Pulmonary vascular endothelial growth factor and nitric oxide interaction during total cardiopulmonary bypass in neonatal pigs

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Objective: Lung injury after cardiopulmonary bypass includes pulmonary hypertension and lung edema. Both complications are related to endothelial pulmonary vascular dysfunction, leukocyte sequestration, and increased capillary permeability. This study was done in an attempt to better define the endothelial dysfunction and the cause of edema.

Methods: Twenty-five neonatal piglets were subjected to total cardiopulmonary bypass for 90 minutes without crossclamping of the aorta. After weaning from cardiopulmonary bypass, they were allowed to survive 2 hours, at which time they were killed. Preoperative and postoperative hemodynamic studies, lung ($n = 16$) and muscular ($n = 5$) vascular endothelial growth factor contents, and exhaled nitric oxide ($n = 8$) were recorded. Immediately after the animals were killed, pulmonary arterial rings were obtained from 12 piglets and mounted in organ chamber for assessment of endothelial function with receptor-dependent (acetylcholine) or non-receptor-dependent (calcium ionophore A23187) studies and compared with control pulmonary arterial rings. The left lungs of 13 piglets were mounted in isolated perfused lung chambers for filtration coefficient assessment and comparison with control preparations.

Results: After cardiopulmonary bypass, pulmonary vascular resistance increased from $953.7 \pm 302.6 \text{ dyne} \times \text{s} \times \text{cm}^{-5}$ to $1973.6 \pm 925.4 \text{ dyne} \times \text{s} \times \text{cm}^{-5}$ ($P = .03$). This was associated with an increase in lung vascular endothelial growth factor content from $91.07 \pm 5.314 \text{ pg/100 mg tissue}$ to $151.6 \pm 11.4 \text{ pg/100 mg tissue}$ ($P < .0001$), an increase in muscle vascular endothelial growth factor from $76.02 \pm 11.53 \text{ pg/100 mg tissue}$ to $81.58 \pm 7.7 \text{ pg/100 mg tissue}$ (P not significant), and a decrease in exhaled nitric oxide from $6 \pm 1.7 \text{ ppb}$ to $3.12 \pm 1.4 \text{ ppb}$ ($P = .003$). The filtration coefficient was statistically significantly higher after cardiopulmonary bypass than in control preparations (0.259 ± 0.02 vs 0.525 ± 0.07 , $P < .0001$). Variations in lung vascular endothelial growth factor accumulation were statistically significantly higher than in muscular vascular endothelial growth factor accumulation (60.5 ± 9.1 vs 5.5 ± 5.9 , $P = .0008$). In addition, a statistically significant correlation was found between postbypass lung vascular endothelial growth factor

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and lung filtration coefficient ($P = .0058$), as well as between change in lung vascular endothelial growth factor and change in lung filtration coefficient ($P = .03$). Pulmonary vascular endothelial receptor-dependent (acetylcholine) function was statistically significantly blunted after bypass relative to control values ($15.44\% \pm 4.8\%$ vs $55.5\% \pm 5.96\%$ of maximal relaxation, $P = .0001$), whereas non-receptor-dependent endothelial function was unaffected by cardiopulmonary bypass ($110.77\% \pm 8.9\%$ vs $120.63\% \pm 15.46\%$ of maximal relaxation, P not significant).

Conclusions: These findings suggest that lung ischemia that occurs during cardiopulmonary bypass affects the signal transduction from membrane receptors to intracellular calcium mobilization and nitric oxide synthase activation. Lung edema after bypass is probably due in part to lung accumulation of vascular endothelial growth factor, a finding that was not found in systemic muscular nonischemic territories.

Cardiac surgery for repair of acquired or congenital heart defects mandates in the vast majority of cases the use of cardiopulmonary bypass (CPB). In the early days of CPB, pulmonary complications caused a significant percentage of the attendant morbidity and mortality.¹ In the last decades, during which time CPB has become commonplace, improvements in technology and better understanding of the pathophysiology of lung injury after these procedures have reduced the incidence of pulmonary complications; they still remain a problem, however, particularly in neonates and small children. Acute pulmonary hypertension with decreased gas exchange and low cardiac output are not uncommon, and microscopic alterations of the lung parenchyma have been demonstrated.² In addition, post-CPB ventilation may be more difficult because of subclinical lung edema. Post-CPB lung dysfunction is probably multifactorial; the inflammatory response to CPB and the lung ischemia-reperfusion injury during CPB have been demonstrated to be the main causes for this dysfunction.³ One of the most common feature after CPB is an elevation of pulmonary vascular resistance (PVR),³ which has been attributed to the inability of pulmonary vascular endothelium to produce nitric oxide to maintain a low tone in the pulmonary arterial tree. The reasons for that endothelial dysfunction, although probably multifactorial, remain unknown.

