APOCYNIN ATTENUATES LIPOPOLYSACCHARIDE-INDUCED LUNG INJURY IN AN ISOLATED AND PERFUSED RAT LUNG MODEL

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ABSTRACT—Apocynin (Apo) suppresses the generation of reactive oxygen species that are implicated in lipopolysaccharide (LPS)-induced lung injury (LPSLI). We thus hypothesized that Apo may attenuate LPSLI. In addition, we explored the cellular and molecular mechanisms of Apo treatment in LPSLI. Lipopolysaccharide-induced lung injury was induced by intratracheal instillation of 10 mg/kg LPS in isolated and perfused rat lung model. Apocynin was administered in the perfusate at 15 min before LPS was administered. Hemodynamics, lung injury indices, inflammatory responses, and activation of apoptotic pathways were assessed. There was an increase in lung vascular permeability associated with lung weight gain after LPS exposure. The levels of interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), macrophage inflammatory protein 2, H2O2, and albumin increased in the bronchoalveolar lavage fluid. Adhesion molecule of neutrophil (CD31) was upregulated. The expression of TNF-α, IL-1β, glutathione, myeloperoxidase, JNK, P38, caspase 3, p-AKT, and plasminogen activator inhibitor 1 in lung tissue was greater in the LPS groups when compared with the control group. Upregulation and activation of nuclear factor κB occurred along with increased histopathologic lung injury score in LPSLI. The Apo attenuated these inflammatory responses including the levels of CD31, H2O2, TNF-α, IL-1β, myeloperoxidase, P38, and nuclear factor κB along with downregulation of apoptosis as reflected by caspase 3 and p-AKT. In addition, Apo attenuated the increase in lung weight, bronchoalveolar lavage fluid albumin content, and the histopathologic lung injury score. In conclusion, LPSLI is associated with increased inflammatory responses, apoptosis, and coagulation. The administration of Apo attenuates LPSLI through downregulation of the inflammatory responses and apoptosis.

KEYWORDS—Apocynin, LPS, ARDS, inflammation, NADPH, MAPK, NF-κB

Sepsis is a significant public health problem and represents the 10th leading cause of death in the United States. Acute lung injury (ALI) and acute respiratory distress syndrome commonly complicate sepsis (1) and often result in prolonged mechanical ventilatory support with a mortality of approximately 30% to 50% (1) despite substantial advances in the management of these disorders (2, 3). Health care expenditures in the care of these patients remain high (1, 4).

Sepsis related to bacterial infection is characterized by an overwhelming systemic inflammatory response (5). Toll-like receptor 4 (TLR4) recognizes gram-negative bacteria and bacterial components (lipopolysaccharide [LPS]) and thereby mediates innate immune cell activation (6). Upon LPS binding, TLR4 undergoes homodimerization and, through the cytoplasmic Toll/interleukin 1R (IL-1R) homology domain (TIR) domain, recruits the adapter molecule MYD88 (myeloid differentiation marker 88) and/or TIR (Toll/IL-1 receptor) domain-containing adaptor (TRIF) to initiate signal transduction (6). Recent studies have shown that reactive oxygen species (ROS) play crucial roles in TLR4 activation and the pathology of sepsis by regulating immune cell activation and end-organ injury (7). Excess intracellular and extracellular ROS (superoxide, hydrogen peroxide) have the ability to prime the phagocytes (macrophages and neutrophils) toward an inflammatory response (8, 9). Host factors that regulate cellular ROS levels may act as important modifiers in the pathogenesis of sepsis (10).

The NADPH oxidase complex is a major source of intracellular ROS generation in macrophages and neutrophils (11). The NADPH oxidase complex is composed of two transmembrane proteins: flavocytochrome b components (gp91phox and p22phox) and four cytosolic proteins (p47phox, p67phox, p40phox, and Rac) (11). Upon activation, the cytosolic components translocate to the transmembrane catalytic protein gp91phox, which results in the formation of functional NADPH oxidase complex. Reactive oxygen species have been implicated in multiple physiological and pathological processes as a secondary messenger.

