Effect of NADPH oxidase inhibition on cardiopulmonary bypass-induced lung injury

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diphenyleneiodonium; apocynin; reflection coefficient; pig; endothelium; hypoxia-reoxygenation; tumor necrosis factor; interleukin-6

Cardiopulmonary bypass (CPB) causes significant lung injury characterized by abnormal gas exchange and pulmonary edema (61). Pulmonary dysfunction results in a mortality rate of nearly 0.7% following cardiac surgery (12, 28), accounting for over half of the 1.3% overall mortality associated with this procedure (11). Proposed mechanisms behind CPB-induced lung injury include the adverse effects of pulmonary vascular ischemia and reperfusion (18), and, if the lung is deflated during CPB, tissue hypoxia and reoxygenation (18, 33). The generation of reactive oxygen species (ROS) is common to all of these injurious mechanisms and is thought to play an important role in the ensuing injury. Sources of ROS include activated inflammatory cells, such as neutrophils (44) and macrophages, as well as vascular endothelial cells subjected to ischemia-reperfusion (I/R) (17) or hypoxia-reoxygenation (66). One of the major pathways of ROS production in these cells involves an NADPH-dependent oxidase. In neutrophils, this enzyme consists of a heterodimeric transmembrane protein flavocytochrome b245 formed by the combination of gp91phox and p22phox and multiple cytoplasmic components, including the proteins p47phox, p67phox, and p40phox as well as the regulatory protein p21 rac (6). With appropriate stimuli, the cytosolic and membrane proteins assemble, and the enzyme generates superoxide anion by a one-electron transfer from NADPH to O2. The endothelial form of the enzyme appears to differ by having an intracellular location, constitutive activity, and lower ROS production (23, 35, 54).

The goal of the present study was to determine the role of NADPH oxidase in the pulmonary vascular dysfunction after CPB. To inhibit NADPH oxidase, we used two structurally unrelated inhibitors. Apocynin, a methoxy-substituted catechol, blocks phagocytic NADPH oxidase in neutrophils (13, 57) and macrophages (40, 57) as well as systemic endothelial NADPH oxidase activity (26) through inhibition of p47phox translocation (39). Other actions include inhibition of thomboxane synthase (13) and cytochrome P450 (48). Additional experiments were performed with diphenyleneiodonium (DPI), which impairs NADPH oxidase by flavoprotein inhibition (15). Unlike apocynin, DPI is also capable of inhibiting nitric oxide synthase (25) and mitochondrial electron transport (57).

We previously found that apocynin completely prevented the increased pulmonary vascular permeability and edema in an isolated lung model of I/R injury (13). In that study, however, ischemia and reperfusion occurred in the absence of hypoxia-reoxygenation because ventilation was maintained during ischemia. In the lung, the mechanisms of ROS gener-
ation secondary to ischemia and reperfusion may differ from those of hypoxia and reoxygenation. For example, NADPH oxidase-derived ROS from neutrophils and endothelial cells have been implicated in I/R, whereas xanthine oxidase (XO)-derived ROS played an important injurious role in some models of lung hypoxia-reoxygenation (1, 2, 62). By contrast, cell culture models of hypoxia-reoxygenation utilizing pulmonary endothelial cells demonstrated extracellular hydrogen peroxide release that was blocked by DPI but not by XO inhibition, suggesting that a significant component of ROS production under these circumstances was derived from an endothelial NADPH oxidase (64). The effect of apocynin was not evaluated in these studies.

To determine the role of NADPH oxidase in CPB-induced lung injury, we examined the effects of apocynin and DPI on the pulmonary vascular injury caused by 2 h of CPB in pigs. We performed warm CPB (58) with an occluded pulmonary artery (4), a ligated bronchial artery (14), and deflated, non-ventilated lungs (43, 47, 52, 60) to maximize lung injury. We assessed lung injury over the first 25 min following CPB because we previously found that the peak alveolar-arterial oxygen tension difference occurred over this time period (22, 49). In the present study, arterial oxygenation, pulmonary hemodynamics, plasma cytokine concentrations, extravascular lung water, and pulmonary vascular permeability were measured to assesslung injury. Additional experiments were performed to determine the effects of apocynin and DPI on ROS production in cultured pulmonary artery endothelial cells exposed to hypoxia-reoxygenation.

