Apocynin attenuates ischemia-reperfusion lung injury in an isolated and perfused rat lung model

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Apocynin suppresses the generation of reactive oxygen species (ROS) that are implicated in ischemia-reperfusion (I/R) lung injury. We thus hypothesized that apocynin attenuates I/R. Furthermore, we explored the mechanisms by which apocynin may attenuate I/R. I/R was induced in an isolated and perfused rat lung model with ischemia for 1 h followed by reperfusion for 1 h. Apocynin was administered in the circulating perfusate at the onset of ischemia. Hemodynamics, lung injury indices, inflammatory responses, and activation of apoptotic pathways were determined. An increase in lung permeability and lung weight gain was noted after I/R. Peak airway pressure was increased, and pH of circulating perfusate was decreased. The adhesion molecule of neutrophil (CD31) in perfusate was upregulated. The levels of albumin, white blood cell count, and inflammatory cytokines including interleukin-1β, tumor necrosis factor-α, and macrophage inflammatory protein-2 increased in lung lavage fluid; the concentrations of carbonyl and thiobarbituric acid reactive substances were greater in the circulating perfusate; and the expression of myeloperoxidase, JNK, P38, and caspase-3 in lung tissue was greater in the control group. Upregulation and activation of nuclear factor-κB (NF-κB) in nuclei were found in I/R. The administration of apocynin attenuated these inflammatory responses and lung permeability associated with decreased activation of NF-κB. We conclude that I/R is associated with inflammatory responses including the generation of ROS, adhesion protein of neutrophil, cytokines, and the activation of mitogen-activated protein kinase and NF-κB cascade. The administration of apocynin attenuates the inflammatory responses and I/R in the isolated, perfused rat lung model. (Translational Research 2011;158:17–29)

Abbreviations: Apo = Apocynin; BALF = bronchoalveolar lavage fluid; BCA = bicinchoninic acid; EDTA = ethylenediaminetetraacetic acid; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-1β = interleukin-1β; I/R = ischemia/reperfusion; LWG = lung weight gain; MAPK = mitogen-activated protein kinase; MIP-2 = macrophage inflammatory protein 2; MPO = myeloperoxidase; NADPH = nicotinamide adenine dinucleotide phosphate; NF-κB = nuclear factor-kappa B; PBMC = peripheral blood mononuclear cell; PCNA = proliferating cell nuclear antigen; PMSF = phenylmethylsulfonyl fluoride; ROS = reactive oxygen species.

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Ischemia/reperfusion (I/R) injury is a common clinical problem encountered in myocardial or cerebral ischemia, shock, and organ transplantation. The lack of effective therapeutic strategies to treat this disorder remains an important issue. The pathogenesis of I/R lung injury is still unclear. Hypoxia and mechanotransduction1 (no blood flow) during ischemia induces macrophages, endothelial cell, or other cells to generate reactive oxygen species (ROS) as well as the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nuclear factor-kappa B, signal transduction, cytokines, ROS, and adhesion molecule of neutrophil. We found Apo to have the potential to be a drug to protect or treat I/R especially in lung transplantation.

**METHODS**

**Animal preparation.** The study protocol was approved by our institutional board for animal care and use. The in situ isolated-perfused lung model has been described previously.17 Briefly, male Sprague-Dawley rats weighing 250 g to 350 g were anesthetized with an intraperitoneal injection of sodium pentobarbital. A tracheotomy was performed and mechanical ventilation was applied (Rodent ventilator Model 683; Harvard Apparatus, South Natick, Mass) at a tidal volume of 5 mL/kg and positive end-expiratory pressure of 2 cmH2O. After a sternotomy, heparin (1 unit/g) was injected into the right ventricle through which the pulmonary artery was catheterized. A tracheotomy was performed and mechanical ventilation was applied (Rodent ventilator Model 683; Harvard Apparatus, South Natick, Mass) at a tidal volume of 5 mL/kg and positive end-expiratory pressure of 2 cmH2O. After a sternotomy, heparin (1 unit/g) was injected into the right ventricle through which the pulmonary artery was catheterized. The left atrium was catheterized at the apex of the heart. The pulmonary venous outflow was diverted into a reservoir. To prevent backflow into the ventricles, an additional ligation was performed above the atriocentric junction. The lungs were perfused with 10 mL blood mixed with 20 mL 0.9% normal saline (Minipulse 2; Gilson Medical Electronic, Middleton, Wis) at a constant flow of 30 μL/min/g body weight. Pulmonary arterial pressure ($P_{pa}$), pulmonary venous pressure ($P_{pv}$), pH in circulating perfusate, and peak airway pressure were monitored.