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in cell signaling (12). Numerous studies have demonstrated the role of NADPH oxidase–dependent ROS generation in modulating TLR4 signaling, inflammatory response (13), and disease pathogenesis (14). The pathogenesis of sepsis appears to involve the generation of ROS. An oxidative stress-induced damage is suggested to serve as a critical mechanism of septic injury.

Apocynin (Apo) is a naturally occurring methoxy-substituted catechol and has been used as an inhibitor of NADPH oxidase, other antioxidant effect, or other unknown effects. Apocynin has been shown to decrease the production of O$_2^-$ from activated neutrophils and macrophages (15). Previously, Wang et al. (16) showed that Apo prevents the increased vascular permeability caused by septic lung injury in guinea pigs, but the underlying mechanisms of Apo in this lung injury model remain unclear. We hypothesized that Apo attenuates septic lung injury by downregulating ROS production and the downstream signaling pathways.

**MATERIALS AND METHODS**

**Animal preparation**

The study protocol was approved by the institutional board for animal care and use. The in situ isolated-perfused lung model has been previously described (17). Briefly, male Sprague-Dawley rats weighing 250 to 350 g were anesthetized with 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 6 mL/kg and positive end-expiratory pressure of 2 cmH$_2$O. After a sternotomy, heparin (1 U/g) was injected into the right ventricle through which 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 flow back into the ventricles, an additional ligation was performed above the atriocentric junction. The lungs were perfused with 10 mL blood mix with 20 mL 0.9% normal saline (Minipulse 2; Gilson Medical Electronic, Middleton, Wis) at a constant flow at 30 µL/min per gram of body weight. Pulmonary artery pressure ($P_{pa}$), pulmonary venous pressure ($P_{pv}$), pH in circulating perfusate, and peak airway pressure were monitored. The rat weight was determined to reflect lung weight in the in situ system, and the lung weight gain (LWG) was continuously recorded. Pulmonary arterial resistance ($R_a$) and venous resistance ($R_v$) were calculated using the following equations: $R_a = (P_{pa} - P_{pc}) / Q$, and $R_v = (P_{pv} - P_{pc}) / Q$, where $Q$ is perfuse flow, and $P_{pc}$ is pulmonary capillary pressure.

**Determination of $P_{pc}$**

The $P_{pc}$ was estimated by using the double-occlusion method (18). Arterial inflow and venous outflow lines were occluded simultaneously, and the equilibrium $P_{pa}$ and $P_{pv}$ were measured. This equilibrium pressure is well correlated with isogravimetric measurements of $P_{pc}$ and also reflects the prevailing capillary pressure when the lung is not isogravimetric.

**Measurement of microvascular permeability**

Pulmonary capillary filtration coefficient ($K_{fc}$) was used as an index of microvascular permeability. The $K_{fc}$ was measured by using the method described previously (18). Briefly, after an isogravimetric period, $P_{pc}$ was rapidly elevated to 6 to 8 cmH$_2$O 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 (D WT/DT) during the 6- to 14-min interval was analyzed by using linear regression of the log10-transformed rates of weight changes per minute. The instant 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 change in $P_{pc}$ that occurred after venous outflow pressure was increased then normalized using the baseline wet-lung weight and expressed as milliliters per minute per centimeter H$_2$O per 100 g of lung tissue.

**Experiment protocols**

Three groups: (a) sham (control), (b) intratracheal LPS (LPS), and (c) intratracheal LPS plus Apo (LPS + Apo) of isolated lung preparations were ventilated with tidal volume settings at 6 mL/kg in all groups. A group ventilated with low tidal volume of 6 mL/kg and not exposed to LPS served as sham for the control group. The protocol for LPS-induced injury was performed as follows: LPS with 10 mg/kg was administered intratracheally as group with LPS-induced lung injury. Apocynin was administered in the perfusate at 15 min before LPS was begun. Apocynin (Biomol, Enzo Life Sciences, Farmingdale, NY) was administered at 0.1 mmol/L in a total volume of 30 mL of circulating perfusate. Hemodynamics were continuously monitored for 2 h in all groups. Vascular permeability was measured by determination of $K_{fc}$ as previously described (19).

