Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion lung injury

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Dodd-o, Jeffrey M., and David B. Pearse. Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion lung injury. Am J Physiol Heart Circ Physiol 279: H303–H312, 2000.—Apocynin (4-hydroxy-3-methoxy-aceto-phenone) inhibits NADPH oxidase in activated polymorpho-nuclear (PMN) leukocytes, preventing the generation of re-active oxygen species. To determine if apocynin attenuates ischemia-reperfusion lung injury, we examined the effects of apocynin (0.03, 0.3, and 3 mM) in isolated situ sheep lungs. In diluent-treated lungs, reperfusion with blood (180 min) after 30 min of ischemia (ventilation 28% O2, 5% CO2) caused leukocyte sequestration in the lung and increased vascular permeability [reflection coefficient for albumin ($\sigma_{\text{alb}}$) 0.47 ± 0.10, filtration coefficient ($K_f$) 0.14 ± 0.03 g min⁻¹ mmHg⁻¹ 100 g⁻¹; mmHg⁻¹ 100 g⁻¹] compared with nonreperfused lungs ($\sigma_{\text{alb}}$ 0.77 ± 0.03, $K_f$ 0.03 ± 0.01 g min⁻¹ mmHg⁻¹ 100 g⁻¹; P < 0.05). Apocynin attenuated the increased protein permeability at 0.3 and 3 mM ($\sigma_{\text{alb}}$ 0.69 ± 0.05 and 0.91 ± 0.03, respectively, P < 0.05); $K_f$ was decreased by 3 mM apocynin (0.05 ± 0.01 g min⁻¹ mmHg⁻¹ 100 g⁻¹; P < 0.05). Diphenyleneiodonium (DPI, 5 μM), a structurally unrelated inhibitor of NADPH oxidase, worsened injury ($K_f$ 0.32 ± 0.07 g min⁻¹ mmHg⁻¹ 100 g⁻¹; P < 0.05). Neither apocynin nor DPI affected leukocyte sequestration. Apocynin prevented ischemia-reperfusion lung injury, but the mechan-ism of protection remains unclear.

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An injurious role for PMN leukocyte-derived NADPH oxidase has been inferred from the protective effect of PMN leukocyte depletion (30) and adhesion molecule inhibition (25, 26) on the increased pulmonary vascular permeability after ischemia (25) and ischemia-reperfusion (26, 30). For example, in ischemic rat lungs, ROS production and increased capillary protein permeability were observed in the vicinity of adherent PMN leukocytes (25). Other investigators have suggested that ROS production during pulmonary ischemia may originate from a NADPH oxidase in pulmonary endothelial cells (3). Pulmonary endothelial or macrophage-derived ROS production from this enzyme complex could explain the presence of a PMN leukocyte-independent com-ponent of lung ischemia-reperfusion injury (2, 22, 36). Alternatively, ROS from sources other than NADPH oxidase, such as xanthine oxidase (1) or cytochrome P-450 (6), may play a role.

Apocynin is a methoxy-substituted catechol derived from the root extract of the medicinal herb Picrorhiza kurroa (39). Apocynin has been shown to confer protec-tion in animal models of arthritis (43) and in ozone (37)- and endotoxin-induced (40, 48) lung injury. These protective effects were attributed to the ability of apo-cynin to inhibit NADPH oxidase in PMN leukocytes by reacting with thiol groups required for enzyme assem-bly (39). Apocynin is thought to require activation by reacting with hydrogen peroxide and extracellular per-

ISCHEMIA-REPERFUSION LUNG INJURY is a well-known clinic-ical phenomenon characterized by increased pulmo-nary vascular permeability, edema, and resistance to blood flow (31). The pathogenesis of this injury appears to involve the generation of reduced oxygen species (ROS), which can be detected during both ischemia (3, 25) and reperfusion (17). The ability of superoxide dismutase to attenuate ischemia-reperfusion lung in-

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oxidase, possibly limiting the effect of apocynin to stimulated PMN leukocytes (39, 44).

We hypothesized that apocynin would attenuate ischemia-reperfusion lung injury. To test this hypothesis, we determined the effect of 0.03, 0.3, and 3 mM apocynin on the pulmonary vascular injury caused by 30 min of ischemia and 180 min of reperfusion in isolated sheep lungs (32). Additional experiments were performed with diphenyleneiodonium (DPI), a compound that is structurally unrelated to apocynin (16) and that impairs NADPH oxidase by flavoprotein inhibition (9), and indomethacin, a blocker of cyclooxygenase, to elucidate the mechanisms of the observed apocynin effects.

