Diazoxide Decreases Ischemia-Reperfusion Injury in a Rat Model of Lung Transplantation

W. Guo, D. Ge, Q. Wang, S. Xu, L. Xue, C. Lu, and L. Tan

ABSTRACT

Background. Ischemia-reperfusion injury (IRI) is a significant factor contributing to primary graft failure in lung transplantation. Given a pivotal role of mitochondria in IRI-related molecular events, the effects of diazoxide, a selective opener of mitochondrial adenosine-5′-triphosphate (ATP)-sensitive potassium channels (mitoK_{ATP}), on IRI were investigated in a rat model of lung transplantation.

Methods. The 108 rats were randomly assigned to 5 groups; a sham-operated, 2 control, and 2 experimental groups that received either diazoxide alone or a combination of diazoxide with 5-hydroxydecanoate sodium salt. Lung injuries were assessed by multiple parameters at 2 hours or 24 hours after reperfusion, including oxygenation index, wet/dry weight ratio of transplanted lungs, lung morphology, as well as measurements of myeloperoxidase, malondialdehyde, total antioxidant capacity, tumor necrosis factor-α, and interleukin-6.

Results. Compared with the sham group, the 2 control groups revealed significant changes among most parameters of lung injury measured at either 2 hours or 24 hours after reperfusion. The extent of the changes was dramatically reduced by the administration of diazoxide. Importantly, the protective effect of diazoxide was almost completely reversed by co-administration of 5-hydroxydecanoate sodium salt, a selective blocker of mitoK_{ATP}.

Conclusions. These data provide evidence for substantial protective effects of diazoxide in an in vivo rat lung IRI model. Pharmacological modulation of mitoK_{ATP} may be a potential strategy to reduce IRI-induced primary graft failure in lung transplantation.

LUNG transplantation is considered to be the final treatment effective for end-stage lung diseases.[1,2] However, despite significant improvements in surgical skills, lung preservation, and immunosuppressive strategies, primary graft failure remains a significant issue in this field, especially compared with other organ transplantations, such as the liver, kidney, and heart.[3,4] A well-documented single dominant cause of primary graft failure is ischemia-reperfusion-induced lung injury (IRI), which is characterized by nonspecific pulmonary alveolar damage, lung edema, and hypoxemia within the first 72 hours after transplantation.[5] IRI represents the complex result of multiple events associated with cold ischemic storage of donor lungs; oxidative stress, intracellular calcium overload, apoptosis, and release of inflammatory mediators play critical roles.[5] Given a pivotal role of mitochondria in these molecular events, it is reasonable to hypothesize that modulation of functional mitochondrial enzymes may improve the clinical results of IRI. In this setting, mitochondrial adenosine-5′-triphosphate (ATP)-sensitive potassium channels (mitoK_{ATP}) represent an attractive target. The mitoK_{ATP}, first discovered in 1991, shows K^+ channel activity similar to surface K_{ATP} channels.[6] It has been proposed to be a major effector of autoprotective mechanisms, known as ischemic precondi-

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Table 1. Description of 5 Groups in This Study

Group	Definition	Surgery	Injection	Time Points After the Reperfusion 2 h	24 h
1	Control	Sham operation	None	n = 6	n = 6
2	Control	Transplantation	NaCl	n = 6	n = 6
3	Control	Transplantation	DMSO	n = 6	n = 6
4	Experimental	Transplantation	DA	n = 6	n = 6
5	Experimental	Transplantation	DA + 5-HD	n = 6	n = 6

Note: There is a sham operation group, and 4 transplantion groups (NaCl, DMSO, DA, and DA + 5-HD). Each group was further divided into 2 subgroups representing 2 time points, 2 hours and 24 hours after the reperfusion.

tioning (IPC), in which a brief period of ischemia provides protection against subsequent longer ischemic periods.[7,8] Using a well-established lung transplantation model, we investigated the putative interference of mitoK_{ATP} on IRI through application of diazoxide (DA) and 5-hydroxydecanoate (5-HD), the selective opener and blocker, respectively, of mitoK_{ATP} channels.[9]

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200–250 g) were purchased from Sina-British SIPPR/BK Lab Animals Co. (Shanghai, China). The experimental protocol was approved by our Committee of Animal Care. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Lung Transplantation

Left unilateral lung orthotopic transplantation was performed as detailed previously.[10,11] Rats were randomly assigned into 5 groups (Table 1). Group 1 were the sham-operated group, undergoing a left thoracotomy without transplantation. Groups 2 and 3 received an intraperitoneal (i.p.) injection of either 0.5 mL of 0.9% NaCl or dimethyl sulfoxide (DMSO) prior to lung transplantation, respectively. DA (Sigma, St. Louis, Mo, United States; 5 mg/kg) was dissolved in 0.5 mL DMSO and was administered i.p. to rats in Group 4. Group 5 hosts were injected i.p. with 5-HD sodium salt (Sigma; 5 mg/kg), which was dissolved in 0.5 mL of 0.9% NaCl at 15 minutes prior to the DA injection.