**Measurement of albumin concentration and white cell count in bronchoalveolar lavage fluid**

All experiments were terminated after 120 min of closed extracorporeal perfusion, the lungs were removed, and wet weights were measured. The lungs were lavaged twice with saline (2.5 mL/lavage) in the left upper lobes. Mixture of fluid was done, and recovery rate of bronchoalveolar lavage fluid (BALSE) needed more than 85%. Lavage samples were centrifuged at 1,500g at room temperature for 10 min. The concentrations of albumin and white cell count were determined as previously described (17, 20).

**CD31 expression**

Peripheral blood mononuclear cells were isolated from heparinized blood by centrifuging for 15 min at 3,000 revolutions/min (rpm). Peripheral blood mononuclear cells were then stained with phycoerythrin-coupled CD31 antibodies (1:100) (BD Pharmingen, Franklin Lakes, NJ) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, Calif).

**Myeloperoxidase 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 (20).

**Measurement of H$_2$O$_2$ levels in BALF**

Samples were centrifuged 1,000g within 30 min, and the supernatants retained. Fifty microliters of the H$_2$O$_2$ reaction mix (46 µL assay buffer, 2 µL OxiRed Probe solution, and 2 µL horseradish peroxidase solution) (BioVision)

<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>
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<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>LPS</td>
<td>7</td>
<td>7.70 (3.65)</td>
<td>4.63 (0.49)</td>
<td>5.96 (3.10)</td>
<td>0.03 (0.05)</td>
<td>0.03 (0.04)</td>
</tr>
<tr>
<td>LPS + Apo</td>
<td>7</td>
<td>9.50 (2.00)</td>
<td>4.79 (0.27)</td>
<td>6.86 (0.86)</td>
<td>0.05 (0.02)</td>
<td>0.04 (0.02)</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 (2.23)</td>
<td>0.05 (0.05)</td>
<td>0.04 (0.05)</td>
</tr>
<tr>
<td>LPS</td>
<td>7</td>
<td>8.85 (7.08)</td>
<td>4.88 (0.20)</td>
<td>5.98 (3.10)</td>
<td>0.06 (0.06)</td>
<td>0.05 (0.05)</td>
</tr>
<tr>
<td>LPS + Apo</td>
<td>7</td>
<td>10.54 (3.47)</td>
<td>4.89 (0.19)</td>
<td>6.72 (2.23)</td>
<td>0.05 (0.05)</td>
<td>0.04 (0.05)</td>
</tr>
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Values are mean (SD). $P_{pa}$ and $P_{pv}$ (in mmHg) indicates pulmonary arterial and venous pressure, respectively; $P_{pc}$ (in mmHg), isogravimetric capillary pressure; $R_a$ and $R_v$ (in mm Hg/min per mL), arterial and venous resistance, respectively.
to each sample and H$_2$O$_2$ standards were added, mixed well, then incubated at room temperature for 10 min. The absorbance was read at 570 nm (SpectraMax M5; Molecular Devices, Silicon Valley, Calif).

### Glutathione assay in lung tissue

In a separate group of animals, the right lung was used for glutathione (GSH) assay. The lung was washed in phosphate-buffered saline, pH 7.2, blotted in 10 mL of ice-cold 5% metaphosphoric acid solution, then thoroughly homogenized for 2 min and centrifuged at 10,000 g for 10 min at 4°C. The upper clear aqueous phase was collected and assayed within 4 h. The protein content of the lysate was measured using a DC protein assay (Bio-Rad Laboratories, Hercules, Calif). Total lung GSH was measured using a colorimetric microplate assay kit (Oxford Biomedical Research, Oxford, Mich) based on the oxidation of GSH by 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The GSH standards and treated samples were added to microtiter plate wells, followed by DTNB and GSH reductase. Addition of NADPH initiated the progressive reduction of DTNB by GSH, causing an increase in absorbance that was monitored at 405 nm. The rate of change in absorbance over 4 min is proportional to the GSH concentration, which was reported as mg GSH/mg of protein.

### Cytokines assays

The levels of IL-1β, tumor necrosis factor α (TNF-α), and macrophage inflammatory protein 2 (MIP-2) in lavage fluids were measured using enzyme-linked immunosorbent assay kits (R&D Systems, Oxon, UK). The absorbance was read at 450 nm (SpectraMax M5; Molecular Devices).