METHODS

Blood Studies

Effects of apocynin and DPI on whole blood luminol-enhanced chemiluminescence. To compare the effects of apocynin (Aldrich Chemical, Milwaukee, WI) and DPI (Colour Your Enzyme, Ontario, Canada) on the capacity of whole sheep blood to generate ROS, heparinized blood was diluted 1:10 in Hanks’ balanced salt solution (HBSS), and 0.78 ml was added to the stirred, warmed (37°C) cuvette of a Chronolog 560-CA luminometer (Chronolog Instruments, Haverton, PA). Apocynin (3, 0.3, and 0.03 mM), DPI (5 μM), or diluent (0.1 ml) was added, and the mixture was allowed to incubate for 30 min. Luminol (Sigma Chemical, St. Louis, MO) stock solution (62.5 mM in DMSO) was added to achieve a final concentration of 0.5 mM, and baseline chemiluminescence was recorded for 2 min. Serum-treated zymosan (STZ) was added to achieve a final solution of 1.2 mg/ml, and peak chemiluminescence was recorded. STZ was prepared by boiling zymosan A (Sigma Chemical) in HBSS for 20 min. It was then incubated two times (30 min each) in serum (normal human donors) and reconstituted in HBSS (10 mg/ml). STZ was frozen and stored at −80°C until use.

Effects of apocynin and DPI on resazurin reduction by activated PMN leukocytes. Luminol-enhanced chemiluminescence detects both intra- and extracellular ROS but is dependent on the generation of hypochlorous acid from superoxide-derived hydrogen peroxide (46). Therefore luminol-enhanced chemiluminescence could be attenuated by mechanisms other than NADPH oxidase inhibition, such as scavenging hydrogen peroxide or hypochlorous acid. We therefore examined the effects of apocynin and DPI on resazurin reduction. Resazurin (Sigma Chemical) is directly reduced by activated NADPH oxidase to form the fluorescent product resorufin (13). Isolated PMN leukocytes (5 × 10^6) were incubated at 37°C for 75 min with resazurin (0.03 mg/ml), STZ (1 mg/ml), and apocynin or diluent to achieve concentrations of 3, 0.3, 0.03, or 0 mM apocynin. The effect of apocynin was compared with increasing concentrations of DPI (0.003–0.3 mM). To determine if the assay was affected by superoxide anion scavenging, the effect of superoxide dismutase (bovine erythrocyte; Sigma Chemical) was also examined. Resorufin production was monitored (excitation 530 nm, emission 590 nm) with a CytoFluor 2350 fluorometer (Millipore, Bedford, MA).

PMN isolation technique. PMN leukocytes were isolated from peripheral blood of healthy human donors. Briefly, whole blood was collected in a syringe containing 10 U heparin/ml and diluted 1:2 with HBSS. Diluted whole blood (6 ml) was centrifuged (1,850 rpm) over a Ficoll-Histopaque gradient (3 ml Histopaque 1077 over 3 ml Histopaque 1119) for 30 min. The PMN leukocyte layer was washed two times in HBSS and reconstituted in NaHCO₃- and MgCl₂-containing HBSS.

Lung Studies

Isolated lung preparation. ISCHEMIA-REPERFUSED LUNGS. Studies were performed in isolated sheep lungs perfused and ventilated in situ. Young sheep (20–37 kg) were anesthetized with intramuscular ketamine (30 mg/kg) and atropine (0.5 mg). Anesthesia was maintained by intravenous infusion of pentobarbital sodium (20 mg·kg⁻¹·h⁻¹). A tracheostomy was performed, and mechanical ventilation was begun with oxygen-supplemented room air at a tidal volume of 12 ml/kg and respiratory rate of 15 breaths/min. A sternotomy was performed, heparin (10,000 units) was injected intravenously, and the animal was exsanguinated from a left atrial cannula into the perfusion circuit. After exsanguination, ventilation was maintained with warmed, humidified gas at a tidal volume of 12 ml/kg and a frequency of 10 breaths/min. Initially, O₂ and CO₂ concentrations were 28 and 5%, respectively. End-expiratory tracheal pressure was maintained at 5 mmHg.