Recently a strong body of publications have shown a tight relation between nitric oxide production and vascular endothelial growth factor (VEGF). VEGF is a soluble 46-kd angiogenic glycoprotein that exerts its biologic function through high-affinity tyrosine kinase receptors.⁴ VEGF has been shown to have several biologic functions. In addition to its involvement in angiogenesis, it induces an endothelium-dependent vasodilatation that is inhibited by nitric oxide

synthase (NOS) inhibitors.⁵ Brock and colleagues⁶ demonstrated that VEGF increased cytosolic calcium, which promotes calmodulin binding to the endothelial isoform of NOS and stimulates nitric oxide production.⁷ More recently, this effect of VEGF on nitric oxide production has been associated with upward regulation of endothelial cell NOS activity.⁸ In addition, VEGF has been recognized to be encoded by the very same gene that encodes for vascular permeability factor, a protein that promotes extravasation of proteins from tumor-associated blood vessels.⁹ In addition, VEGF has been demonstrated to prevent endothelial cell apoptosis through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway¹⁰ and inhibits leukocyte-endothelium interactions.¹¹

Hypoxia and ischemia are major stimulators of VEGF expression.¹² Hypoxia-induced transcription of VEGF messenger RNA is apparently mediated at least in part by the binding of hypoxia-inducible factor 1 to a hypoxia-inducible factor 1 binding site located in the VEGF promoter.¹³ Interleukin 1 β and interleukin 6 are also strong stimulators of VEGF production.¹⁴

We therefore hypothesize that during CPB, strong stimulation for VEGF lung production or receptors expression might be present through hypoxia-ischemia mechanisms as well as through the production of various cytokines. Therefore an increase in pulmonary vascular lung nitric oxide production should be observed, which is in contrast with previous observations, namely decreases in nitric oxide production¹⁵ and endothelial cell apoptosis^{16,17} and increased leukocyte-endothelium interactions.³ This study was an attempt to demonstrate that during CPB there is a stimulus for lung VEGF accumulation but that signal transduction to produce nitric oxide fails. In addition, if VEGF is produced in excess, capillary permeability should be increased.

Material and Methods

The experimental protocol was divided into two parts. The first part was an in vivo hemodynamic study before and after CPB. Measurements of exhaled nitric oxide and assay of biopsy specimens for VEGF lung and skeletal muscle quantification were obtained before and after CPB. The second part assessed pulmonary vascular reactivity with particular pharmacologic dissection of the signal transduction for nitric oxide production by endothelial cells after CPB versus a control group. Finally, in a last group of experiments, post-CPB pulmonary capillary permeability was assessed in isolated perfused lungs and compared with that in control lungs not subjected to CPB.

Twenty-five neonatal piglets (mean age 7 days, mean weight 3.2 kg) were purchased from a local farmer. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. They were kept without food for 12 hours before the operation, sedated with intramuscularly injected ketamine (10-15 mg/kg), and anesthetized with thiopental (3-5 mg/kg intravenously). Analgesia was maintained throughout the experimentation with fentanyl (3-5 $\mu\text{g}/[\text{kg} \cdot \text{min}]$). Subsequently, the animals were intubated and general anesthesia was maintained with nitrous oxide (60%) and oxygen (40%) under intermittent positive-pressure ventilation (MMS 107 ventilator; S.N.E.H., Gonesse, France) at 40 breaths/min with a tidal volume of 15 mL/kg. With the animal in a supine position, the right femoral artery was isolated. After femoral arterial cannulation, ventilatory rate and tidal volume were adjusted to establish a normal pH and PCO_2 , as determined by arterial blood gas measurement (ABL2; Radiometer Medical A/S, Brønshøj, Denmark). After midline sternotomy and pericardiotomy, the ductus was ligated and the great vessels were controlled. A pulmonary artery catheter (Intracath, Deseret Medical, Inc, Sandy, Utah) was introduced into the main pulmonary artery through the right ventricular infundibulum, and cardiac output was recorded with a Doppler probe placed around the pulmonary artery trunk (model T106 M; Transonic Systems, Inc, Ithaca, NY). The central venous and left atrial pressures were monitored after placement of indwelling catheters in the right and left atria. Pressure recording was performed with the P23 ID Statham pressure transducer (Statham, Paris, France). After heparin administration (3 mg/kg), CPB was instituted between the right atrium with the arterial return directed into the ascending aorta. The bypass circuit consisted in a cardiectomy reservoir and a membrane oxygenator (Safe Micro; Polystan A/S, Værløse, Denmark), a heat exchanger, and a roller pump. No arterial filter was used. The circuit was primed (400 mL) with whole blood obtained from a donor pig the day before surgery. Within the first 5 minutes of CPB, a vent was introduced through the left ventricular apex. Mean systemic arterial pressures were maintained to prebypass values with a mean flow of 500 mL/min. After a steady state was obtained under CPB, ventilation was stopped and cooling to 33°C was started. The pulmonary artery trunk was crossclamped to avoid any antegrade flow to the lungs, and CPB was maintained for 90 minutes without aortic crossclamping. After this period mechanical ventilation was reinstituted and the piglets were weaned

from CPB. They were allowed to survive 2 hours and then killed. The pulmonary arterial vessels were dissected beyond the second-generation branch and mounted in an organ chamber for vascular reactivity evaluation.