**AT A GLANCE COMMENTARY**

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**Background**

Reactive oxygen species (ROS) plays a key role in ischemia reperfusion lung injury (I/R), and Apocynin (Apo) suppresses the generation of ROS; therefore, it may attenuate I/R.

**Translational Significance**

Our study explored the mechanism of I/R. We are the first to explore the mechanism of attenuation on I/R by Apo treatment that is based on physiology, pathology, and molecular-level evidence (mitogen-activated protein kinase, nuclear factor-kappa B, signal transduction, cytokines, ROS, and adhesion molecule of neutrophil). We found Apo to have the potential to be a drug to protect or treat I/R especially in lung transplantation.
The rat weight was determined to reflect lung weight in the in situ system and recorded the lung weight gain (LWG) continuously.

Pulmonary arterial resistance (Ra) and venous resistance (Rv) were calculated using the following equations: 
\[ Ra = \frac{(P_{pa} - P_{pc})}{Q} \] 
\[ Rv = \frac{(P_{pc} - P_{pv})}{Q}, \] 
where Q is perfusate flow.

**Determination of pulmonary capillary pressure (Ppc).** The Ppc was estimated by using the double-occlusion method.16 Arterial inflow and venous outflow lines were occluded simultaneously, and the equilibrium \( P_{pa} \) and \( P_{pv} \) were measured. This equilibration pressure correlated well with isogravimetric measurements of \( P_{pc} \) and reflected the prevailing capillary pressure when the lung is not isogravimetric.

**Measurement of microvascular permeability.** The pulmonary capillary filtration coefficient \( (K_{fc}) \) was used as an index of microvascular permeability to water. \( K_{fc} \) was measured by using the method described previously.14 Briefly, after an isogravimetric period, \( P_{pv} \) was rapidly elevated to 6 cmH2O to 8 cmH2O for 15 min. The increase in lung weight was recorded, and a characteristic rapid weight gain (vascular filling) was followed by a slower rate of weight gain. The rate of weight change \( (DWt/Dt) \) during the 6- to 14-min interval was analyzed using linear regression of the log10-transformed rates of weight changes per minute. The initial rate of weight gain was calculated by using extrapolation of \( DWt/Dt \) to time 0. \( K_{fc} \) was calculated by dividing \( DWt/Dt \) at time 0 by the changes in \( P_{pc} \) that occurred after the venous outflow pressure was increased, normalized using the baseline wet lung weight, and expressed as mL/min/cm H2O per 100 g of lung tissue.

**Experiment protocols.** Three groups (control, I/R, and I/R + Apo) of isolated lung preparations were ventilated with tidal volume settings at 5 mL/kg in all groups. The protocol for I/R injury induction was as follows. Ventilation and perfusion were discontinued for 60 min (ischemia) in the isolated lung and then re instituted (reperfusion) for 60 min at room temperature. Apo was administered in the perfusate at the onset of ischemia. Apo (Biomol, Philadelphia, Pa) was administered at 0.1 mmol/L in a total volume of 30 mL of circulating perfusate at the onset of mechanical ventilation. Vascular permeability was measured by determining the pulmonary capillary filtration coefficient \( (K_{fc}) \) as previously described.18–20