The pulmonary artery was cannulated, and the pulmonary vasculature was flushed with 300 ml of warmed 3% dextran 70 (Sigma Chemical) in normal saline. The solution was allowed to dwell for 3 min before being drained by gravity from the left atrium. After 30 min of ischemia, the lungs were perfused in situ with a mixture of autologous blood (~1,000 ml) and 3% dextran in Ringer lactate solution (~300 ml) through an extracorporeal circuit described previously (32). The perfusate was warmed (38–39°C) and continuously filtered of clots and bubbles. The desired flow was achieved over 3–5 min by gradually increasing roller pump speed. The lungs were perfused for 180 min, with time 0 defined as the time a flow of 90 ml·kg⁻¹·min⁻¹ was achieved. Tracheal, pulmonary arterial (Pₐₐ), and left atrial pressures were measured with Statham P50 transducers referenced to the level of the left atrium. Pₐₐ was determined at end expiration while the lung was mechanically ventilated. Left atrial pressure was kept subatmospheric. Pressures were continuously recorded (Grass model 7D polygraph; Astro-Med, West Warwick, RI). Perfusate O₂ and CO₂ tensions and pH were measured at regular intervals using standard electrode techniques. Perfusate pH was adjusted to 7.35–7.45 at 30-min intervals with 1 N NaHCO₃. Perfusate glucose concentrations were monitored with Dextro-stix and kept within 90–130 mg/dl by periodic addition of 50% glucose in water.

Perfusate samples were obtained from the reservoir immediately before reperfusion and from the left atrium at fixed intervals during reperfusion and were analyzed for total leukocyte, PMN leukocyte, and platelet counts as previously described (30). Plasma samples were frozen for later analysis of thromboxane B₂ (TXB₂), the stable metabolite of thromboxane A₂, 5-keto-PGF₁α, the stable metabolite of prostacyclin, PGF₂α, PGE₂, and PGD₂ by gas chromatography-mass spectrometry as previously described (33).

Pulmonary vascular permeability was assessed after reperfusion by estimation of the filtration coefficient (Kᵢ) and reflection coefficient for albumin (σᵢ) as previously described (32, 34). Briefly, perfusion and ventilation were stopped, and the lungs were rapidly removed from the chest and suspended from a force transducer. Ventilation was resumed, the pulmonary arterial and left atrial cannulas were connected to a pressurized stirred reservoir filled with perfusate, and intravascular pressure (referenced to the level of the middle of the lung) was increased from 20 mmHg to 30,
35, and 40 mmHg at 10-min intervals. The rate of lung weight gain over the last 5 min at each vascular pressure was plotted against vascular pressure, and the slope of this relationship was used to estimate $K_f$. The $\sigma_{m_b}$ was determined by the filtered volumes method, as modified for a nonflowing system, by Becker and co-workers (5). The right lung was then resected for measurement of extravascular lung water (EVLW) and blood-free dry weight (BFDW) as previously described (30). In some experiments, tissue sections from the left lung were obtained for light microscopy after fixing the lung via the vasculature with 4% paraformaldehyde while maintaining a constant alveolar pressure of 10 mmHg. The lung tissue was imbedded in paraffin and sectioned and stained with hematoxylin and eosin. Note that all EVLW measurements and lung histology were performed on lung tissue subjected to the period of increased static vascular pressure required to measure $\sigma_{m_b}$ and $K_f$.

NONREPERFUSED LUNGS. Pulmonary vascular permeability and EVLW were measured in 10 lungs that were excised without reperfusion. Ventilation was maintained throughout an ischemic time of ~30 min of ischemia, and $K_f$ and $\sigma_{m_b}$ were measured as previously described (32, 34).

Pharmacological intervention. Six groups of ischemic-reperfused lungs were studied. In three groups, apocynin was added to the vascular flush solution and the reservoir blood before reperfusion to achieve pulmonary vascular concentrations of 3 mM ($n = 6$), 0.3 mM ($n = 5$), or 0.03 mM ($n = 5$). A fourth group of lungs ($n = 4$) was treated with 5 $\mu$M DPI in the vascular flush and perfusate. Because apocynin is known to inhibit thromboxane formation, an additional group of five lungs was treated with 56 $\mu$M indomethacin (Sigma Chemical). Drug-treated lungs were compared with five diluent-treated control lungs.

Apocynin and indomethacin were dissolved in the 300-ml vascular flush solution by heating. To achieve the appropriate perfusate concentrations, additional drug was dissolved in 100 ml normal saline containing 10 mM NaHCO$_3$, which was added to the ~1,500 ml perfusion circuit just before initiating reperfusion. A solution of DPI (0.5 mg/ml) was prepared in distilled water by sonication for 30–60 s. Two milliliters of this stock solution were added to the vascular flush solution; 10 ml were added to the reservoir just before reperfusion.

Statistics

All time course data were compared with a two-factor (group, time), split-plot ANOVA (38). The peak pulmonary artery pressure and perfusate prostanooid concentrations and lung water and pulmonary vascular permeability were analyzed by one-way ANOVA. The mean values of the prostanooid concentrations were found to be directly proportional to SD, indicating nonhomogeneity of variance; therefore, these data were transformed to logarithms before statistical analysis to comply with the assumptions of the ANOVA. When significant variance ratios were obtained, least-significant differences were calculated to allow comparison of individual means. Values presented in the text are means ± SE. Differences were considered significant at $P \leq 0.05$.