METHODS

Animal Preparation

CPB, SURGICAL PROCEDURE. All animals received care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Pigs (35–50 kg) were fasted for 12 h before surgery. They were anesthetized with ketamine (30 mg/kg im, then 3–6 mg iv every 10–20 min) and inhaled halothane. After intubation, they were mechanically ventilated (Harvard Ventilator) with 100% oxygen at an initial rate of 10 breaths/min, a tidal volume of 12 ml/kg, and no positive end-expiratory pressure. Ventilation was stopped, and the endotracheal tube was disconnected from the ventilator. Warm CPB (37°C) was continued for 2 h with a blood flow of 70–80 ml/kg and a targeted systemic MAP of 50–60 mmHg. At termination of CPB, atelectasis was reversed with a single hyperinflation, mechanical ventilation with 100% O₂ was resumed, and the pigs were weaned from CPB. Throughout the experiment, pH was maintained 7.35–7.45 by the administration of NaHCO₃ and the adjustment of the ventilator rate. Intravenous fluids and α-agonists were used to treat systemic hypotension (MAP < 50 mmHg). Atroine and epinephrine were utilized to reverse bradycardia. The pigs were killed by rapid exsanguination from the femoral artery cannuas 30 min after separation from CPB.

HEMODYNAMIC MEASUREMENTS AND ARTERIAL BLOOD GASES. Hemodynamic parameters, airway pressure (Spacelabs Medical, Instrumentarium; Issaquah, WA), arterial blood gases (model 348 Blood Gas Analyzer, Electrolyte, Hematocrit Analyzer; Chiron Diagnostics; Norwood, MA), and blood samples for cell counts were obtained at baseline before CPB, at the end of CPB just before separation, and at 10-min intervals (starting at 5 min) following separation from CPB. Cardiac output (CO) was measured by thermodilution by using a 5-ml injection of cold saline (mean of three measurements). The pulmonary vascular resistance (PVR) was calculated as:

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PVR = \frac{P_{PA} - P_{LA}}{CO}
\]

where \(P_{PA}\) is pulmonary artery pressure and \(P_{LA}\) is left atrial pressure. Systemic vascular resistance (SVR) was calculated as:

\[
SVR = \frac{P_{SA} - P_{RA}}{CO}
\]

where \(P_{SA}\) is systemicartery pressure and \(P_{RA}\) is right atrial pressure.

BLOOD CELL COUNTS. Hematocrit (Hct), white blood cell counts, and platelet counts were measured by machine (Coulter Counter, Beckman Coulter; Fullerton, CA). Neutrophil counts were determined by manual evaluation of Wright-stained blood smears.

INDUCIBLE BLOOD CHEMILUMINESCENCE. To determine the effect of NADPH oxidase inhibitor treatment on the capacity of pig blood samples to generate ROS, blood samples were obtained at the time intervals described above as well as immediately before and after infusions of drug or diluent. Heparinized blood was diluted 1:10 in enzyme-linked immunosorbent assay (R&D Systems; Minneapolis, MN).

REFLECTION COEFFICIENT FOR ALBUMIN. After exsanguination, the pulmonary artery and left atrium were cannulated, and the pulmonary vasculature was flushed with 200 ml of a mixture of autologous blood and 3% dextran 70 (Hct = 20%) as previously described (46). The pulmonary arterial and left atrial cannuas were connected to a pressurized stirred reservoir filled with the blood-dextran mixture, and static intravascular pressure (referenced to the level of the middle of the lung) was increased to 35 mmHg for 15 min. Intravascular blood was then pumped retrograde and collected in serial 12-ml samples. Reflection coefficient for albumin (σₐ) was estimated by...
the filtered volumes method modified for a nonflowing system (8). Briefly, Hct and albumin concentration were determined in duplicate for each sample, and $\sigma_{an}$ was estimated iteratively from the relationship

$$\frac{C}{C_0} = \frac{1 - Hct - \sigma}{1 - Hct - \sigma} \left( \frac{1 - Hct}{1 - Hct - \sigma} \right)^{-nHct}$$  (3)

where C represents albumin concentration and $C_0$ and $Hct_0$ represent initial reservoir values. The $\sigma_{an}$ for each lung was determined from the sample with the greatest change in Hct from $Hct_0$.

**EXTRAVASCULAR LUNG WATER.** Extravascular lung water (EVLW) and blood-free dry lung weight (BFDLW) were determined after the period of increased intravascular pressure by using hemoglobin concentration as the intravascular marker, as previously described (46).

**Experimental protocol.** Pigs were administered an equal volume of diluent (Ringer-lactate solution, $n = 8$), low-dose apocynin (200 mg/kg, $n = 6$; Aldrich Chemical; Milwaukee, WI), high-dose apocynin (400 mg/kg, $n = 6$), or DPI (8 mg/kg, $n = 6$). An additional group of pigs was treated with indomethacin (10 mg/kg, $n = 3$). All drug dosages were given in two equal dosages. The first dose was administered by intravenous infusion 5 min after the initiation of CPB. The second dose was added to the CPB circuit 5 min before CPB ended. The high apocynin dose was chosen to achieve 95% inhibition of blood leukocytes after a single administration. A 50% reduction in dose was used for the low-dose group. Apocynin was dissolved in 500 mL warmed Ringer-lactate solution. DPI (Colour Your Enzyme; Ontario, Canada) was dissolved in water by sonication for 30–60 s to create a stock solution (0.5 mg/mL) that was then diluted in 0.9% normal saline for intravenous administration. The dose was chosen based on the study by Vlessis et al. (58). Indomethacin (Sigma Chemical) was prepared by dissolving 100 mg in 50 mL normal saline and 10 mL of 1 M NaHCO$_3$. The dose of indomethacin was chosen to prevent cytochrome oxidase metabolism, as indicated by inhibition of the transient pulmonary hypertensive response to intrapulmonary artery injection of arachidonic acid in anesthetized sheep (45).