**Measurement of albumin concentration and white blood cell (WBC) count in bronchoalveolar lavage fluid (BALF).** All experiments were terminated after 120 min of closed extracorporeal perfusion, and the lungs were removed and wet weights were measured. The lungs were lavaged twice by instilling saline (2.5 mL/lavage) in the left upper lobe. Lavage samples were centrifuged at 1500 g at room temperature for 10 min. The concentration of albumin and white blood cell (WBC) count were determined as previously described.19

**CD31 expression.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by centrifuging for 15 min at 3000 rpm. PBMCs then were stained with phycoerythrin-coupled CD31 antibodies (1:100) (BD Pharmingen, Franklin Lakes, NJ) and analyzed using a FACSCalibur flow cytometer (BD Pharmingen).

**Myeloperoxidase (MPO) assay.** The concentration of myeloperoxidase (MPO), an index of neutrophil sequestration in the lungs, was measured as previously described in the right middle lung tissue.21

**Carbonyl and thiobarbituric acid reactive substances (TBARS) assay in circulating perfusate, BALF, and lung tissue.** ROS was assessed by protein carbonyl and thiobarbituric acid reactive substances (TBARS) assays that are indices of protein and lipid peroxidation and oxidative stress, respectively. Protein carbonyl content was measured by Protein Carbonyl Assays (Geneteks Biosciences, Inc, San-Chong City, Taipei). The TBARS level was measured using OxiSelect TBARS Assay Kit (Geneteks Biosciences Inc). Carbonyl and TBARS of circulating perfusate, bronchoalveolar lavage fluid (BALF), and lung tissue were measured, respectively. The carbonyl protein concentration of soluble protein fractions was determined according to the method of Levine et al.22 The protein carbonyl content was measured by forming labeled protein hydrazone derivatives using 2,4-dinitrophenylhydrazide. These derivatives were sequentially extracted with 10% (vol/vol) TCA followed by treatment with 1:1 ethanol/ethy lacetate (vol/vol), and reextraction with 10% TCA. The resulting precipitate was dissolved in 6M urea hydrochloride. The spectrophotometric difference from a 2,4-dinitro phenylhydrazide protein blank was used to calculate the nmol of 2,4-dinitrophenylhydrazide incorporated per milligram of protein. The results are shown for each sample read at 370 nm. The TBARS level in the serum was determined using a method described by Ledwozyw et al23 with some modifications. To acidify 0.5 mL of serum, 2.5 mL of 1.22M trichloracetic acid (TCA)/0.6M hydrochloric acid was used and the mixture was left to stand at room temperature for 15 min. Next, 1.5 mL of 0.67% thiobarbituric acid/0.05M sodium hydroxide was added. The samples were incubated in a 100 °C water bath for 30 min. They were left to cool at room temperature before adding 4 mL of n-butanol. After thorough mixing, the mixture was centrifuged for 10 min at 3000 rpm. The absorbance was measured spectrophotometrically at 532 nm.
Cytokines assays. The levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and macrophage inflammatory protein 2 (MIP-2) in BALF were measured using commercial enzyme-linked immunosorbent assay kits (R&D Systems, Oxon, UK). The absorbance was read at 450 nm (SpectraMax M5; Molecular Devices, Silicon Valley, Calif).

Western blotting analysis. Lung tissues were homogenized using lysis buffer containing a protease inhibitor cocktail (Roche, Pleasanton, Calif) and a phosphatase inhibitor cocktail (Roche). The total protein concentration in the extract was determined with a bicinechonic acid (BCA) protein assay (Pierce, Rockford, Ill). Eight micrograms of protein were separated on 10% sodium dodecyl sulfate polyacrylamide gel and electrotransferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, Mass). The membrane was blocked with 5% nonfat dry milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Antibodies against phospho-p44/42 mitogen-activated protein kinase (MAPK; ERK1/2), phospho-SAPK/JNK, phospho-p38 MAPK, anti-p44/42 MAPK (ERK1/2), anti-SAPK/JNK, and anti-p38 MAPK each [1:1000; Cell Signaling Technology, Beverly, Mass] were used. Antibodies against GADPH [1:10000; Lab Frontier, Abfrontier, Seoul, Korea], JNK1 [1:1000; Santa Cruz Biotechnology, Santa Cruz, Calif], Caspase-3 [1:2000; Cell Signaling Technology], and PAI-1 [1:1000; Cell Signaling Technology] were used. The appropriate secondary antibodies were used (1:10,000 horseradish peroxidase antirabbit) at room temperature for 1 h. Visualization was performed by enhanced chemiluminescence (Visual Protein Biotechnology Crop). The protein bands on the destained gels were quantified with the Kodak 1D Image Analysis Version 3.5 software package (Eastman Kodak Company). Anti-PCNA antibody was used as a loading control to correct the pixel values for NF-κB.