RESULTS

Sheep body weight averaged 26.5 ± 0.7 kg and was not different among groups. There were no differences in perfusate cell concentrations between groups of reperfused lungs. Perfusate hematocrit and total leukocyte, PMN leukocyte, and platelet concentrations averaged 21.5 ± 0.7% and 3,710 ± 268, 1,165 ± 111, and 463,000 ± 57,000 cells/mm$^3$, respectively, in reservoir blood at time 0. As in previous studies (32), perfusate PMN leukocytes fell to near 0 cells/mm$^3$ in all lungs by 30 min of reperfusion. Platelet concentrations were unchanged.

Blood Studies

As shown in Fig. 1, all three doses of apocynin significantly inhibited luminol-enhanced chemiluminescence of zymosan-stimulated sheep blood in a dose-dependent fashion. For example, 3 mM apocynin inhibited 97 ± 1% of the peak chemiluminescence generated by zymosan in diluent-treated blood, whereas 0.3 mM attenuated the signal by 75 ± 4%. DPI (5 $\mu$M) produced an effect similar to 3 mM apocynin, blocking 97 ± 1% of the luminol-enhanced signal.

Figure 2 shows the effects of apocynin, DPI, and superoxide dismutase on the reduction of resazurin by STZ-stimulated PMN leukocytes. Both apocynin and DPI inhibited resazurin reduction in a dose-dependent manner. Similar to the results obtained with whole blood chemiluminescence, 0.03 and 0.3 mM apocynin attenuated resazurin reduction by 31 ± 7 and 83 ± 3%, respectively. Unlike whole blood chemiluminescence, 3 mM apocynin (82 ± 2%) provided no additional inhibition compared with 0.3 mM. DPI was less effective at inhibiting resazurin reduction than whole blood chemiluminescence; 3 $\mu$M DPI inhibited resazurin reduction by 69 ± 4% compared with 97 ± 1% inhibition of chemiluminescence by 5 $\mu$M DPI. Superoxide dismutase had no significant effect on PMN leukocyte-induced resazurin reduction at either concentration studied.

![Graph showing inhibitory effect of apocynin (n = 8 sheep) and 5 $\mu$M diphenyleneiodonium (DPI; n = 4 sheep) on zymosan-stimulated, luminol-enhanced, peak chemiluminescence of whole sheep blood. Values are means ± SE. *$P < 0.05$ compared with diluent control. # $P < 0.05$ compared with 0.03 mM apocynin. + $P < 0.05$ compared with 0.3 mM apocynin. Statistical comparisons between apocynin and DPI were not made.](http://ajpheart.physiology.org/)

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**APOCYNIN IN ISCHEMIA-REPERFUSION LUNG INJURY**

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Lung Studies

Effect of ischemia and reperfusion. Figure 3 shows the effect of ischemia and reperfusion on pulmonary vascular permeability. Compared with nonreperfused lungs (mean and 95% confidence limits indicated in Fig. 3), the diluent-treated ischemic-reperfused lungs had decreased $\sigma_{alb}$ ($0.47 \pm 0.10$ vs. $0.77 \pm 0.03$) and increased $K_f$ ($0.14 \pm 0.03$ vs. $0.03 \pm 0.01$ g $\cdot$ min$^{-1}$ $\cdot$ mmHg$^{-1}$ $\cdot$ 100 g$^{-1}$), indicating increased vascular permeability to protein and water. The increase in water permeability was corroborated by an increase ($P < 0.05$) in EVLW normalized to BFDW in the ischemic-reperfused compared with the nonreperfused lungs ($7.97 \pm 0.28$ vs. $4.77 \pm 0.20$ g/g BFDW, data not shown).

Light microscopy of lung sections from ischemic-reperfused lungs demonstrated PMN leukocytes in the alveolar capillaries, interstitial and alveolar edema, and prominent peribronchial hemorrhage (Fig. 4). Two of the diluent-treated lungs could only be reperfused for 30 min because of massive edema formation and loss of reservoir volume.

As shown in previous studies (32), blood perfusion of isolated sheep lungs causes the release of prostanoids into the perfusate and an associated transient pulmonary hypertension. Peak perfusate concentrations of TxB$_2$, 6-keto-PGF$_{1\alpha}$, and PGE$_2$ occurred at 30 min and are shown in Fig. 5. Measurable amounts of neither PGD$_2$ nor PGE$_2$ were detected. $P_{pa}$ peaked at 30 min of reperfusion ($33 \pm 7$ mmHg; Fig. 5). $P_{pa}$ decreased to $16 \pm 3$ mmHg at 60 min and remained at this level for the remainder of the reperfusion period (data not shown).