An additional group of control pigs in which the bronchial artery was left patent was concurrently studied and compared with the bronchial artery-ligated control animals to examine the effect of bronchial artery perfusion on CPB-induced lung injury. These data have been published separately (14).

**Effect of Apocynin on Endothelial ROS Production After Hypoxia-Reoxygenation**

**Pulmonary artery endothelial cultures.** Bovine pulmonary artery endothelial cells were obtained between passages 6 and 10 (Coriell Cell Repositories; Camden, NJ) and grown to confluence (DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 U/mL penicillin, 50 μg/mL streptomycin, and 250 μg/mL fungizone; 37°C, 5% CO$_2$, 95% air) in 150-mm dishes. For the experimental studies, monolayers were gently washed [10 mL phosphate-buffered saline (PBS)], incubated with 2 mL of 0.1% trypsin for 10 min, followed by quenching with medium containing 10% fetal bovine serum (10 mL). The cells were centrifuged (100 g × 3 min), washed twice (10 mL PBS), and resuspended in the desired volume of diluent.

**Electron paramagnetic resonance measurement of ROS.** Electron paramagnetic resonance (EPR) of endothelial cells was determined as previously described (65). Briefly, with the use of purified 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO, 100 mM) as the spin trap, EPR spectra were recorded in flat cells at room temperature with a Bruker IBM ER 300 spectrometer (Bruker Instruments; Bellerica, MA) operating at X-band with 100-kHz modulation frequency and a TM 110 cavity. The microwave frequency and magnetic field were precisely measured with an EIP 575 microwave frequency counter (EIP Microwave; San Jose, CA) and a Bruker ER 035M NMR gaussmeter. Serial EPR acquisitions were performed at 1-min intervals, blocked, and stored in digital form at 10-min acquisitions. Quantification of the observed DMPO-OH spin adduct was performed as previously described (54).

**Experimental protocol.** Cells were exposed to room air or to severe hypoxia (vigorous stream of 100% nitrogen) at room temperature for 60 min. In addition, one of the following was added to cells subjected to hypoxia: 1) 200 U/mL recombinant human copper-zinc superoxide dismutase (n = 4; Biotechnology General; New York, NY); 2) 400 U/mL bovine liver catalase (n = 6; Sigma Chemical); 3) 3 mM apocynin (n = 4); 4) 20 μM DPI (n = 7); 5) 0.5 mM oxyxurinol (n = 6; Sigma Chemical); 6) 10 μM indomethacin (n = 5); or 7) drug diluent. After the 60-min incubation, cells were reoxygenated by exposure to air. Additional diluent or drug was added to create a cell suspension of 5 × 10⁶ cells/mL, and 900 μL of the solution were rapidly placed in a flat cell (open to room air) for EPR measurement over an additional 60 min. The SOD, catalase, and oxyxurinol concentrations were chosen based on previous studies in hypoxia-reoxygenation of systemic endothelial cells (65, 66). These apocynin and DPI concentrations were shown to inhibit neutrophil NADPH oxidase by >80% (13).

On each study day, multiple experimental replicates were performed with the same drug or diluent treatment (n = 2–6). These data were averaged to generate a single paired replicate (drug vs. diluent) for each experimental group for that day, which was then used in the subsequent statistical analysis. In addition, the average normoxic EPR spectra time course obtained on each study day was subtracted from each hypoxia-reoxygenation time course obtained on the same day to allow spectra comparisons between drug and diluent-treated cells that originated solely from the effects of hypoxia and reoxygenation.

**Statistics**

All time-course data were analyzed with two-factor (group, time), split-plot analysis (ANOVA) (53). Non-time-course data were compared by one-factor randomized ANOVA. When significant variance ratios were obtained, least significant differences were calculated to allow comparison of individual means. The mean values of arterial PO$_2$ (Pa$_{O_2}$), inducible blood chemiluminescence, TNF-α, and IL-6 were found to be directly proportional to standard deviations, indicating nonhomogeneity of variance; therefore, these data were transformed to logarithms before statistical analysis. The relationships between LEC and $P_{A_{CO_2}}$, $P_{A_{O_2}}$, and plasma cytokine concentrations and $\sigma_{an}$ were analyzed by least-squares linear regression. Values are presented as means ± SE. Differences were considered significant when P < 0.05.