### Western blotting analysis

Lung tissues were homogenized using lysis buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche, Pleasanton, Calif). The total protein concentration in the extract was determined with a bicinchoninic acid protein assay (Pierce, Rockford, Ill); 80 μg protein was separated on 10% sodium dodecyl sulfate polyacrylamide gel and electro-transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, Mass). The membrane was blocked with 5% nonfat dry milk in TBS containing: 0.1% Tween 20 (TBST) for 1 h. Antibodies against phospho-p44/p42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [ERK] 1/2), phospho-SAPK/JNK, phospho-p38 MAPK, (1:1,000, Cell Signaling Technology, Beverly, Mass) were used. Antibodies against GADPH (1:10,000; Lab Frontier, Abfrontier, Seoul, Korea), JNK1 (1:1,000; Cell Signaling Technology, Santa Cruz, Calif), caspase 3 (1:2,000; Cell Signaling Technology), p-AKT (1:1,000; Cell Signaling Technology), AKT (1:1,000; Cell Signaling Technology), plasminogen activator inhibitor 1 (PAI-1) (1:1,000; Cell Signaling Technology), AP-1 (1:1,000; Abcam, Cambridge, Mass), IL-1β (1:1,000; Abcam), and TNF-α (1:1,000; Abcam) were used. The appropriate secondary antibodies were used (1:10,000 horseradish peroxidase anti–rabbit [Jackson ImmunoResearch Laboratories, West Grove, PA]). Visualization was performed by enhanced chemiluminescence (Visual Protein Biotechnology Corp, New Taipei City, Taiwan). The protein bands were quantified with the Kodak 1D Image Analysis (Eastman Kodak Company, Rochester, NY).

#### Nuclear factor κB analysis of nuclear protein

Lung tissue was homogenized with a Dounce tissue homogenizer in 5-mL solution A (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM p-nitrophenylsulfonyl fluoride). The homogenates were centrifuged for 30 s at 2,000 rpm, and the supernatants were collected and centrifuged for 5 min at 5,000 rpm. The pelleted nuclei were resuspended at 4°C in 300 μL solution B (25% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 μg/mL pepstatin A, 5 μg/mL leupeptin, 5 μg/mL aprotinin) and incubated on ice for 20 min. Samples were centrifuged at 15,000 rpm for 10 min. The total protein concentration in the extract was determined with a bicinchoninic acid protein assay (Pierce). The membrane was blocked for 1 h. Anti–nuclear factor κB (NF-κB) antibody (1:1,000; Cell Signaling Technology) and anti-PCNA antibody (1:1,000; Cell Signaling Technology) were diluted in TBST buffer (Tris-buffered saline/0.1% Tween 20) and incubated at 4°C overnight. The appropriate secondary antibody was used (1:10,000 horseradish peroxidase anti–rabbit [Jackson ImmunoResearch Laboratories) at room temperature in 1 h. Visualization was performed by enhanced chemiluminescence (Visual Protein Biotechnology Corp). 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.

### Lung histopathology

After the termination of each experiment, the right lower lobe of the lungs were dissected and fixed immediately in 10% neutral buffered formalin. After fixation, the lung tissue was 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 and of perivascular, interstitial, and alveolar cell infiltration were examined by a score system. We developed a scoring method to measure the severity of ALI, as follows: perivascular edema = 1, peribronchial edema = 2, interstitial edema = 2, alveolar edema = 3, perivascular cell infiltration = 2, interstitial cell infiltration = 3, and alveolar cell infiltration = 4. A total of 20 scope views were examined for each lung tissue. 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 two pathologists, and the mean of these two scores was taken as the final score (17, 19).

#### 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 method of post hoc testing. Comparison between baseline and post-LPS values within group was conducted using Student paired t test. Values are expressed as mean (SD). $P < 0.05$ was considered statistically significant.

### RESULTS

#### LPSL1 model

There was no significant difference in hemodynamics among the groups at baseline and the end of study (Table 1). Lung...
weight gain was higher in the LPS groups than in the control group, but the weight gain was attenuated in the presence of Apo (Table 2). The LWG was in agreement with increased lung potassium (Table 2) in LPS groups. Concentration of albumin in BALF increased in LPS groups, but this increase was attenuated in the presence of Apo (Table 2).