For each group, 2 times—2 hours and 24 hours—after the reperfusion when applicable, were chosen for detailed examination of physiological dynamics associated with IRI. Rats assigned the 24-hour time were administered DA or DA-5-HD every 6 hours after reperfusion, seeking to maintain efficient blood concentrations based on the lung pharmacokinetics.[12]

Sample Collection

At 2 hours postreperfusion, blood was sampled from the right carotid artery (RCA) using a 22-gague deep venous catheter after 10 minutes of ventilation, followed by immediate blood gas analysis. Blood sampling from the left pulmonary vein (LPV) was achieved through a left thoracotomy. Serum samples from the RCA were stored at −80°C until use. The animals were then exsanguinated and the entire heart-lung block was removed. Transplanted lungs were sliced into 3 parts that were snap-frozen in liquid nitrogen and stored at −80°C. In animals assigned to the 24-hour postreperfusion groups, tracheal intubation was removed upon the recovery of spontaneous breathing. The animals were then returned to single cages with an oxygen supply

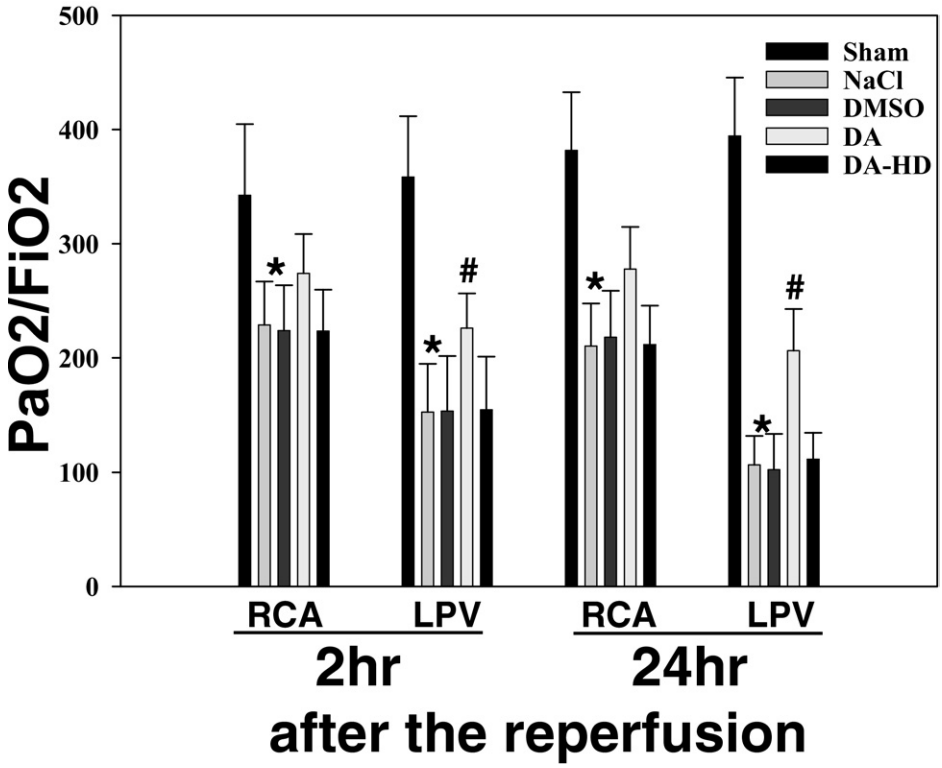

Fig 1. Comparison of PaO_2/FiO_2 among the 5 groups. Values are expressed as mean ± SD (n = 6). The PaO_2/FiO_2 was significantly decreased in the control groups (NaCl and DMSO) than the sham group (*, $P < .05$). The administration of DA reversed the decrease of PaO2/FiO2 with statistical significance seen in the LPV (#, $P < .05$).

for the first 12 hours. At the time of sampling, animals were anesthetized again and ventilated for 10 minutes, followed by the sampling procedure as described above. Sampling from the LPV was more difficult due to the expected adhesions.