**RESULTS**

**Animal Experiments**

**Hematological parameters.** The mean body weight did not differ between experimental groups averaging 41 ± 1 kg. Baseline Hct, total leukocyte, neutrophil, and platelet concentrations in control pigs were 31 ± 2% and 15,300 ± 1,700, 8,000 ± 1,600, and 491,000 ± 49,000 cells/mm$^3$, respectively. All hematological cell counts in this group decreased significantly by the end of CPB and then remained unchanged through the entire post-CBP period averaging 25 ± 2% and 5,100 ± 1,200, 2,200 ± 900, and 231,000 ± 41,000 cells/mm$^3$, respectively (data not shown). The time courses of total leukocyte, neutrophil, and platelet concentrations were not different between control and treatment groups. The time course of Hct values were similar in control, DPI, or indomethacin-treated pigs, but Hct decreased by a greater amount in the
apocynin-treated pigs. The Hct at the end of CPB averaged 19 ± 1 and 17 ± 2% in the low- and high-dose apocynin animals, respectively, and remained unchanged in the post-CPB period (P < 0.05 vs. control; data not shown). The decreased Hct associated with apocynin treatment was secondary to hemodilution from increased intravenous fluid administration during CPB to maintain target MAP. One low-dose and two high-dose apocynin-treated animals developed refractory shock and succumbed after the 15-min time point. All data through this time point were included in the analysis.

Effect on LEC. Figure 1 shows the time course of inducible whole blood LEC throughout the experiment. Apocynin decreased blood LEC in a dose-response fashion. For example, the low-dose apocynin treatment did not effectively decrease LEC compared with LEC in diluent-treated pigs until the second dosage was administered at the end of CPB. By contrast, the first administration of high-dose apocynin treatment decreased LEC before CPB by 96% (compared with the before-drug value). The LEC in the high-dose apocynin group remained lower than baseline and lower than the corresponding LEC in the other groups at each measurement time (P < 0.05) with the exception of DPI-treated pigs, which exhibited a similar degree of inhibition. Indomethacin treatment had no effect on LEC compared with control values (data not shown).

Effect on gas exchange. As shown in Fig. 2, control CPB resulted in severe gas exchange abnormalities. The PaO₂ decreased from 611 ± 18 mmHg before CPB to 64 ± 9 mmHg at 5 min after CPB and remained severely depressed through the end of the experiment (P < 0.05). This was accompanied by an increased minute ventilation (V̇_{\text{Etot}}), an unchanged arterial Pco₂ (PaCO₂), and a significant decrease in pH from pre-CPB levels. Indomethacin treatment had no effect on gas exchange compared with control values.

Apocynin treatment attenuated the post-CPB hypoxemia in a dose-response manner. Specifically, the PaO₂ values at 15 and 25 min in the high-dose apocynin group, which averaged 387 ± 77 and 335 ± 88 mmHg, respectively, and the low-dose apocynin group, which averaged 130 ± 42 and 162 ± 54 mmHg, differed significantly from each other and from the corresponding PaO₂ values in the control pigs (60 ± 9 and 52 ± 5 mmHg; P < 0.01). DPI treatment reproduced the protective effect of high-dose apocynin on arterial oxygenation with PaO₂ values that were significantly greater than control at all post-CPB time points and greater than low-dose apocynin at 5 and 15 min. The salutary effects of apocynin and DPI on oxygenation were accompanied by significantly lower PaCO₂ values at the same level of V̇_{\text{Etot}} compared with control pigs (P < 0.05). There was a significant linear relationship (R = −0.60, P < 0.005) between the log of blood LEC measured after the first dosage of apocynin, DPI, or diluent and the log PaO₂ measured 25 min after the discontinuation of CPB. There were no differences between the groups in arterial pH.

Fig. 1. Inducible blood chemiluminescence before and after each dose of apocynin (Apo), diphenyleneiodonium (DPI), or diluent and following separation from cardiopulmonary bypass (CPB) (n = 5–7). All drugs were administered twice: before and at the end of CPB. Values are means ± SE. †P < 0.05 vs. Pre-CPB before drug value. *P < 0.05 vs. other treatments at same time point by ANOVA interaction effect. #P < 0.05 vs. immediate corresponding before drug value. ‡P < 0.01 vs. Low Apo by ANOVA interaction effect.