The lung weight data were further supported by the histological analysis showing perivascular edema, intra-alveolar hemorrhage, interstitial and intra-alveolar leukocytic infiltrates, and proteinaceous intra-alveolar exudates in the LPS groups (Fig. 1B) as compared with the control group (Fig. 1A). Addition of Apo group reduced the histological alterations associated with LPSLI (Fig. 1C). Lung injury scores of LPSLI groups were higher than that in the control group, and Apo treatment decreased the lung injury score (Fig. 1D).

**Inflammatory responses**

The concentration of MPO (Fig. 2A) in LPS groups was higher, but antioxidant GSH (Fig. 2B) was lower than that in the control group. Expression of neutrophil adhesion molecule (CD31) (Fig. 3) increased in the LPS groups compared with the control group. Apocynin treatment decreased the expression of MPO (Fig. 2A) and CD31 (Fig. 3) to a level comparable to the control group. Glutathione level of the Apo treatment group was higher than that in LPS group (Fig. 2B), but no significant difference was found between the Apo treatment group and the control group.

The concentration of H$_2$O$_2$ in LPS groups was higher than that in the control group, and Apo treatment attenuated this increase in BALF (Fig. 4A). The levels of TNF-α, IL-1β, and MIP-2 in lavage fluids were higher in the LPS groups compared with the control group. The administration of Apo attenuated these cytokine responses (Fig. 4, B–D). Interleukin 1β and TNF-α in lung tissue (Fig. 5, A and B) of LPS groups were higher than in control, but the group treated with Apo had lower levels of IL-1β and TNF-α compared with the LPS group (Fig. 5, A and B).

**Plasminogen activator inhibitor 1**

Upregulation of the PAI-1 content occurred in LPS groups and did not differ with Apo treatment (Fig. 5C).

**MAPK and NF-κB signaling pathways**

There was an increase in JNK and P38 activation in response to LPS (Fig. 6, B and C). Apocynin treatment attenuated the P38 activation (Fig. 6C). The expression of ERKs was not different among the three groups (Fig. 6A). Upregulation NF-κB expression was seen in the LPS group, but this response was attenuated by Apo treatment (Fig. 7A).

**Apoptosis**

The caspase 3 and p-AKT contents in lung tissue were higher in the LPS groups compared with the control group, but this increase was attenuated by the Apo treatment (Fig. 7, B and C).

**DISCUSSION**

In our animal model, LPS-induced lung injury increased pulmonary vascular permeability, infiltration of inflammatory cell, pulmonary edema, generation of ROS, cytokine responses, neutrophil adhesion molecule (CD31) expression, MAPK activation, NF-κB expression, and expression of apoptotic enzymes (caspase 3 and p-AKT) and PAI-1. The Apo treatment efficiently suppressed ROS (H$_2$O$_2$) production and increased antioxidant (GSH) capacity while attenuating the inflammatory and apoptosis responses, although PAI-1 expression was unchanged.
Our LPS model reproduces many features reported in other studies of ALI, including inflammatory responses and structural lung damage (21).

There are a number of studies that have tested the effects of pharmacological interventions in an attempt to reduce LPSLI. These previous studies have largely reported attenuation of inflammatory responses (22). It should be noted that the Apo treatment given at the onset of LPS showed attenuation of LPSLI. Our results suggest that the oxidative stress associated with LPSLI may be a significant therapeutic target in the management of ALI. These results are consistent with a previous study (13) and are in agreement with another study showing an inability of natural host defenses to increase antioxidant capacity leading to damaging sequelae of ALI (23).

Apocynin is a strong oxidative inhibitor that has been demonstrated to block NADPH oxidase in neutrophils (24), macrophages (25), and endothelium (26) through inhibition of p47phox translocation, without interfering with other immune biological function of the cell system. This may be an important mechanism underlying the therapeutic effects of Apo as NADPH plays a crucial role in the complex cell-cell interaction influencing oxidative status (27). In addition, Apo contains a phenol group with possible ROS-scavenging capacity; previous study showed Apo acts as an inhibitor of NAPDH oxidase restricted only to MPO-expressing leukocytes, but as a scavenger of ROS in MPO-free vascular cells or skeletal muscle cells suggested by some studies (28, 29).