Physiologic Measurements

Hemodynamic measurements were made before institution of CPB and 120 minutes after cessation of CPB. Cardiac output was determined as pulmonary blood flow (Q_{pa}) in liters per minute. PVR was calculated as follows:

$$\text{PVR} = (\text{PAP} - \text{LAP})/Q_{pa} \times 79.9$$

where PAP is the mean pulmonary arterial pressure and LAP is the mean left atrial pressure.

Biopsy specimens were obtained from the lungs ($n = 16$) and from skeletal muscle ($n = 5$) before and 120 minutes after CPB for VEGF assessment. They were frozen at -80°C until processing.

Evaluation of Lung Nitric Oxide Production

Exhaled nitric oxide was measured as follow. The expiratory circuit was disconnected from the ventilator and connected to a polypropylene bag (150 mL). Nitric oxide was immediately measured within the bag with a rapid-response chemiluminescent analyzer (NOX 4000; SERES, Aix en Provence, France). The analyzer was calibrated before use with serial dilutions of a standard nitric oxide gas. The lower limit of the sensitivity was 2 ppb. Measurements of inspired gas in the room were taken as a prelude to the investigation and suggested a level of 2 ppb nitric oxide. At each measurement the mean of three samples was recorded. Although this method does not eliminate upper airway nitric oxide metabolism, prebypass and postbypass values were assumed to include the same bias.

Lung and Muscular VEGF Assessment

VEGF assessment was performed according to the method described by Turnage and coworkers.¹⁸ Tissues were pulverized and homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc, Westbury, NY) in a buffer solution (tris[hydroxymethyl]aminomethane hydrochloride at 0.05 mol/L and ethylenediaminetetraacetic acid at 0.1 mmol/L, pH 7.40) containing protease inhibitors (leupeptin at 10 $\mu\text{mol/L}$, pepstatin at 1 $\mu\text{mol/L}$, phenylmethylsulfonyl fluoride at 1 mmol/L) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate at 20 mmol/L. The homogenate was sonicated on ice for 15 seconds, frozen (at -70°C) and thawed two times, and then centrifuged at 40000g for 20 minutes. VEGF concentration in the supernatant was then assessed with specific antibody for human VEGF₁₆₅ (Quantikine; R&D Systems, Minneapolis, Minn). Results are expressed in picograms per 100 mg tissue.

Isolated Pulmonary Arterial Ring Studies

At the end of each experiment, left and right intrapulmonary arterial segments were dissected out and placed in warm Krebs-Henseleit buffer composed of 118.3-mmol/L sodium chloride, 4.7-mmol/L potassium chloride, 2.5-mmol/L calcium chloride, 1.2-mmol/L potassium phosphate, 1.2-mmol/L magnesium sulfate, 25-mmol/L sodium hydrogen carbonate, 0.03-mmol/L ethyl-

enediaminetetraacetic acid, and 11.1-mmol/L glucose. Isolated pulmonary arteries were cleaned and cut into rings 3 to 4 mm in length (1–2 mm outer diameter). From 12 to 16 rings were obtained from each animal. The rings were then mounted on stainless steel hooks, suspended in 10-mL tissue baths, and connected to force displacement transducers (LB-5; Showa-Sokki Corp, Tokyo, Japan) to record changes in force by the use of chart recorder (LR 4210; Yokogawa Corporation of America, Newman, Ga). The baths were filled with 10 mL Krebs-Henseleit buffer and aerated at 37°C with a gas mixture of 95% oxygen and 5% carbon dioxide. Pulmonary arterial rings were initially stretched to produce a preload of 1g of force and equilibrated for 60 to 90 minutes. A preload of 1g was proved to be the optimal resting tension by length-tension analyses performed in pilot studies. This value is close to the optimal resting tension ($1.060g \pm 0.040g$) found by Liu and coworkers¹⁹ in piglet neonatal pulmonary arterial rings. During this period, the Krebs-Henseleit buffer in the tissue baths was replaced every 10 minutes. After incubation with indomethacin (10^{-5} mol/L) for 60 minutes, a concentration-response curve to phenylephrine was obtained. The rings were then washed, and the developed force was allowed to return to baseline. The rings were then precontracted with phenylephrine to generate 1g of developed force. Once a stable contraction was obtained, different pharmacologic protocols were performed.