Immunohistochemistry. Lung slides coated with poly-L-lysine (Sigma, St. Louis, Mo) were deparaffinized and rehydrated using xylene and ethanol and then placed in 3% H2O2 for 15 min. The slides were incubated with a 1:60 dilution of monoclonal NF-κB (Cell Signaling Technology), incubated at 4 °C overnight, and stained with diaminobenzidine (Dako, Glostrup, Denmark) and Mayer’s Hematoxylin (Dako). Analysis was performed under an Eclips 80i microscope (Nikon, Tokyo, Japan) using Image Pro Plus 5.0 (Media Cybernetics, Silver Spring, Md). The cells with positive staining NF-κB in nuclei were counted in 100 cells in 3 slides of immunohistochemical stain in each animal lung tissue.

Lung histopathology. After the termination of each experiment, lung tissue in the right lower lobes were dissected and fixed immediately in 10% neutral buffered formalin. After fixation, the lung tissues were dehydrated through a graded series of alcohol, cleared in xylene, and embedded in paraffin. All sections were cut to 5 μm and stained with hematoxylin/eosin. The severity of perivascular, peribronchial, septal, and alveolar edema as well as perivascular, interstitial, and alveolar cell infiltration was examined by a scoring system. We developed the following scoring method to measure the severity of acute lung injury: perivascular edema = 1; peribronchial edema = 2; interstitial edema = 2; alveolar edema = 3; perivascular cell infiltration = 2; interstitial cell infiltration = 3; alveolar cell infiltration = 4. A total of 20 scope views were examined for each lung tissue specimen. The sum of all the pathological scores was the score for each scope, and then we calculated the mean score of 20 scopes as the injury score for this lung tissue. Blind reviews were carried out by 2 pathologists, and the mean of these 2 scores was taken as the final score.13

Statistical analysis. Systat 10.0 (Systat Software Inc, San Jose, Calif) was used for statistical analysis.
Comparisons among all groups were conducted using analysis of variance followed by Dunnett’s method of post hoc testing. Comparison between the baseline and the post-I/R values within the group was conducted using the student paired t test. Values are expressed as mean ± standard deviation (SD). P < 0.05 was considered statistically significant.

RESULTS

I/R model. No significant difference was found in hemodynamics among the groups at baseline and at the end of study (Table I). LWG was higher in the I/R groups than in the control group, which decreased with Apo treatment (Table II). The LWG was in agreement with increased lung \( K_{fc} \) (Table II). The peak away pressure and WBC count increased in I/R but exhibited a trend to decrease with Apo treatment (Table III). The concentration of albumin in BALF increased in the I/R group, which decreased with Apo treatment (Table II). The concentrations of protein carbonyl in BALF were higher in the I/R group than in the control group but decreased in the presence of Apo (Table III).

The lung weight data were supported by the histological analysis showing perivascular edema, intraalveolar hemorrhage, interstitial and intraalveolar leukocytic infiltrates, as well as proteinaceous exudates in the I/R group (Fig 1, B). The Apo group showed less histological alterations (Fig 1, C). The lung injury scores of I/R groups were higher than those of the control group, and Apo treatment decreased the lung injury score (Fig 1, D).