Measurements

Oxygenation Index (PaO_2/FiO2). Oxygenation index was measured by blood gas analysis of blood samples from either the RCA or the LPV.

Wet/dry Weight Ratio of Transplanted Lungs. As described above, transplanted left lungs removed at either 2 hours or 24 hours after the reperfusion were sliced into 3 parts. After the upper one third was weighed immediately, it was dried at 56°C for 72 hours to calculate the wet/dry weight ratio (W/D).

Lung Morphology. A tissue sample from the middle of the transplanted lung was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. The severity of lung injury was scored based on pulmonary alveolar and interstitial edema, neutrophil infiltration, and bleeding.[13] Histological scores of 1, 2, 3, 4, or 5 were assigned to specimens with (1) no or very minor, (2) modest and limited, (3) intermediate, (4) widespread or prominent, and (5) widespread and most prominent injury, respectively.[13] For each lung sample, the final value was the mean of the scores for the 3 separate slides.

Myeloperoxidase, Malondialdehyde, and Total Antioxidant Capacity Measurement. Myeloperoxidase (MPO), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) were quantitated in serum and lung tissue samples using commercial kits from Jiancheng Bioengineering Research Institute (Nanjing, China). MDA was measured using the thiobarbituric acid method. MPO and T-AOC were determined with the colorimetric method as described by the supplier. MDA and T-AOC were expressed as nanomoles and units per milligram protein (lung) or per milliliter serum, respectively, whereas MPO was calculated as the units per milligram wet tissue film.

Cytokine Measurement. Total protein concentration was determined using the bicinchoninic acid method.[14] Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured in serum (pg/mL) and lung tissue (pg/mg) samples using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minn, United States). Frozen lung tissues were homogenized in 0.9% NaCl (4°C) to generate a 10% homogeneous suspension. After a brief centrifugation, 200 μL of supernate was used for the ELISA.

Statistical Analysis

All parameters were expressed as mean values with standard deviations (SD). Comparisons among multiple groups were performed using one-way analysis of variance. The Bonferroni post hoc test was used to compare any 2 groups. The analysis of semiquantitative histological data used the nonparametric Mann-Whitney test. $P < .05$ was considered significant. To avoid a type I statistical error, $P < .025$ was considered to be statistically significant for triple-group comparisons. All statistical analyses were performed using the SPSS package, version 15.0 (Chicago, Ill, United States).