- Cumulative doses of acetylcholine (10^{-9} – 10^{-4} mol/L) were added to the bath to assess changes in endothelium-dependent relaxation.
- Cumulative doses of calcium ionophore (10^{-9} – 10^{-5} mol/L) were added.
- Cumulative doses of acetylcholine with the NOS inhibitor N-monomethyl-L-arginine (L-NAME (10^{-3} mol/L) were added.
- Cumulative doses of calcium ionophore with L-NAME (10^{-3} mol/L) were added.

In addition to the change in force, responses were assessed by determining the concentration that produced 50% of the maximal response (EC_{50}), extrapolated from a plot of log concentration versus percentage of maximal response. The contractile response to phenylephrine were expressed in absolute values (g), and the maximum relaxations to acetylcholine and calcium ionophore were expressed as percentages of the phenylephrine-induced precontraction, with 0% indicating no relaxation and 100% indicating a relaxation equals to that of the precontraction. Post-CPB pulmonary arterial rings were compared with control pulmonary arterial rings obtained from matched piglets that did not undergo CPB.

Isolated Perfused Lung

The left lung was harvested and suspended by the left main bronchus to a force-displacement transducer placed in a humidified chamber to allow monitoring of weight changes. An MMS 107 respirator (S.N.E.H., Gonesse, France) set at 20 breaths/min with a tidal volume of 200 mL and a positive end-expiratory pressure of 2 cm H₂O was used to ventilate the left lung with a humidified warm gas mixture (20% oxygen, 75% nitrogen, and 5% carbon dioxide). The lung was perfused with 300 mL of heparinized autologous blood through a cannula placed in the left pulmonary artery. A constant flow of 0.03 mL/g body weight was maintained with a peristaltic pump (Ismatec; Bioblock, Strasbourg, France)

placed in series with the arterial line. Venous blood was collected by gravity into a reservoir through a cannula in the left atrium and was recirculated for 60 minutes. To prevent loss of reservoir volume through retrograde perfusion of the bronchial circulation, the bronchial arteries were ligated. PAP and pulmonary venous pressure (PVP) were continuously monitored with pressure transducers (model P23 ID) placed proximal to the lung, on the arterial line and on the venous line, respectively. Pressure signals were amplified (model M52; Telco) and recorded on a polygraph recorder (Solar 8000, Marquette, Milwaukee, Wis). Zone 3 conditions (arterial > venous > alveolar pressures) were maintained throughout all experiments.

Measurements of Pulmonary Hemodynamics and Microvascular Lung Permeability

Pulmonary capillary pressure (Pc') was estimated with the double-occlusion method.²⁰ With this method, after simultaneous occlusion of the arterial and venous line, PAP and PVP equilibrate to the same pressure, which is well correlated with Pc' . Pulmonary arterial resistance (Ra) and venous resistance (Rv) were calculated as follows:

$$Ra = (PAP - Pc')/Q$$

$$Rv = (Pc' - PVP)/Q$$

where Q is the flow.

The filtration coefficient (K_{fc}) was used as an index of endothelial permeability to fluid and was measured by the isogravimetric method described by Hakim and Kelly.²¹ Briefly, after an isogravimetric period of 30 minutes, PVP was rapidly increased by 20 cm H₂O for 20 minutes by raising the outflow end of the venous reservoir connected to the left atrial cannula. The resultant increase in lung weight was recorded. The characteristic rapid weight gain from vascular bed filling was followed by a phase of slower weight gain reflecting filtration of fluid into the pulmonary interstitium. The rate of slow weight change ($\Delta W/\Delta t$) was analyzed with linear regression of the $\log_{10}$ -transformed weight changes per minute. The initial rate of weight gain was calculated by extrapolating $\Delta W/\Delta t$ to time 0. K_{fc} was obtained by dividing $\Delta W/\Delta t$ at time 0 by the change in Pc' that occurred after the venous outflow pressure increase, normalizing the result for the baseline wet lung weight, and expressing it in milliliters per minute per centimeter of water per 100 g lung tissue. Baseline wet lung weight was estimated by measuring the weight of the left lung at the beginning of the experiment and subtracting the weight of the extrapulmonary tissue measured after reperfusion.

Statistical Analysis

Results are expressed as mean $\pm$ SD. Comparison were performed with paired and unpaired 2-way analysis of variance. The Spearman rank correlation test was performed to assess any correlation between lung VEGF values and K_{fc} .

Results

Hemodynamic Values

Two hours after weaning from 90 minutes of CPB, only PAP and PVR were statistically significantly increased relative to pre-CPB measures (Table 1).