Effect of apocynin. Apocynin inhibited the increased pulmonary vascular permeability caused by ischemia and reperfusion in a dose-dependent manner (Fig. 3). Treatment with 0.3 mM apocynin was associated with a $\sigma_{alb}$ of $0.69 \pm 0.05$ and a $K_f$ of $0.07 \pm 0.01$ g $\cdot$ min$^{-1}$ $\cdot$ mmHg$^{-1}$ $\cdot$ 100 g$^{-1}$, values near the 95% confidence limits for the nonreperfused lungs. Moreover, 3 mM apocynin completely prevented the increase in permeability, as evidenced by a $\sigma_{alb}$ of $0.91 \pm 0.03$, which exceeded the 95% confidence limits for nonreperfused lungs. The $K_f$ in the 3 mM apocynin group ($0.05 \pm 0.01$ g $\cdot$ min$^{-1}$ $\cdot$ mmHg$^{-1}$ $\cdot$ 100 g$^{-1}$) was significantly less than diluent-treated lungs and was not different from the $K_f$ for nonreperfused lungs. Apocynin treatment also decreased EVLW, with 3 mM apocynin resulting in $5.34 \pm 0.26$ g/g BFDW, which was less than the diluent-treated lungs ($P < 0.05$) and not different from the nonreperfused group ($P = 0.06$, data not shown). As shown in Fig. 4, lungs treated with 3 or 0.3 mM apocynin had no histological evidence of peribronchial hemorrhage compared with multiple areas of extravascular erythrocytes observed on tissue sections from diluent-treated lungs. Although 0.03 mM apocynin significantly attenuated luminol-enhanced chemiluminescence of sheep blood ($29 \pm 9\%$, Fig. 1) and resazurin...
Fig. 4. A: histological section from lung treated with diluent after ischemia and reperfusion showing erythrocytes in peribronchial interstitium (arrow). Histological section from 0.3 mM (B) and 3 mM (C) apocynin-treated lung after ischemia and reperfusion (hematoxylin and eosin; original magnification ×400).
Unlike apocynin, DPI did not attenuate the pulmonary hypertension and increased vascular protein permeability; $P_{pa}$ at 30 min (45 ± 4 mmHg) and $\sigma_{alb}$ (0.42 ± 0.13) were not different from the diluent-treated lungs, whereas $K_f$ (0.32 ± 0.07 g · min$^{-1}$ · mmHg$^{-1}$ · 100 g$^{-1}$) and EVLW (9.97 ± 1.25 g/g BFDW) were significantly greater than diluent-treated lungs (Figs. 3 and 5).

To determine if any of the protective effects of apocynin were secondary to inhibition of cyclooxygenase, we studied five lungs treated with indomethacin. The indomethacin dose was previously shown in this preparation to decrease perfusate levels of Tx$B_2$ and 6-keto-PGF$\text{I}_\alpha$ to near 0 ng/ml and to eliminate the early transient pulmonary hypertension (29). As shown in Fig. 3, indomethacin treatment did not prevent vascular injury inasmuch as $\sigma_{alb}$ (0.51 ± 0.06) and $K_f$ (0.10 ± 0.03 g · min$^{-1}$ · mmHg$^{-1}$ · 100 g$^{-1}$) were not different from diluent-treated lungs. As expected, indomethacin blocked the early pulmonary hypertension (Fig. 5) previously shown to be dependent on the generation of thromboxane (29).

**DISCUSSION**

The major finding of the present study was that apocynin, a novel inhibitor of NADPH oxidase, prevented the increased vascular permeability caused by ischemia and reperfusion in isolated lung. As shown in Fig. 3, 30 min of ischemia followed by 180 min of reperfusion decreased $\sigma_{alb}$ and increased $K_f$ compared with a separate group of nonreperfused lungs. Apocynin treatment attenuated both the decrease in $\sigma_{alb}$ and the increase in $K_f$ caused by ischemia-reperfusion injury. $\sigma_{alb}$ is a dimensionless index that describes the ability of the vascular endothelium to maintain an oncotic pressure gradient during convective fluid movement; a $\sigma_{alb}$ of zero indicates free movement of albumin across the vessel wall, whereas a $\sigma_{alb}$ of one indicates impermeability to albumin. We have found $\sigma_{alb}$ to be more sensitive to small changes in vascular permeability than $K_f$ (47). $K_f$ is an index of water permeability which, unlike $\sigma_{alb}$, is affected by changes in vascular surface area, confounding the interpretation of $K_f$ as an index of injury (16). We previously showed that the decrease in $\sigma_{alb}$ in this preparation was dependent on the combination of ischemia and reperfusion; neither ischemia (30 min with ventilation) without reperfusion nor extracorporeal perfusion without ischemia decreased $\sigma_{alb}$ (32) compared with estimates of $\sigma_{alb}$ in intact sheep lungs (28).

