increase of ET-1 over the pulmonary vascular bed, change is positive; Figure 1). The possibility of this shift is supported by similar findings in healthy rats with normal mPAP²³ and in patients with elevated mPAP and PVR.^{6,8} In healthy rats with normal mPAP, the pulmonary vascular bed was found to be a net ET-1 clearance site.²³ In contrast, in patients with elevated mPAP and PVR, the pulmonary vascular bed was found to be a net ET-1 production site.^{6,8} The net ET-1 production in these patients was due to decreased clearance of ET-1.^{6,8} Studies investigating the pulmonary clearance of ET-1 in patients with shunt-related CHD are needed to confirm these implications.

Furthermore, one can speculate that the results of this study may explain the clinical experience that increased pulmonary blood flow does not in itself lead to increased PVR. Clinical experience shows that even longstanding increased pulmonary blood flow, as in patients with atrial septal defects, mostly does not lead to increased PVR. On the other hand, increased pulmonary blood flow accompanied by increased pulmonary blood pressure, as in patients with large ventricular septal defects or a large patent ductus arteriosus, leads to drastically increased PVR. Additionally, the important role of ET-1 in patients with increased PVR has been shown.^{6,9} These observations, in combination with our finding that pressure and not flow is the major trigger for net ET-1 production in patients with CHD and normal PVR, may explain why patients with increased flow alone do not have increased PVR, whereas patients with combined increased flow and pressure do.

Limitations

A limitation of our study is that the observed ET-1 concentrations were very low. Thus the detected differences could have been due to systematic measurement problems. However, we carefully ensured that the measured concentrations

reflected the genuine situation by carrying out dual measurements. A coefficient of variation of at least 0.15 between the two measurements led to a second dual measurement or to exclusion of the patient if not enough plasma was available. Furthermore, we carefully analyzed the data by multiple regression analysis. We think that multiple linear regression is the appropriate way to detect interdependence of hemodynamic variables with concentrations of circulating substances, because no single hemodynamic variable is independent of the others (some are even calculated from others). Additionally, the immunoassay kit that we used in this study is quite accurate in detecting low concentrations and small concentration differences without extraction. For this kit, cross-reactivity with the ET-1 precursor big ET-1 is < 1%. In contrast, nearly all studies investigating the relationship of ET-1 and hemodynamic variables in patients with CHD and increased pulmonary blood flow or pressures have used ET-1 assays with large cross-reactivities (up to 80%) with big ET-1.^{6,11,12,14,15,22} These large cross-reactivities make the interpretation of the results problematic. The reason is that active ET-1 is produced by ET-1 converting enzyme from the precursor big ET-1. It is known that ET-1 converting enzyme is strongly up-regulated in lambs with in utero placement of an aortopulmonary shunt,²⁴ so measuring ET-1 with an assay that has a strong cross-reactivity with big ET-1 in this setting must lead to confusing data.

Another limitation of our study is that ET-1 is not a single-acting mediator but is regulated in a fine-tuned manner.²⁴ Therefore future studies should investigate the entire ET-1 cascade in patients with shunt-related CHD.

A final limitation of this study is the variety of types of CHD in the study population. However, we believe that this problem was appropriately addressed by carrying out a multiple regression analysis, and we showed that the type of CHD was not related to ET-1 concentrations. Notably, after multiple linear regression, ET-1 concentrations in the largest group (patients with secundum type atrial septal defect) did not act differently than the ET-1 concentrations of the other groups. This finding suggests that the underlying hemodynamic condition does not contribute to the changes in ET-1 concentrations in pulmonary arterial and venous blood. This finding is supported by a previous study, which also did not find any significant differences in ET-1 concentrations in patients with different types of CHD.¹⁴ If the study population had been more uniform, and therefore smaller, any influence of various hemodynamic conditions would have been avoided; however, the smaller number of blood samples would have very likely made a statistical analysis impossible.

Conclusion

In this study we investigated the effects of hemodynamic and demographic variables on ET-1 concentrations in a

large number of patients with CHD by carrying out dual measurements of ET-1 concentration and applying multiple regression analysis. We found that pulmonary blood pressure and not flow is associated with net ET-1 production in patients with CHD and normal PVR, because of all of the hemodynamic variables studied, mPAP had the strongest effect on ET-1 concentrations. Studying agents that manipulate the ET-1 cascade should reveal whether a therapeutic approach that influences the PVR in this setting can be developed. ET receptor antagonists promise to play an important role.

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