TABLE 1. Hemodynamic values before and after CPB

	Before CPB (n = 12)	60 min after CPB (n = 12)
Systemic pressure (mm Hg, mean $\pm$ SD)	45 $\pm$ 5.04	49.8 $\pm$ 11.5
Left atrial pressure (mm Hg, mean $\pm$ SD)	4.5 $\pm$ 1.3	5.4 $\pm$ 1.8
Right atrial pressure (mm Hg, mean $\pm$ SD)	3.4 $\pm$ 1.2	3.75 $\pm$ 1.5
PAP (mm Hg, mean $\pm$ SD)	11.42 $\pm$ 2.14	16.5 $\pm$ 4.23*
PVR (dyne $\cdot$ s $\cdot$ cm ⁻⁵ , mean $\pm$ SD)	982.8 $\pm$ 327.6	1829.7 $\pm$ 862.9†
Cardiac output (mL/min)	780	733

P* = .01.†*P* = .03.TABLE 2. Assessment of lung VEGF content and exhaled nitric oxide before and after CPB**

	Animals assessed	Before CPB	After CPB
Lung VEGF (pg/100 mg tissue)	16	91.07 $\pm$ 5.31	151.6 $\pm$ 11.4*
Muscle VEGF (pg/100 mg tissue)	5	76.02 $\pm$ 11.53	81.6 $\pm$ 7.7
Exhaled nitric oxide (ppb)	8	6 $\pm$ 1.7	3.12 $\pm$ 1.4†

All values are mean $\pm$ SD.**P* = .01.†*P* = .003.**Exhaled Nitric Oxide and VEGF Quantification**

After CPB there were a statistically significant decrease in exhaled nitric oxide (6 $\pm$ 1.7 ppb before bypass versus 3.12 $\pm$ 1.4 ppb after bypass, *P* = .003), a statistically significant increase in lung VEGF (from 91.07 $\pm$ 5.314 pg/100 mg tissue to 151.6 $\pm$ 11.4 pg/100 mg tissue, *P* < .0001), and an increase in muscle VEGF (from 76.02 $\pm$ 11.53 pg/100 mg tissue to 81.58 $\pm$ 7.7 pg/100 mg tissue, *P* not significant). Variation in lung VEGF accumulation was statistically significantly higher than that in muscular VEGF accumulation (60.5 $\pm$ 9.1 vs 5.5 $\pm$ 5.9, *P* = .0008; Table 2).

Pulmonary Vascular Reactivity

In pulmonary vessels, similar maximal contractions to phenylephrine were observed in all groups of vascular rings. The EC₅₀ to phenylephrine after CPB was not different from that in control animals not subjected to CPB.

Maximal relaxation response to acetylcholine was reduced after bypass to 30% of control values (*P* < .0001; Figure 1). EC₅₀ for acetylcholine was nearly unchanged before and after CPB, ranging from 8.5 $\times 10^{-8}$ to 1.8 $\times 10^{-7}$ mol/L. These results indicate that endothelium-dependent relaxation of pulmonary artery was impaired after CPB. Addition of L-NAME significantly reduced the relaxation in control vascular rings, but to a less extent than in post-CPB pulmonary vessels (from 55.5% $\pm$ 5.96% to 18.5% $\pm$ 4.1% of relaxation in control pulmonary arterial rings vs when L-NAME was added and from 15.44% $\pm$ 4.8% to 6.26% $\pm$ 3.7% in post-CPB rings, *P* = .04).

In another series of pulmonary arterial rings, after pre-

contraction with phenylephrine, cumulative doses of calcium ionophore were added to the bath. Maximal relaxation response to calcium ionophore was unchanged from control values (120.63% $\pm$ 15.46% in control rings vs 110.77% $\pm$ 8.9% in post-CPB rings, *P* not significant; Figure 2). EC₅₀ values for calcium ionophore were similar before and after CPB. Addition of L-NAME in the bath reduced the relaxation in both control and post-CPB pulmonary arterial rings to the same extent (from 120.63% $\pm$ 15.46% to 62.8% $\pm$ 7.35% in control rings and from 110.77% $\pm$ 8.9% to 43.37% $\pm$ 6.3% in post-CPB rings, *P* not significant).

Isolated Lung Study

This approach demonstrated statistically significant increases in PVR and in K_{fc} after CPB versus control (PVR 1.235 $\pm$ 0.31 dyne $\times$ s $\times$ cm⁻⁵ vs 0.422 $\pm$ 0.25 dyne $\times$ s $\times$ cm⁻⁵, *P* = .01, K_{fc}: 0.525 $\pm$ 0.07 vs 0.249 $\pm$ 0.02, *P* < .0001; Table 3). In addition, statistically significant correlations were found between postbypass lung VEGF and lung K_{fc} (*P* = .0058) and between change in lung VEGF and change in lung K_{fc} (*P* = .03).