Inflammatory responses. The concentration of MPO in lung tissue (Fig 2) and the expression of CD31 of neutrophil (Fig 3) increased in the I/R group compared with the control group. Treatment with Apo decreased the expression of CD31 to a level observed in the control group.

The concentrations of carbonyl and TBARS in the I/R groups were higher than those in the control and Apo treatment groups and decreased these levels in BALF and lung tissue at the end of the experiment (Fig 4, A and C). The concentrations of protein carbonyl in the circulating perfusate peaked 10 min after the onset of ischemia; the values were higher in the I/R group than in the control and I/R + Apo groups, but the value for I/R + Apo group returned to that of the control group in 75 min. The concentration of TBARS was higher in I/R groups than in the control group 10 min into ischemia; the values were higher in the I/R group than in the control, I/R and C groups compared with the I/R group (Fig 4, B and D).

The levels of MIP-2, IL-1β, and TNF-α in lavage fluids were higher in the I/R group than in the control group. The administration of Apo attenuated the cytokine responses (Fig 5, C–E). IL-1β and TNF-α in lung tissue were higher in the I/R group than in the control, but the group treated with Apo had lower levels of these cytokines (Fig 5, D and E).

MAPK and NF-κB signaling pathways. An increase was noted in JNK and P-38 activation in response to I/R. The use of Apo attenuated this activation (Fig 6, B and C). The expression of extracellular signal-regulated kinases tended to be higher in the I/R group, but the differences among the 3 groups were not statistically significant (Fig 6, A).

Nuclear translocation of NF-κB was observed in epithelial, endothelial, and inflammatory cells after I/R (Fig 7, A–C). The NF-κB activation was attenuated in the Apo-treated groups (Fig 7, C). The ratio of cells with positive NF-κB immunohistochemical staining in

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**Table I.** Hemodynamics*

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>( P_{pa} )</th>
<th>( P_{pv} )</th>
<th>( P_{pc} )</th>
<th>( R_a )</th>
<th>( R_v )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before injury</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>10.00 ± 1.12</td>
<td>4.64 ± 0.84</td>
<td>6.65 ± 1.51</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>I/R</td>
<td>7</td>
<td>11.08 ± 1.85</td>
<td>4.90 ± 0.19</td>
<td>6.94 ± 1.96</td>
<td>0.05 ± 0.05</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>I/R + Apo</td>
<td>7</td>
<td>10.04 ± 2.40</td>
<td>4.46 ± 0.54</td>
<td>6.29 ± 1.82</td>
<td>0.05 ± 0.05</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>After injury</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>9.70 ± 1.60</td>
<td>4.67 ± 0.72</td>
<td>6.58 ± 1.42</td>
<td>0.04 ± 0.05</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>I/R</td>
<td>7</td>
<td>10.45 ± 2.06</td>
<td>5.07 ± 0.44</td>
<td>6.12 ± 2.21</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>I/R + Apo</td>
<td>7</td>
<td>9.50 ± 2.49</td>
<td>4.32 ± 0.66</td>
<td>6.00 ± 1.73</td>
<td>0.04 ± 0.05</td>
<td>0.03 ± 0.04</td>
</tr>
</tbody>
</table>

*Values are mean ± SD.

**Table II.** LWG and pulmonary capillary filtration coefficient (\( K_{fc} \))

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>LWG (g)</th>
<th>Baseline</th>
<th>After injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.09 ± 0.06</td>
<td>0.17 ± 0.07</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>I/R</td>
<td>7</td>
<td>0.89 ± 0.30*</td>
<td>0.19 ± 0.04</td>
<td>0.43 ± 0.12*</td>
</tr>
<tr>
<td>I/R + Apo</td>
<td>7</td>
<td>0.42 ± 0.22,†</td>
<td>0.20 ± 0.07</td>
<td>0.24 ± 0.08*†</td>
</tr>
</tbody>
</table>

*\( P < 0.05 \) compared with Control (2h).
†\( P < 0.05 \) compared with I60/R60.
nuclei of I/R was higher than that in the control group. The total number of cells with a positive stain in the Apo-treated groups was less than that observed in the I/R group (Fig 7, D). Upregulation of NF-κB expression was observed in the I/R group but was lower in the I/R + Apo group as assayed in the nuclear protein of lung tissue (Fig 8).