Fig. 2. Time course of arterial PaO₂ (PaO₂), minute ventilation (V̇_{\text{Etot}}), arterial Pco₂ (PaCO₂), and arterial pH before and after 2 h of CPB in anesthetized pigs (n = 3–7) treated with diluent (control), Low Apo, High Apo, DPI, or indomethacin (Indo). Values are means ± SE. †P < 0.05 vs. Pre-CPB value. *P < 0.01 vs. Control by ANOVA interaction effect. #P < 0.05 vs. Control by ANOVA group effect. ‡P < 0.05 vs. Low Apo by ANOVA interaction effect.
Effect on airway and vascular pressures. The profound arterial hypoxemia in control animals was accompanied by a progressive increase in peak tracheal pressure (P_tr) from 8.2 ± 1.2 to 39.2 ± 6.5 mmHg by 25 min post-CPB and a sustained increase in P_PA and PVR over the post-CPB period compared with baseline (Fig. 3; P < 0.05). Baseline cardiac output and MAP (0.085 ± 0.006 l·min⁻¹·kg⁻¹ and 83 ± 6 mmHg, respectively) decreased by nearly 40% at 5 min post-CPB (0.053 ± 0.006 l·min⁻¹·kg⁻¹ and 55 ± 6 mmHg, respectively, P < 0.05) and then remained unchanged (data not shown). P_LA and P_RA were unaltered over the entire experimental period, averaging 10.3 ± 1.8 and 7.8 ± 1.5 mmHg, respectively (data not shown). Results were essentially identical in indomethacin-treated animals.

Neither apocynin nor DPI treatment altered the time courses of P_tr, P_LA, or P_RA, but both blocked the increase in P_PA observed in control pigs to a similar degree (Fig. 3). Both low- and high-dose apocynin and DPI also resulted in a decreased MAP at 25 min post-CPB to 39 ± 3, 42 ± 4, and 39 ± 4 mmHg, respectively (P < 0.05 vs. control). Apocynin-treated animals required more intravenous fluids during and after CPB to maintain MAP in the target range; thus, both doses of apocynin were associated with a 15% increase in cardiac output at 5 min post-CPB. These cardiac outputs were significantly greater than the cardiac output measured in control at this time point and, because MAP did not differ, indicated a decrease in SVR (data not shown). Subsequent cardiac outputs in apocynin-treated pigs did not differ from control values but remained greater than the average cardiac output in DPI-treated animals (0.034 ± 0.006 l·min⁻¹·kg⁻¹) resulting in the observed differences in PVR between apocynin and DPI (Fig. 3).

Effect on cytokine concentrations. As shown in Fig. 4, plasma TNF-α and IL-6 concentrations in control animals increased to 1.2 ± 0.6 and 2.2 ± 1.3 ng/ml, respectively, by 5 min post-CPB and remained elevated compared with baseline levels (P < 0.05). Despite the significant improvements in gas exchange and pulmonary vascular hemodynamics, neither apocynin nor DPI treatment had a significant effect on circulating plasma cytokine concentrations (Fig. 4). Cytokine measurements were only available for two indomethacin-treated pigs but were not statistically different from control animals (data not shown).

Effect on pulmonary EVLW and σ_alb. EVLW-to-BFDW ratio averaged 11.73 ± 1.27 in control lungs at the end of the experiment (following the period of increased intravascular pressure, Fig. 5). The σ_alb of the pulmonary circulation measured at the same time point was 0.53 ± 0.10, indicating increased pulmonary vascular protein permeability (Fig. 5). Neither NADPH oxidase nor cyclooxygenase inhibition affected EVLW or σ_alb, which averaged 0.53 ± 0.11, 0.54 ±
0.13, 0.23 ± 0.06, and 0.39 ± 0.002 in the low-dose apocynin, high-dose apocynin, DPI, and indomethacin groups, respectively (P = 0.16).

Relationship among PaO₂, cytokines, and σalb. There was no significant correlation between the final PaO₂ and either σalb (R = −0.40, P = 0.08) or the concentrations of plasma TNF-α (R = −0.25, P = 0.27) or IL-6 (R = 0.12, P = 0.61). There were significant correlations between the plasma TNF-α (R = −0.96, P = 0.01) and IL-6 concentrations (R = −0.95, P = 0.02) measured 25 min post-CPB and σalb in the control animals, but these correlations were lost with inclusion of the apocynin and DPI experiments.

Pulmonary Endothelial Cell Hypoxia-Reoxygenation

ROS production, as measured by DMPO-OH adduct generation, was increased by 1,400 ± 400% in bovine pulmonary artery endothelial cells after 60 min of reoxygenation following 60-min exposure to severe hypoxia (Fig. 6, inset; P < 0.001). This signal was significantly attenuated (P < 0.05) by incubation with SOD (90 ± 10% inhibition), catalase (94 ± 6% inhibition), apocynin (56 ± 14% inhibition), or DPI (35 ± 10% inhibition). The effect of apocynin was not statistically different from the inhibition provided by either SOD or catalase, whereas the inhibitory effect of DPI was significantly less (P < 0.01). The effect of oxypurinol on ROS generation was quantitatively similar to DPI but, like indomethacin, failed to achieve statistical significance compared with diluent control.