Discussion

This study confirms some previously demonstrated findings and opens new perspectives on CPB pathophysiology. As previously demonstrated, CPB is responsible for pulmonary hypertension through complex mechanisms associated with ischemia-reperfusion and cytokine production and blunts the pulmonary vascular endothelial ability to maintain a low

Figure 1. Relaxation curves to endothelial receptor-dependent agonist acetylcholine in pulmonary arterial rings after CPB (filled circles) and in non-CPB control rings (open circles). Asterisk indicates $P = .0001$.

Figure 2. Relaxation curves to endothelial non-receptor-dependent agonist calcium ionophore in pulmonary arterial rings after CPB (filled circles) and in non-CPB control rings (open circles).

vascular tone within the pulmonary arterial bed. Although this information has led to the clinical use of inhaled nitric oxide, few authors have tried to define the exact sequence of events that lead to endothelial dysfunction. This work shows that during CPB there is an accumulation of VEGF within the lungs and, as previously described elsewhere,¹⁵ there is a decrease in exhaled nitric oxide. Although the method used to assess the quantity of exhaled nitric oxide was not accurate for true alveolar nitric oxide production, in both settings the same bias was introduced before and after CPB, and the variations in nitric oxide production were thus taken into account. In addition, measurements of lung VEGF were obtained from tissue homogenate, which includes epithelial and smooth muscles cells as well as endothelial cells. Although this measurement does not reflect the true pulmonary endothelial production of VEGF, the variations of this measure were considered to represent a global growth factor production or accumulation that could have vascular effects. These observations are in contrast with the physiologic experiments, which showed that VEGF production is responsible for vasodilatation through an endothelial and nitric oxide-mediated mechanism. Therefore in the setting of CPB, accumulation of VEGF in the lungs tissue and vasculature should be responsible for a pulmonary arterial tree vasodilatation associated with an increase in nitric oxide production.

In vitro experiments on arterial rings showed that endothelium-dependent vasodilatation to acetylcholine was blunted after CPB. Acetylcholine, through membrane mus-

TABLE 3. Hemodynamic values and K_{fc} assessment in isolated perfused lungs after CPB and in non-CPB control animals

	After CPB (n = 13)	No CPB (control, n = 10)
PAP (mm Hg)	29.3 ± 8	16.6 ± 3.9
Capillary pressure (mm Hg)	10.1 ± 4.04	8.7 ± 3.56
K_{fc}	0.525 ± 0.07	$0.249 \pm 0.02^*$
PVR (dyne $\cdot$ s $\cdot$ cm ⁻⁵)	1.235 ± 0.31	$0.422 \pm 0.25^*$

All values are mean $\pm$ SD.

* $P = .01$.

carinic receptors, activates G protein, which subdivides in α and β subunits. This in turns activates the phospholipase C and inositol pathway, which eventually mobilizes calcium from intracellular stores. Increases in intracellular calcium then activate NOS, leading to nitric oxide production. On the other side, calcium ionophore is not a receptor-dependent agonist and itself leads to an increase in intracellular calcium. Therefore in pulmonary arterial rings after total CPB it appears that the signal transduction from membrane receptors to increased intracellular calcium is impaired. This approach was previously used by Evora and associates²² in coronary arteries rings to demonstrate G protein dysfunction after myocardial ischemia and reperfusion in the dog. In addition, Chang and colleagues²³ showed that G protein dysfunction could be attenuated by continuous warm blood cardioplegia. This finding has, however, to the

best of our knowledge not been demonstrated in the pulmonary vascular bed after CPB. As a preliminary report, one may conclude that CPB through lung ischemia-reperfusion injury is responsible for endothelial G protein dysfunction, leading to an impairment of signal transduction from the membrane receptor to the intracellular calcium mobilization.