**DISCUSSION**

A major finding of the present study is that increased generation of ROS (assessed by protein carbonyl and
TBARS concentrations), cytokine responses, increased expression of adhesion molecule of neutrophil (CD31), and activation of MAPK and NF-κB were associated with I/R lung injury. The administration of Apo reduced ROS production and attenuated the inflammatory responses associated with I/R.

Our I/R model reproduces many features reported in other studies, including inflammatory responses and structural lung damage. Several studies including animals and humans have tested the effects of pharmacological intervention in an attempt to reduce I/R injury. Previous studies of I/R treatment were based largely on the attenuation of inflammatory responses. It is important to note that treatment with Apo at the onset of ischemia attenuates...

![Graph showing MPO release](image1)

**Fig 2.** Apo reduced MPO release. Apo reduced the level of MPO in the lung tissue. *P < 0.05 versus other groups. Seven control, I/R, and I/R + Apo experiments were performed for each group.

![Graph showing CD31 expression](image2)

**Fig 3.** The effect of Apo and I/R on adhesion protein of neutrophil (CD31). CD31 of I/R group was higher than the control and I/R + Apo groups. These results indicate that I/R induced overexpression of adhesion molecules on neutrophil, and Apo treatment inhibited CD31 expression. Seven control, I/R, and I/R + Apo experiments were performed for each group.
I/R lung injury. Our study suggests that the oxidative stress implicated in I/R is a significant therapeutic target in the context of I/R lung injury. These results support the previous study, and our results are in agreement with those reported by others showing an inability of natural host defenses to increase antioxidant capacity leading to failure of recovery from acute lung injury.

NADPH oxidase is a unique family of enzymes whose physiologic function is to generate ROS and is ubiquitously distributed. During I/R in the lung, likely cellular sources of NADPH oxidase–derived ROS include leukocytes, endothelial cells, epithelial cells, and dendritic cells. Hypoxia- or ischemia-induced ROS production reportedly is derived from pulmonary epithelial or endothelial NADPH oxidase.

Apo is a strong oxidative inhibitor that has been demonstrated to block NADPH oxidase in neutrophils, macrophages, and endothelium through inhibition of p47phox translocation without interfering with other immune biological functions of the cell system. We demonstrate that treatment with the antioxidant Apo dramatically attenuated ROS production as well as the inflammatory responses including cytokines and MAPK activation. Although it is difficult to know the exact timing for the generation of cytokines and ROS in our in vivo model, it has been demonstrated that cytokine stimulation leads to an accumulation of ROS, which is essential for prolonged MAPK activation and cell death, and in turn, ROS generation results in additional cytokine production. Thus, the observed decrease in cytokines responses along with reduced activation of MAPK activation and NF-κB after the Apo treatment could be interrelated. It has been shown that cytokine and chemokine secretion is dependent on the activation of MARKs. Our previous study...
showed that inhibition of NF-κB produced an attenuation of I/R lung injury as well as a downregulation of cytokine production. In this study, we found that NF-κB was activated in I/R and that treatment with Apo induced downregulation of NF-κB and cytokines. Taken together, Apo seems to have antiinflammatory as well as antioxidative effects.