We demonstrated that the treatment with the antioxidant Apo dramatically attenuated ROS production and the inflammatory responses including cytokines and MAPK activation occurring in LPSLI. Although it is difficult to know the exact time course for generation of cytokines and ROS in our in vivo model, it has been demonstrated that cytokine stimulation leads to accumulation of ROS, which is essential for prolonged MAPK activation and cell death and, in turn, ROS generation resulting in cytokine production (30). Thus, the observed decrease in cytokines and reduced activation of MAPKs and NF-κB would be interrelated after the treatment with Apo. It has been shown that secretion of cytokines and chemokines is dependent on the activation of MAPKs (31). Our previous study showed inhibition of NF-κB produced attenuation of ischemia/reperfusion injury and ventilator-induced lung injury and down-regulation of cytokine production (17). In this study, we found NF-κB was activated in LPSLI, and Apo treatment induced

**Fig. 4.** Effects of Apo on oxidative responses (H2O2), TNF-α, IL-1β, and MIP-2 in bronchoalveolar fluids at the end of the study. The levels of H2O2, TNF-α, IL-1β, and MIP-2 are shown in A to D, respectively.

**Fig. 5.** Cytokines (IL-1β, TNF-α) and PAI-1 of LPS groups in tissue was higher than those of the control and LPS + Apo (A–C) groups. However, the group treated with Apo had the lower levels of IL-1β and TNF-α (A, B).
downregulation of NF-κB and cytokines. Taken together, Apo appears to have anti-inflammatory and antioxidative effects. Furthermore, we have shown for the first time that Apo has an antiapoptotic effect by inhibiting AKT and caspase 3 while increasing antioxidant capacity by increasing the GSH content in the lung.

Effects of Apo have not been well studied at the molecular level in the context of LPSLI. Previously, Wang et al. (16) showed that Apo prevents increased vascular permeability caused by septic lung injury in guinea pigs. These authors did not explore cellular or molecular mechanisms. Viačková et al. (32) showed the failure of Apo to prevent lung inflammation induced by endotoxin in mice. In contrast to our study, Viačková et al. applied 50 μL of 1 mM Apo delivered through the nasal route to mice. Our previous study showed higher Apo dose to induce greater suppression of ROS production in ventilator-induced lung injury model (33). The antioxidant effect of Apo appears to be dose-dependent. The discrepant results regarding Apo treatment of LPSLI may be due not only to differences in dose of the drug but also the animal model and the route of administration.

In this study, we demonstrated Apo to attenuate LPSLI. To our knowledge, this is the first study to show Apo to reduce ROS as reflected in decreased H₂O₂ content of BALF. We also showed Apo to reduce expression of several cytokines (MIP-2, IL-1β, and TNF-α) in BALF. These results support previous studies (34, 35) showing Apo to reduce TNF-α and ROS of the heart in an LPS-induced heart injury model.

Another finding in this study that has not been previously reported is the ability of Apo to decrease expression of neutrophil adhesion molecule (CD31) in circulating perfusate, downregulate of MPO (as an index of neutrophil sequestration in the lung), MAPK pathway (p38) activity, apoptosis index (caspase 3 and p-AKT), and NF-κB nuclear protein in lung
tissue. We also demonstrated downregulation of cytokines (IL-1β and TNF-α) in lung tissue and BALF with Apo treatment in LPSLI.

There are several limitations in the study. First, we used isolated, perfused lung model to minimize any hemodynamic effects on lung injury. Thus, the model excludes interactions with other organ systems. Second, Apo was administered before LPS to study its effects on lung injury. Thus, the model excludes interactions with other injury models.

In summary, our results show LPS to induce permeability pulmonary edema with capillary leakage and suggests that ROS, MARK, NF-kB, cytokines, apoptosis, and coagulation play important roles in the context of LPSLI. The application of Apo can attenuate LPSLI by inhibition of inflammatory responses and apoptosis occurring with the generation of ROS.

REFERENCES