The effect of apocynin on the changes in vascular permeability after ischemia-reperfusion was dose dependent. Of note, the 3 mM apocynin-treated lungs had a $\sigma_{alb}$ that was significantly greater than the nonreperfused lungs and comparable to a measurement of $\sigma_{alb}$ in intact sheep lungs (0.84) (28). This suggests that 3 mM apocynin was able to maintain normal endothelial albumin permeability during ischemia and reperfusion. In addition, 3 mM apocynin decreased the $K_f$ to within the 95% confidence limits of the nonreperfused lungs, suggesting that the component of increased wa-

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**Fig. 5.** Effect of diluent, apocynin, DPI, and Indo on perfusate concentrations of cyclooxygenase metabolites and pulmonary artery pressure at 30 min of reperfusion. Tx$B_2$, thromboxane $B_2$; $P_{pa}$, pulmonary arterial pressure. Values are means ± SE ($n = 6$ for 3 mM apocynin and DPI; all other groups, $n = 5$). *$P < 0.05$ compared with diluent control. #*$P < 0.05$ compared with 0.03 mM apocynin.

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reduction by activated PMN leukocytes (31 ± 7%, Fig. 2), this dose had no effect on the decrease in $\sigma_{alb}$ (0.48 ± 0.05) and increase in $K_f$ (0.17 ± 0.04 g · min$^{-1}$ · mmHg$^{-1}$ · 100 g$^{-1}$) caused by ischemia and reperfusion. Similar to the diluent-treated lungs, two of the 0.03 mM apocynin groups were reperfused for <60 min because of massive edema formation, whereas all of the lungs treated with 0.3 or 3 mM apocynin were reperfused for 180 min.

As shown in Fig. 5, all three doses of apocynin decreased the peak perfusate concentrations of the vasoconstricting prostanooids Tx$B_2$ and PGF$\text{I}_{2\alpha}$, whereas only 3 mM apocynin decreased perfusate 6-keto-PGF$\text{I}_\alpha$ concentration. Although Tx$B_2$ production was significantly attenuated by 0.03 mM apocynin, the 30-min $P_{pa}$ was decreased only by 0.3 and 3 mM apocynin to 20 ± 4 and 11 ± 2 mmHg, respectively.

To determine if the ameliorating effect of apocynin on the vascular permeability change was secondary to inhibition of NADPH oxidase, we studied six lungs treated with the structurally unrelated inhibitor DPI.
ter permeability from the interaction of ischemia and reperfusion was also completely eliminated. Although we did not quantify lung hemorrhage, the histology suggested that apocynin decreased erythrocyte extravasation into the interstitium (Fig. 4). The peribronchial hemorrhage observed in the diluent-treated lungs most likely corresponds to the nearly 100% incidence of blood-stained lung lymph we previously reported in this preparation (30). Moreover, reperfusion with PMN leukocyte-depleted blood significantly decreased the incidence of hemorrhagic lung lymph (30), supporting the possibility that the attenuating effect of apocynin on lung hemorrhage was mediated by an inhibitory effect on PMN leukocyte function. Of note, the protective effect of apocynin was not due to inhibition of PMN leukocyte sequestration in the lung because this occurred to the same degree in all lungs (data not shown).

The protective effect conferred by apocynin on ischemia-reperfusion lung injury correlated with the capacity of apocynin to inhibit inducible chemiluminescence in our whole blood studies (Fig. 1). Luminol-enhanced chemiluminescence reflects, predominantly, the free radical production by PMN leukocytes (46). Apocynin, at concentrations similar to those used in the present study, has indeed been shown to inhibit the ability of isolated PMN leukocytes to utilize oxygen after activation as well as the capacity of isolated PMN leukocytes to induce lucigenin-enhanced luminescence after stimulation by STZ (39). The resazurin assay data (Fig. 2) provided further support that apocynin directly impairs the activity of the NADPH oxidase enzyme. This assay, which measures the reduction of resazurin to resorufin by the NADPH oxidase enzyme, is a direct measure of NADPH oxidase activity (13). The lack of effect of superoxide dismutase on resazurin reduction confirmed that this assay is not affected by superoxide anion scavenging.