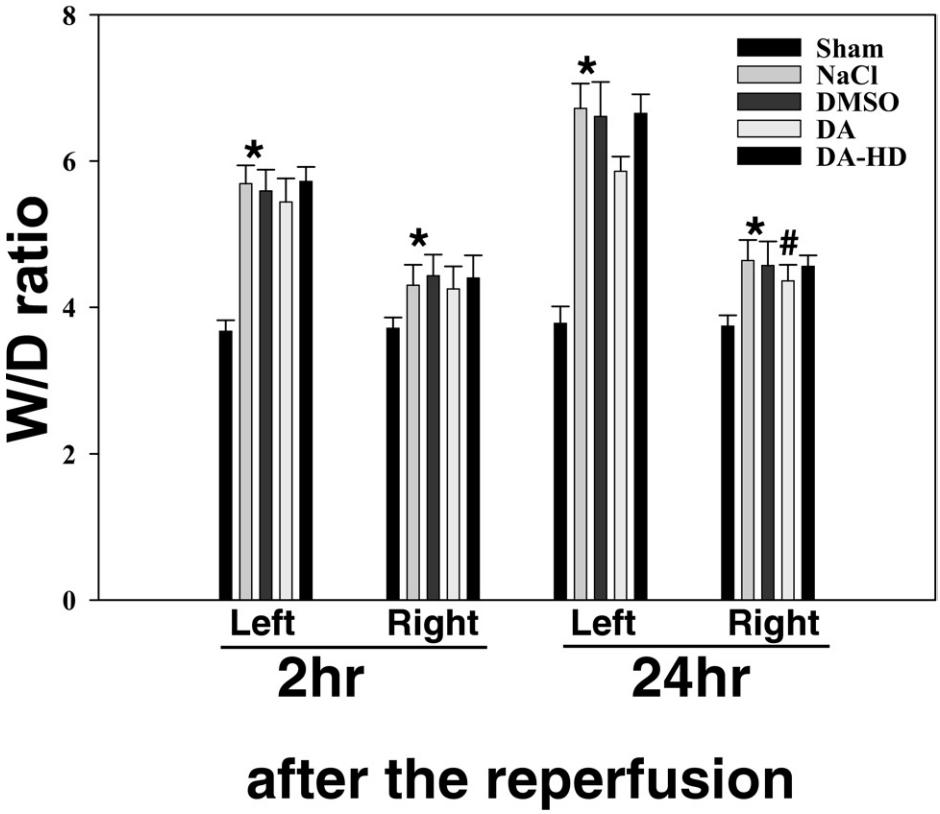

Fig 2. Lung W/D ratios between the experimental and control groups. Values are expressed as mean ± SD (n = 6). In comparison with the sham group, the W/D ratio was significantly higher in the control groups (NaCl and DMSO) (*, $P < .05$). The administration of DA showed a significant decrease of W/D ratio only in the left lung at 24 hours after the reperfusion (#, $P < .05$).

RESULTS

Transplantation Surgery

Lung transplantation was successfully performed in 96/109 rats (48 donors and 48 recipients) with an 88% success rate, which was similar to that in our previous report.[11] No single dominant factor was identified to be responsible for transplant failure. Twelve rats in the sham group underwent left thoracotomy without transplantation. Thus, a total of 108 rats were evaluated in this study.

Effect of DA on Oxygenation Index (PaO_2/FiO2)

Under a 100% inspired oxygen concentration, the sham group showed similar PaO_2/FiO2 levels between the RCA and the LPV at both 2 hours (342.3 ± 62.4 vs 358.3 ± 53.3; $P > .05$) and 24 hours post surgery (381.7 ± 51 vs 394.2 ± 51.3; $P > .05$). In comparison to the sham group, the PaO_2/FiO2 level was significantly decreased at either 2 hours or 24 hours after the reperfusion among the other 4 groups (Fig 1). The NaCl and DMSO groups showed much higher PaO_2/FiO2 values of the RCA than the LPV at both time points. The difference was dramatically minimized after administration of DA, which also yielded an increased PaO_2/FiO2 is the RCA (Fig 1. The effect of DA on PaO_2/FiO2 levels was almost completely counteracted by administration of 5-HD, as shown by similar levels among NaCl, DMSO, and DA-5-HD groups (Fig 1).

Effect of DA on Lung W/D Ratios

At both times apparently lower W/D ratios in either the left or right lung were observed in the sham compared with all other groups (Fig 2). In comparison with the NaCl and the DMSO groups, hosts administered DA showed significantly decreased W/D ratios only in the left lung at 24 hours after the reperfusion (5.86 ± 0.2 vs 6.72 ± 0.34 or 6.61 ± 0.47; $P < .05$). The significance was lost after application of 5-HD (6.65 ± 0.26 vs 6.72 ± 0.34 or 6.61 ± 0.47; $P > .05$; Fig 2). In addition, except for the DA group the transplanted left lung displayed higher W/D ratios at 24 hours than at 2 hours after reperfusion (Fig 2).

Morphological Evaluation of Transplanted Lungs

Transplanted lungs displayed obvious injuries as evidenced by alveolar collapse, interstitial vascular dilation and congestion, widened alveolar septa, pulmonary edema, and local hemorrhage (Fig 3). Accordingly, semiquantitative morphological scores were significantly higher among the transplanted (NaCl, DMSO, and DA-5-HD) than the sham group at both time points ($P < .025$; Fig 3). All symptoms of lung injury in the DA group were alleviated as documented by reduced semiquantitative morphological scores, 4.50 ± 1 and 6.08 ± 0.74 at both 2 hours and 24 hours after reperfusion.