On the other side, VEGF through its membrane tyrosine kinase receptors activates the phospholipase C and inositol pathway that mobilizes calcium from intracellular stores without any activation of G protein.²⁴ Therefore the finding that after CPB there is a pulmonary accumulation of VEGF that does not in turn promote endothelial nitric oxide secretion means that post-CPB pulmonary artery endothelial dysfunction is due to endothelial membrane lesion. In vitro dissection of these processes to point out the impaired sequence of signal transduction is still under evaluation. Indeed, cellular membrane lesions have been documented after ischemia-reperfusion.²⁵ The following sequence of events can be therefore proposed. During CPB, the lungs are subjected to an ischemic process because there is no more antegrade flow through the main pulmonary artery, mimicked in our model by crossclamping of the pulmonary artery, and bronchial collaterals do not have enough flow to provide adequate lung perfusion. This phenomenon triggers the production of hypoxia-inducible factor 1,^{13,26} which in turns activates production of VEGF and expression of VEGF receptors within the ischemic territory.²⁷ Endothelial cell ischemia-reperfusion leads to membrane lesions and induces a dysfunction in signal transduction leading to nitric oxide production. Attempts at producing pulmonary arterial ring vasodilatation with increasing doses of VEGF were unsuccessful in this model either before or after CPB. Therefore no pharmacologic in vitro data were available to demonstrate the interaction between VEGF and nitric oxide. Tofukuji and colleagues²⁸ showed that during myocardial ischemia VEGF messenger RNA is upwardly regulated, as is flk-1 messenger RNA. They also demonstrated that, despite ischemia and reperfusion, relaxation of precontracted coronary arterioles to VEGF was statistically significantly augmented and remained unchanged after use of receptor-mediated endothelium-dependent vasodilator adenosine diphosphate, which contrasts with the results of Chang and associates.²³ The difference in results may be multifactorial. First, microvessels contain less smooth muscle and might be more sensitive to vasodilator agonists than 2-mm diameter pulmonary arterial rings. Second, in their model Tofukuji and colleagues²⁸ showed this particular type of vascular reactivity after use of cardioplegia, whereas in this study lungs were not subjected to any type of pharmacologic protection. Finally, coronary and pulmonary circulations may display different responses to vasodilators after ischemia and reperfusion.

Other possible explanations for the lack of nitric oxide production related to VEGF accumulation might be an endothelial NOS dysfunction, which was ruled out by the calcium ionophore data; a downward regulation of VEGF receptors, but none of our data can confirm or disconfirm this hypothesis; or a questionable relationship between VEGF and nitric oxide in the pulmonary vasculature, which might be answered by use of a VEGF blocker; however, this type of molecule is under experimentation in other medical fields and was not available for our work. Finally, the fact that acetylcholine is unable to relax pulmonary arterial rings after CPB may simply be due to a downward regulation of muscarinic receptors.

An additional probable effect of lung VEGF accumulation was an increase in K_{fc} . Indeed, VEGF was previously known to be the vascular permeability factor, a protein that promotes extravasation of proteins from tumor-associated blood vessels. This observation is consistent with the finding of increased lung water content after CPB and explains some postoperative ventilatory problems. A statistically significant correlation between these findings was found. VEGF has already been investigated as a potential source of edema formation in the heart²⁸ and brain²⁹ after the ischemia-reperfusion phenomenon. This correlation was even more relevant when muscular VEGF was shown not to be statistically significantly increased after bypass. This suggests that because skeletal muscles are not subjected to ischemia during CPB, only ischemic territories express VEGF accumulation, either through production or through higher receptor expression or affinity. A recent work by Abrahamov and coworkers³⁰ showed an independent correlation between plasma VEGF and capillary leak syndrome in neonates after CPB. This finding is not in contrast with those of our work, because some soluble VEGF can be found in the systemic circulation as a result of the excess in the vicinity of the stressed territories.

In conclusion, this study demonstrates that total CPB induces pulmonary hypertension with a pulmonary vascular endothelial dysfunction through a multifactorial mechanism that mainly includes ischemia-reperfusion phenomenon and cytokine production. In addition to vasoconstrictors cytokines endothelin 1 and thromboxane previously demonstrated to intervene in this reaction,³¹ this work shows that it is also probably related to membrane alterations or G protein dysfunction, which in turns impairs signal transduction, leading to nitric oxide production with an accumulation of upstream products. One of these products is VEGF, which also increases vascular permeability and is probably (in addition to other factors) responsible for post-CPB pulmonary edema, a finding that is less pronounced in the systemic nonischemic territories.