DISCUSSION

CPB has long been known to cause acute lung injury characterized by arterial hypoxemia and decreased lung compliance as well as increased pulmonary vascular resistance, permeability, and edema (41). ROS generated by the NADPH oxidase in sequestered neutrophils and pulmonary macrophages are thought to play a significant role in this injury (41). More recently, a vascular nonphagocytic NADPH (or NADH)-dependent oxidase in endothelial and smooth muscle cells has been hypothesized to contribute to oxidant-mediated vascular injury (34). Excessive ROS production from this enzymatic source could occur in association with CPB because of complement activation, pulmonary vascular I/R (18), or hypoxia-reoxygenation (41). ROS can directly cause endothelial barrier dysfunction and pulmonary edema. ROS can also trigger a cascade of other potentially injurious mediators known to be increased during and after CPB, including prostaglandins such as thromboxane (32) and cytokines such as TNF-α and IL-6 (3).

From these considerations, we hypothesized that inhibition of NADPH oxidase would significantly attenuate CPB-induced lung injury. To test this hypothesis, we administered two structurally unrelated NADPH oxidase inhibitors and utilized blood LEC to monitor the activity of circulating phagocytic NADPH oxidase as a surrogate for the NADPH oxidase activity of sequestered and resident lung phagocytes as well as nonphagocytic vascular cells. Both apocynin and DPI inhibited inducible blood LEC throughout the protocol, suggesting that we successfully attenuated NADPH oxidase activity in both circulating and sequestered neutrophils, and possibly in pul-

Fig. 5. Extravascular water-to-blood-free dry lung weight ratio (EV LW/ BFDW) and reflection coefficient for albumin (σalb) after 2 h of CPB, 30 min of pulmonary artery reperfusion, and 15 min of static pulmonary vascular pressure adjusted to 35 mmHg relative to the level of the heart in anesthetized pigs (n = 3–7) treated with diluent (control), Low Apo, High Apo, DPI, or Indo. Values are means ± SE.

Fig. 6. Inhibitory effect of superoxide dismutase (SOD), catalase (CAT), APO, DPI, oxypurinol (OXY), or INDO on pulmonary artery endothelial reactive oxygen species (ROS) concentration measured after 60 min of severe hypoxia and 60 min of reoxygenation (n = 4–7). Inset: time course of ROS production in diluent-treated pulmonary artery endothelial cells exposed to hypoxia-reoxygenation or normoxia (inset, n = 23). Values are means ± SE. *P < 0.05 vs. SOD, CAT, or APO. #P < 0.05 vs. Diluent control. Inset: *P < 0.05 vs. normoxia. †P < 0.05 vs. 10 min hypoxia-reoxygenation ROS. §P < 0.05 vs. 30 min hypoxia-reoxygenation ROS.
monary vascular cells. Moreover, the effect of apocynin was dose dependent.

The significant inhibitory effect of apocynin or DPI on blood LEC was accompanied by an attenuation of the severe hypoxemia resulting from CPB performed with a transiently occluded pulmonary artery and ligated bronchial artery (Fig. 2). The degree of post-CPB hypoxemia in control animals was consistent with a shunt fraction of ~50% (assuming normal mixed venous O2 tensions) (42). The estimated shunt fractions of 25% in the low-dose apocynin group and 17% in the high-dose apocynin and DPI groups indicated a dose-dependent reduction in intrapulmonary right-to-left shunt. This improvement was also evident in the decreased PaCO2 values in both apocynin- and DPI-treated animals compared with control values at similar levels of \( V_{\text{min}} \). Of note, the log LEC accounted for only 37% of the variance in log PO2, indicating the presence of other significant factors affecting pulmonary gas exchange.

One possible explanation for this was our use of circulating blood LEC as a surrogate for NADPH oxidase activity within the lung, which may have had a different time course and dose-response relationship to the inhibitors.

Interestingly, apocynin and DPI treatment were also similar in that the improvements in arterial oxygenation and ventilation were not accompanied by significant changes in the post-CPB time course of peak PTr or the measurements of EVLW and \( \sigma_{\text{abs}} \). These results suggest that the protective effects of NADPH oxidase inhibition were not mediated through prevention of CPB-induced surfactant dysfunction (19, 37, 51) or attenuation of the increased pulmonary vascular protein permeability observed in control animals. The interpretation of the EVLW data is more complicated, because the values shown in Fig. 5 were measured after static pulmonary vascular pressure was increased to 35 mmHg for 15 min to allow the measurement of \( \sigma_{\text{abs}} \). Thus we cannot rule out the possibility that the EVLW present at 25 min post-CPB, but before death, could have been less in the presence of NADPH oxidase inhibition. This could explain the improved gas exchange in these animals and the tendency for less EVLW after elevating static pulmonary vascular pressure (Fig. 5).