The ROS scavenging effect of apocynin remains undefined. Stolk et al. (39) showed that apocynin did not scavenge superoxide anion generated by xanthine oxidase. They concluded, however, that apocynin scavenged hydrogen peroxide because of a dose-response inhibition of luminol-enhanced chemiluminescence generated by hydrogen peroxide. But these studies, in which hydrogen peroxide was diluted with PBS buffer, did not rule out the possibility that the effect of apocynin was on metal ions or peroxidases contaminating the PBS buffer. Hydrogen peroxide does not cause luminol-enhanced chemiluminescence in the absence of contaminating metal ions or peroxidase (46).

Interestingly, the incremental protection from ischemia-reperfusion lung injury conferred by the 3 mM apocynin vs. 0.3 mM apocynin was not paralleled by an incremental inhibition of resazurin reduction by 3 mM apocynin. This disparity suggests that the protective qualities of apocynin were not limited to its effects on PMN leukocyte NADPH oxidase. A ROS scavenging effect of 3 mM apocynin, for example, would explain both its incremental effect on ischemia-reperfusion lung injury and its incremental capacity to attenuate luminol-enhanced luminescence of whole blood. Alternatively, the additional inhibitory effect of 3 mM apocynin on vascular injury and inducible whole blood luminescence may reflect the capacity of higher concentrations of apocynin to block NADPH oxidase activity in non-PMN leukocyte cells (14, 39). Monocytes (45), lymphocytes (7), macrophages (15), pulmonary vascular smooth muscle cells (24), and, possibly, endothelial cells (3) were shown to contain an NADPH oxidase enzyme complex. For example, experiments in ischemic rat and mouse lungs have implicated an endothelial NADPH oxidase as a major source of ROS production during ischemia (3). Al-Mehdi et al. (3) demonstrated ROS production from the parenchyma of ischemic rat and mouse lungs that appeared to be of endothelial origin. The ROS signal was attenuated by PR-39 and diphenylidodium, structurally unrelated inhibitors of NADPH oxidase, and was absent in lungs from gp91-phox knockout mice, suggesting a NADPH oxidase source. Pulmonary vascular permeability was not measured during ischemia, however, and the lungs were not subjected to reperfusion, so the relationship between endothelial ROS production and ischemiareperfusion lung injury was unclear.

In contrast to apocynin, DPI not only failed to prevent the increase in vascular permeability, but it may have enhanced the vascular injury associated with ischemia-reperfusion, as evidenced by an increase in $K_f$ (Fig. 3) and EVLW compared with diluent-treated lungs. This concentration of DPI was as potent an inhibitor of whole blood chemiluminescence as 3 mM apocynin (Fig. 1), although larger concentrations of DPI were necessary to produce a comparable inhibition of resazurin reduction (Fig. 2). Therefore, DPI both blocked NADPH oxidase function and either nonspecifically interfered with the chemiluminescence reaction or scavenged ROS. Nevertheless, based on its NADPH oxidase inhibitory activity as determined by the resazurin assay, we expected this dose of DPI to attenuate vascular injury to a similar degree as 0.3 mM apocynin. The inability of DPI to do so suggests that either the protection of apocynin was independent of its effect on NADPH oxidase or that the toxicity of DPI overshadowed the benefits of NADPH oxidase inhibition. In addition to NADPH oxidase inhibition, DPI inhibits nitric oxide synthase (NOS) activity (41). The NOS enzyme complex is, in fact, more sensitive to DPI-induced inhibition than is the NADPH oxidase enzyme (41). Grimminger et al. (14) found that NOS inhibition by DPI enhanced pulmonary vasoconstriction from the thromboxane analog U-46619, and we previously showed that inhibition of constitutive NOS increased pulmonary vascular resistance in our in situ lung preparation (34). In the present study, however, the tendency toward exacerbating pulmonary hypertension was transient (Fig. 5), peaking at 30 min, and was followed by a tendency toward increased vasodilation at 180 min of reperfusion in the apocynin-treated compared with diluent-treated lungs ($6 \pm 1$ vs. $16 \pm 5$ mmHg, respectively, $P = 0.10$). A similar biphasic effect of DPI on vascular tone was observed in precon-
stricted systemic vessels (8). Pulmonary vasodilation would increase the vascular surface available for fluid movement and may partially explain the increase in $K_f$ caused by DPI treatment. This could not have been the entire explanation, however, because the maximal fractional change in hematocrit from the fluid-exchanging region of the DPI-treated lungs, measured for the calculation of $\sigma_{alb}$ was significantly greater than that in diluent-treated lungs (0.93 ± 0.19 vs. 0.42 ± 0.03, respectively; $P < 0.05$). The magnitude and duration of the increased static vascular pressure during the $\sigma_{alb}$ and $K_f$ measurements were the same in all lungs. Thus an increase in the maximal fractional change in hematocrit indicates an increase in vascular water conductance (34).