References

1. Dodrill FD. The effects of total body perfusion upon the lungs. In Allen JG, editor. Extracorporeal circulation. Springfield (IL): Charles Thomas; 1958. p. 327-35.
2. Ratliff NB, Young WG Jr, Hackel DB, Millat E, Wilson JW. Pulmonary injury secondary to extracorporeal circulation. An ultrastructural study. *J Thorac Cardiovasc Surg.* 1973;65:425-32.
3. Serraf A, Robotin M, Bonnet N, Détruit H, Baudet B, Mazmanian MG, et al. Alteration of the neonatal pulmonary physiology after total cardiopulmonary bypass. *J Thorac Cardiovasc Surg.* 1997;114:1061-9.
4. Risau W. Mechanisms of angiogenesis. *Nature.* 1997;386:671-4.
5. Wu HM, Huang Q, Yuan Y, Granger HJ. VEGF induces NO-dependent hyperpermeability in coronary venules. *Am J Physiol.* 1996;271(6 Pt 2):H2735-9.
6. Brock TA, Dvorak HF, Senger DR. Tumor secreted vascular permeability factor increases cytosolic Ca^{++} and von Willebrand release in human endothelial cells. *Am J Pathol.* 1991;138:213-21.
7. Moncada S, Higgs EA. Endogenous nitric oxide: physiology, pathology and clinical relevance. *Pharmacol Rev.* 1991;21:361-74.
8. Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates eNOS message, protein, and NO production in human endothelial cells. *Am J Physiol.* 1998;274(3 pt 2):H1054-8.
9. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science.* 1983;219:983-5.
10. Gerber HP, McMurtrey A, Kowalski J, Yan M, Key BA, Dixit V, et al. Vascular endothelial growth factor regulates endothelial cells survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. *J Biol Chem.* 1998;273:30336-43.
11. Scalia R, Booth G, Lefer DJ. Vascular endothelial growth factor attenuates leukocyte-endothelium interaction during acute endothelial dysfunction: essential role of endothelial-derived nitric oxide. *FASEB J.* 1999;13:1039-46.
12. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature.* 1992;359:843-5.
13. Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem.* 1995;270:13333-40.
14. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* 1999;13:9-22.
15. Beghetti M, Silkoff PE, Caramori M, Holtby HM, Slutsky AS, Adatia I. Decreased exhaled nitric oxide may be a marker of cardiopulmonary bypass-induced injury. *Ann Thorac Surg.* 1998;66:532-4.
16. Serraf A, Sellak H, Herve P, Bonnet N, Robotin M, Detruit H, et al. Vascular endothelium viability and function after total cardiopulmonary bypass in neonatal piglets. *Am J Respir Crit Care Med.* 1999;159:544-51.
17. Cooper WA, Duarte IG, Thourani VH, Nakamura M, Wang NP, Brown WM 3rd, et al. Hypothermic circulatory arrest causes multi-system vascular endothelial dysfunction and apoptosis. *Ann Thorac Surg.* 2000;69:696-702.
18. Turnage RH, Kadesky KM, Bartula L, Myers SI. Intestinal reperfusion upregulates inducible nitric oxide synthetase activity within the lung. *Surgery.* 1995;118:288-93.
19. Liu SF, Hislop AA, Haworth SG, Barnes JP. Developmental changes in endothelium dependent pulmonary vasorelaxation. *Br J Pharmacol.* 1992;106:324-30.
20. Townsley MI, Korthuis RJ, Rippe B, Parker JC, Taylor AE. Validation of double occlusion method for Pci in lung and skeletal muscle. *J Appl Physiol.* 1986;108:1-9.
21. Hakim TS, Kelly S. Occlusion pressures vs. micropipette pressures in th pulmonary circulation. *J Appl Physiol.* 1989;67:1277-85.
22. Evora PB, Pearson PJ, Schaff HV. Impaired endothelium-dependent relaxation after coronary reperfusion injury: Evidence for G-protein dysfunction. *Ann Thorac Surg.* 1994;57:1550-6.
23. Chang CH, Lin PJ, Chu Y, Lee YS. Impaired endothelium-dependent relaxation after cardiac global ischemia and reperfusion: role of warm blood cardioplegia. *J Am Coll Cardiol.* 1997;29:681-7.
24. He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through Flk-1 KDR activation of c-Src. *J Biol Chem.* 1999;274:25130-5.
25. Song C, Al-Mehdi AB, Fisher AB. An immediate endothelial cell signaling response to lung ischemia. *Am J Physiol Lung Cell Mol Physiol.* 2001;281:L993-L1000.
26. Gregg L, Semenza. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J App Physiol.* 2000;88:1474-80.
27. Waltenberger J, Mayr U, Pentz S, Hombach V. Functional upregulation of the vascular endothelial growth factor receptor KDR by hypoxia. *Circulation.* 1996;94:1647-54.
28. Tofukuji M, Metais C, Li J, Franklin A, Simons M, Sellke FW. Myocardial VEGF expression after cardiopulmonary bypass and cardioplegia. *Circulation.* 1998;98(19 Suppl):II242-6.
29. Paul R, Zhang ZG, Eliceiri BP, Jiang Q, Boccia AD, Zhang RL, et al. Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. *Nat Med.* 2001;7:222-7.
30. Abrahamov D, Erez E, Tamariz M, Dagan O, Pearl E, Abrahamov Y, et al. Plasma vascular endothelial growth factor level is a predictor of the severity of postoperative capillary leak syndrome in neonates undergoing cardiopulmonary bypass. *Pediatr Surg Int.* 2002;18:54-9.
31. Pearl JM, Manning PB, McNamara JL, Saucier MM, Thomas DW. Effect of modified ultrafiltration on plasma thromboxane B_2 , leukotriene B_4 , and endothelin-1 in infants undergoing cardiopulmonary bypass. *Ann Thorac Surg.* 1999;68:1369-75.

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