A decrease in EVLW could have occurred from a decrease in the transvascular hydrostatic pressure gradient or a decrease in filtration coefficient. Both apocynin and DPI significantly decreased PPA (Fig. 3). If pulmonary venoconstriction contributed to the increased PPA in control animals, the vasodilation caused by the NADPH oxidase inhibitors would have decreased pulmonary capillary pressure, thereby decreasing the transvascular hydrostatic pressure gradient. Thromboxane production has been shown to play a pathogenetic role in CBP-induced lung injury (59), is known to cause pulmonary venoconstriction (56), and is inhibited by apocynin (13). The inability of DPI to inhibit thromboxane synthesis (13) and indomethacin to prevent CPB-induced hypoxemia or vascular barrier dysfunction (Figs. 2 and 5) suggests that thromboxane inhibition by apocynin is not a viable explanation for the observed effects. Unlike apocynin or DPI, indomethacin did not prevent the increase in PPA, suggesting that a decrease in pulmonary capillary pressure may have still contributed to the improved oxygenation. This was not likely the entire explanation, however, because the vasodilating effect of apocynin did not differ between the low- and high-dose groups (Fig. 3), whereas the improvement in PaO2 was clearly dose related (Fig. 2). These data suggest that the improved gas exchange associated with NADPH oxidase inhibition was not likely due to nonspecific relaxation of vascular smooth muscle (24) or inhibition of thromboxane synthetase (13). Additional experiments designed to measure the time course of EVLW accumulation and filtration coefficient will be necessary to better understand the link between NADPH oxidase inhibition and gas exchange.

These results differed from the effects of apocynin in an isolated sheep lung model of I/R injury (13). In that study, apocynin completely prevented the decreased \( \sigma_{\text{abs}} \), increased filtration coefficient, and increased edema caused by 30 min of ischemia and 180 min of reperfusion with whole blood. Apocynin also blocked an increase in plasma thromboxane and PPA. Indomethacin had similar effects on thromboxane production and PPA but did not prevent the increased pulmonary vascular permeability or edema, suggesting that apocynin protected barrier function via NADPH oxidase inhibition. Interestingly, 5 \( \mu \)M DPI enhanced both the increase in pulmonary vascular filtration coefficient and PPA in association with a mild lactic acidosis, suggesting that the known inhibitory effects of DPI on nitric oxide synthase and cytochrome oxidase more than countered the beneficial effects of NADPH oxidase inhibition (13). We did not observe either pulmonary hypertension or lactic acidosis in the current study, suggesting that the DPI dose administered to the pigs achieved a less-toxic blood concentration (58).

Two significant differences in experimental protocol may explain the differing effects of apocynin between our prior isolated lung study and the current CPB preparation. First, the duration of lung ischemia was three times longer in the current study, suggesting that either a component of the ROS-mediated injury during ischemia became irreversible or that additional, non-ROS mediated injurious effects of ischemia may have played a role (9). Second, the lungs were deflated during CPB in the present study, whereas ventilation was maintained during lung ischemia in the previous study. Thus hypoxia-reoxygenation may provide a metabolic and oxidant insult that is distinct from the increased ROS generation associated with nonhypoxic I/R (2, 62). For example, NADPH oxidase inhibition with DPI prevented increased endothelial ROS production and lipid peroxidation secondary to pulmonary ischemia in isolated rat and mouse lungs but had no effect on ROS and lipid peroxidation generated by severe hypoxia and reoxygenation (2, 62). The converse occurred with XO inhibition, suggesting that I/R preferentially stimulated NADPH oxidase, whereas hypoxia-reoxygenation activated XO. These data are consistent with the protective effect of XO inhibition on barrier function in previous studies of intact lungs subjected to the combination of I/R and hypoxia-reoxygenation (1, 29). In addition to the effects of hypoxia-reoxygenation, the lack of ventilatory lung motion during ischemia adds an additional injurious effect that is independent of ventilatory oxygen concentration (47, 52). If either of these additional pulmonary vascular insults were not mediated through assembly and activation of NADPH oxidase, they could explain the inability of apocynin or DPI to alter \( \sigma_{\text{abs}} \) in the current study.

To determine the effects of apocynin and DPI on ROS generated by hypoxia-reoxygenation, we pretreated pulmonary artery endothelial cells with either agent before subjecting them to 60 min of severe hypoxia followed by 60 min of reoxygenation. Endothelial ROS production was determined.
by EPR rather than chemiluminescence because of the increased sensitivity and specificity of EPR in the detection of vascular NADPH oxidase-derived ROS (7). Bovine cells were chosen to allow comparison with the many in vitro studies of endothelial hypoxia-reoxygenation utilizing this species (2, 63–66). Compared with control normoxic exposures, hypoxia-reoxygenation caused a progressive increase in the concentration of DMPO-OH that began to level off after the first 30 min of oxygen exposure, suggesting persistent ROS production (Fig. 6). DMPO-OH adducts can be formed by either direct trapping of hydroxyl radicals or spontaneous decay of the superoxide-derived adduct DMPO-OOH. The near-complete inhibition of the DMPO-OH adduct in the present study by either SOD or catalase suggests that the signal was produced from extracellular hydroxyl radical, which was generated from hydrogen peroxide and superoxide anion. Although DMPO readily crosses cell membranes, intracellular DMPO-OH is more rapidly degraded than extracellular adduct causing the extracellular signal to predominate (27, 50).