Apo has not been well studied at the molecular level for its therapeutic effects in the context of I/R injury. Previously, Dodd-O et al showed that Apo prevents the increased vascular permeability caused by I/R in isolated sheep lungs. In a separate study, they showed Apo to attenuate the severe hypoxemia resulting from cardiopulmonary bypass performed with a deflated sheep lung as well as from a complete pulmonary artery occlusion and a ligated bronchial artery. However, Apo had no effect on the increased plasma cytokine concentrations. The reasons for the discrepancy in the effect of Apo on cytokine levels between the study by Dodd-O et al and ours are not clear but may be because of differences in animal models and study protocols. The results of previous studies that were in accordance with our results are as follows: In isolated perfused rat lung, Apo attenuated I/R injury with less ROS and TNF-α production, and Apo exerted protective effects on hepatic I/R injury with a lower malondialdehyde level in liver tissues and less expression of TNF-α in serum of mice. In this study, we also demonstrated that Apo attenuates I/R lung injury, which is reflected by decreased LWG and Kf. To our knowledge, our study is the first to show that Apo reduced ROS as reflected in the decreased concentrations of carbonyl and TBARS in circulating perfusate and

**Fig 5.** Cytokines (IL-1β, TNF-α, and MIP-2) in BALF of I/R group was higher compared with the control and I/R + Apo groups (C–E). IL-1β and TNF-α in lung tissue were higher in the I/R group compared with the control, but the group treated with Apo had lower levels of IL-1β and TNF-α in lung tissue (D and E).
Fig 6. Apo attenuated activation of MAPK (JNK and p-38) and caspase-3 expression in lung tissue. Seven control, I/R, and I/R + Apo experiments were performed for each group. These results indicate that I/R induced overexpression of JNK, p-38, and caspase-3, but Apo treatment attenuated these responses.

Fig 7. Apo decreased NF-κB activation in macrophages of ventilated lung tissue with 3 panels (400×) and inside panels (1000×). Active NF-κB was translocated into nuclei of epithelial, endothelial, and inflammatory cells (dark brown stain) in the I/R group (B), but NF-κB did not translocate into the nuclei of these cells in the control (A) or I/R + Apo groups (C) (blue stain). The ratio of cells with positive NF-κB immunohistochemical staining in the nuclei of I/R was higher than that in the control group. The total number of cells with positive stain in the Apo-treated groups was less than that in the I/R group (D).
BALF. We also showed that Apo reduced cytokine (MIP-2, IL-1β, and TNF-α) expression in BALF. In comparing I/R and I/R + Apo groups, we are also the first, to our knowledge, to find reduced expression of adhesion molecule (CD31) on neutrophils in the circulating perfusate, downregulation of MPO (a major release from neutrophil as an index of active sequestration neutrophil in the lungs), MAPK pathway (p-JNK and p38), apoptosis index (caspase-3), and NF-κB nuclear protein in lung tissue. Using immunohistochemistry staining of NF-κB in lung tissue, we found decreased NF-κB translocation into the nucleus. We also demonstrated downregulation of cytokines (IL-1β and TNF-α) in lung tissue and BALF in I/R after Apo treatment.

This study has several limitations. First, we used an isolated perfused lung model to minimize any hemodynamic effects on lung injury, but the model does not provide interactions with another organ system. Second, Apo was administered prior to ischemia for a proof-of-concept study, and future studies will need to address the effects of Apo administered after I/R has occurred. Third, about 10 mL to 13 mL of blood was withdrawn from heart in each rat; therefore, a 10-mL blood + 20-mL 0.9% normal saline as perfusate was used in the isolated-perfused lung model. The circulating perfusate in our model contains less albumin and WBCs. Theoretically, more WBCs may induce more severe I/R injury, and less albumin with less colloid substance in the perfusate may result in more leakage of fluid into intra-alveolar spaces. However, this experimental setup reflects the clinical aspects of a cardiopulmonary bypass.

In summary, our results demonstrate that I/R-induced permeability pulmonary edema with capillary leakage and suggest that ROS, MARK, NF-κB, and cytokines play important roles in the context of I/R lung injury. The administration of Apo effectively can attenuate I/R lung injury by inhibiting inflammatory responses associated with the generation of ROS.

Fig 8. Expression of NF-κB of nuclear protein in lung tissue. Seven control, I/R, and I/R + Apo experiments were performed for each group. Overexpression of NF-κB was found in the I/R group, but Apo attenuated the NF-κB expression.
We appreciate Jay H. Ryu for providing editorial assistance.

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