Loss of constitutive NOS activity may explain the injurious effect of DPI on vascular permeability, independent of its effect on vascular resistance (23, 27). NOS inhibition did not increase vascular permeability during ventilated ischemia in the sheep lung (34), but we have not determined the role of nitric oxide in the reperfusion phase of the injury. Other cellular functions inhibited by DPI include mitochondrial electron transport (20), cytochrome P-450 enzyme activity (35), and ion channel open probability (49). Inhibition of mitochondrial respiration was previously shown to exacerbate pulmonary edema in isolated rat lungs, although neither $\sigma_{alb}$ nor $K_f$ was measured (4). We did not exceed 5 $\mu$M DPI because this concentration was shown to inhibit 80% of macrophage NADPH oxidase activity without affecting mitochondrial respiration (15). Nevertheless, the DPI-treated lungs required more NaHCO$_3$ to maintain a normal perfusate pH (data not shown), suggesting that 5 $\mu$M DPI may have caused partial inhibition of mitochondrial respiration. The lactic acidosis resulting from ventilation with nitrogen during ischemia and reperfusion in a previous study (32) was more severe than that produced by DPI in the present study. Nitrogen ventilation did not worsen vascular permeability (32), arguing against mitochondrial dysfunction as the cause for increased $K_f$ with DPI treatment in this model. Finally, DPI inhibits potassium and calcium channels in vascular smooth muscle (49). Administration of cromakalim, a potassium channel opening drug, attenuates ischemia-reperfusion injury in isolated rat lungs (18), suggesting that closed potassium channels may worsen the injury. Although glibenclamide, a potassium channel closing agent, did not exacerbate ischemia-reperfusion lung injury in the rat lung model (18), species differences could exist.

Regardless of the mechanism behind the enhanced injury caused by DPI, the lack of protection raises the possibility that NADPH oxidase did not play a major role in the ischemia-reperfusion lung injury. The injurious role of PMN leukocyte NADPH oxidase in ischemia-reperfusion lung injury remains controversial, with recent studies showing no effect of PMN leukocyte depletion on this injury (22, 36). Other investigators have identified a PMN leukocyte-independent injury early in reperfusion (<30 min), followed by a later injury (4 h) mediated by PMN leukocytes (11). The early phase of injury appeared to be mediated by macrophage-derived cytokines. The later phase of injury is consistent with our previous observation that reperfusion of untreated ischemic sheep lungs with leukocyte-depleted blood decreased lung hemorrhage after 3 h of reperfusion (30). Unfortunately, vascular permeability was not measured in that study, so a direct comparison of leukopenic reperfusion with apocynin treatment cannot be made.

The protective effect of apocynin may not be limited to NADPH oxidase inhibition. Apocynin has been shown to inhibit cytochrome P-450 (35) and thromboxane synthase (10). Interestingly, both cytochrome P-450 (6) and thromboxane (21, 22) have been implicated as mediators of ischemia-reperfusion lung injury. Cytochrome P-450 inhibition is shared by DPI (42), making this pathway a less likely explanation for the effect of apocynin. As shown in Fig. 5, apocynin inhibited prostanoid production and pulmonary hypertension during reperfusion. Thromboxane production appeared to be more sensitive to the inhibitory effects of apocynin compared with the other measured cyclooxygenase products. This pattern of prostanoid inhibition is similar to that shown by others (10). Indomethacin treatment, with a dose previously shown to inhibit cyclooxygenase in this preparation (29), had no effect on the increased vascular permeability in the present study. These data suggest that the protection conferred by apocynin was not due to thromboxane synthesis inhibition and are consistent with our previous findings of an inverse correlation between the thromboxane-induced pulmonary hypertension and vascular permeability in this model (32). In contrast, the increased vascular protein permeability associated with ischemia-reperfusion was attenuated by indomethacin in rat lungs (21) and thromboxane receptor blockade in rabbit lungs (22), suggesting that the role of thromboxane in this form of injury may depend on the species being studied.

Apocynin was recently shown to attenuate the pulmonary artery vasoconstriction from either hypoxia or the thromboxane analog U-46619 (14), suggesting that apocynin may have an inhibitory effect on vascular smooth muscle contraction. ATP-regulated potassium channel opening agents attenuate ischemia-reperfusion lung injury (18) and inhibit pulmonary vasoconstriction from either hypoxia (50) or U-46619 (12). To our knowledge, the effect of apocynin on endothelial or smooth muscle cell ion channels has not been studied, but an effect on potassium channels could represent an alternative explanation for its remarkable ability to prevent ischemia-reperfusion lung injury.

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