Both apocynin and DPI significantly inhibited pulmonary endothelial ROS production, suggesting that an endothelial NADPH or NADH oxidase was a major source of ROS production following hypoxia-oxygenation. Neither oxypurinol nor indomethacin had a significant effect, suggesting that ROS production from XO or cyclooxygenase played no role. In these experiments, we used the same concentrations of apocynin and DPI that were shown to inhibit neutrophil NADPH oxidase in vitro (13). The successful inhibition of whole blood ROS production in apocynin- and DPI-treated pigs in the present study (Fig. 1) suggests that we achieved the same concentrations in the intact animal and thus likely inhibited any endothelial ROS production from hypoxia-reoxygenation associated with CPB. The inability of either drug to affect pulmonary vascular permeability after CPB therefore suggests that endothelial ROS production by this mechanism did not play an injurious role.

These results are similar to Zulueta et al. (64), who showed that hypoxia and reoxygenation of pulmonary endothelial cells caused extracellular hydrogen peroxide release (measured by a fluorometric assay) that was blocked by DPI but not by XO inhibition. However, they also found intracellular fluorometrically measured ROS formation that was decreased by XO or by nitric oxide synthesis inhibition (63). These data support the hypothesis that ROS production from hypoxia-reoxygenation may occur in a compartmental fashion by multiple pathways. Allopurinol was recently reported to decrease circulating malondialdehyde concentrations in human CPB, suggesting the presence of XO-induced lipid peroxidation (10). The source of XO may not have been from the lung, however, because systemic vascular endothelial cells caused extracellular DMPO-OH adduct formation following hypoxia-reoxygenation (5, 65) that was significantly blocked by XO inhibition (65). To our knowledge, the effect of XO inhibition on CPB-induced lung injury has not been examined. Additional studies to further test this hypothesis are necessary.

In addition to the apocynin- and indomethacin-treated groups described here, we concurrently examined the effect of maintaining a patent bronchial artery in a separate group of animals subjected to warm CPB without ventilation or pulmonary artery blood flow while on pump (14). Similar to apocynin, the presence of bronchial artery blood flow attenuated post-CPB hypoxemia (PaO₂ at 25 min = 312 ± 58 mmHg). Unlike apocynin, however, the presence of bronchial blood flow resulted in a normal pulmonary vascular σabh (0.82 ± 0.05) and attenuated the increase in circulating TNF-α. Moreover, there were significant correlations between both TNF-α (R = −0.95, P < 0.001) and IL-6 (R = −0.82, P < 0.01) and σabh that remained significant when only the controls with ligated bronchial arteries were analyzed, suggesting a possible casual role for cytokines in CPB-induced endothelial barrier dysfunction (14). Both TNF-α and IL-6 can increase endothelial permeability directly (31, 36) or through a priming effect on activated neutrophils (21, 30). Increased plasma cytokine concentrations have been measured in human CPB (59), but a definite causal relationship with CPB-induced lung injury remains to be determined.

In the present study, NADPH oxidase inhibition had no inhibitory effect on either TNF-α or IL-6 (Fig. 4), suggesting that ROS production from NADPH oxidase was not the major stimulus for cytokine production. The inability to prevent cytokine production could represent the explanation for the lack of effect of apocynin or DPI on σabh following CPB. The loss of the significant correlation between cytokine concentration and σabh when all animals were included does not necessarily rule out this possibility. For example, apocynin, as a methoxy-substituted catechol, may mimic the ability of epinephrine to stimulate IL-6 release from skeletal muscle (55). In addition, differences in the distribution of systemic blood flow may alter the half-life of circulating cytokine concentrations (16), and therefore, the relationship between tissue and plasma cytokine levels.

In summary, NADPH oxidase inhibition with either apocynin or DPI attenuated the severe hypoxemia resulting from CPB performed with a deflated lung, complete pulmonary artery occlusion, and a ligated bronchial artery. The ameliorating effect of NADPH oxidase inhibition on PaO₂ correlated with the inhibition of inducible blood chemiluminescence, supporting the hypothesis that inhibition of NADPH oxidase contributed to the dose-related protection. However, NADPH oxidase inhibition had no effect on the increased plasma cytokine concentrations and pulmonary vascular protein permeability observed in the control group, suggesting that cytokine production was not stimulated by NADPH oxidase-derived ROS and was not responsible for CPB-induced hypoxemia. Both apocynin and DPI effectively inhibited ROS production from pulmonary artery endothelial cells exposed to hypoxia-reoxygenation, suggesting that an inability to block endothelial NADPH oxidase was not responsible for the unchanged σabh. The roles of cytokines and ROS from sources other than NADPH oxidase in CPB-induced lung injury require further study.

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