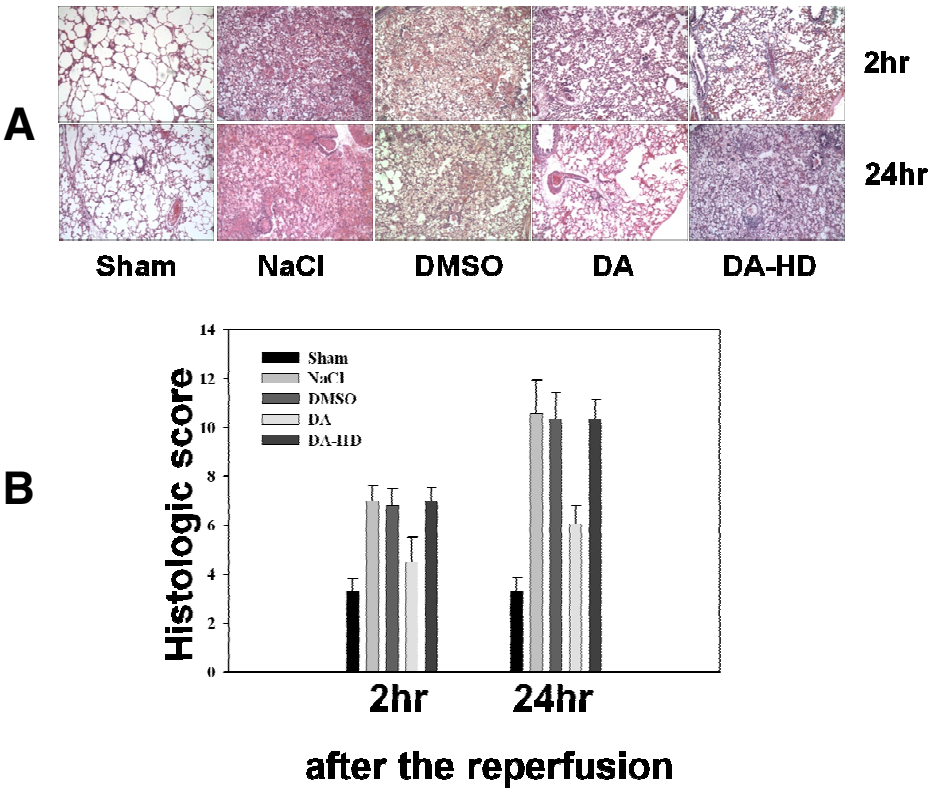

Fig 3. Morphological evaluation of lung injury. **(A)** Representative lung sections of the 5 groups stained with hematoxylin and eosin (20×). **(B)** Histological scores for all 5 groups (mean ± SD). Treatment with DA significantly decreased the histological injury score at 24 hours compared with the NaCl and DMSO control groups, and this effect was blocked by the coadministration of HD ($P < .025$).

Effect of DA on the Changes of MDA, MPO, and T-AOC After Transplantation

Compared with the sham group, the transplanted control groups (NaCl and DMSO) showed significant increases in both MPO and MDA in both the serum and the transplanted lungs. Within the same group, the level of MDA decreased at 24 hours after reperfusion, whereas MPO displayed a steady increase (Fig 4). DA administration facilitated recovery of both parameters to the levels observed among the sham group. Again, the DA effect was almost completely blocked by the application of 5-HD, showing similar levels to the control groups (NaCl and DMSO; Fig 4). Like MDA and MPO, the T-AOC actually showed the same changes but in reverse. The levels of T-AOC were significantly decreased among the transplanted groups (NaCl and DMSO) versus the sham group. DA and 5-HD played similar roles as seen with MDA and MPO (Fig 4).

Alteration of Cytokines in Transplanted Rats

At both time points, serum levels of TNF-α and IL-6 were slightly higher among transplanted groups (NaCl and DMSO) versus the sham group (Fig 5). However, the tissue expressions of both cytokines were significantly increased at 2 hours versus a lesser extent at 24 hours posttransplantation (Fig 5). Pretreatment with DA resulted in an apparent reduction of both TNF-α and IL-6 compared with the control groups (NaCl and DMSO; Fig 5). Finally, controls and DA-5-HD groups showed similar levels of both TNF-α and IL-6, indicating the expected blockade by 5-HD on DA (Fig 5).

DISCUSSION

We investigated potential roles of both DA and 5-HD on IRI in the present experimental rat lung transplantation model. The 5 experimental groups consisted of a sham group and 2 control groups that received NaCl or DMSO (Table 1). DMSO was the solvent for both DA and 5-HD; it showed similar results as those observed in the NaCl group.

Experimental IRI was induced by placing the expanded donor left lungs in low-potassium dextran glucose at 4°C without a flush for 4 hours prior to the transplantation.[11] Lung injuries were assessed by multiple parameters that are mutually linked in mechanism, reflecting physiological and functional alterations of transplanted lungs. For instance, one of the essential pathogenic manifestations of IRI is increased pulmonary capillary permeability that results in excessive intravascular exudation, therefore increasing lung W/D ratios as well as functionally decreasing the oxygenation index (PaO_2/FiO2). MDA is a major product of lipid peroxidation, which results from the interaction between lipid components of cellular membranes and reactive oxygen species (ROS).[15] The formation of ROS, the so-called oxidative stress, is a basic characteristic of IRI with profound impact on cellular injuries, ranging from increased permeability to cell lysis.[16] The MDA is thus an indirect marker to evaluate lung injury. Similarly, T-AOC measures the activities of intrinsic enzymatic antioxidants, including

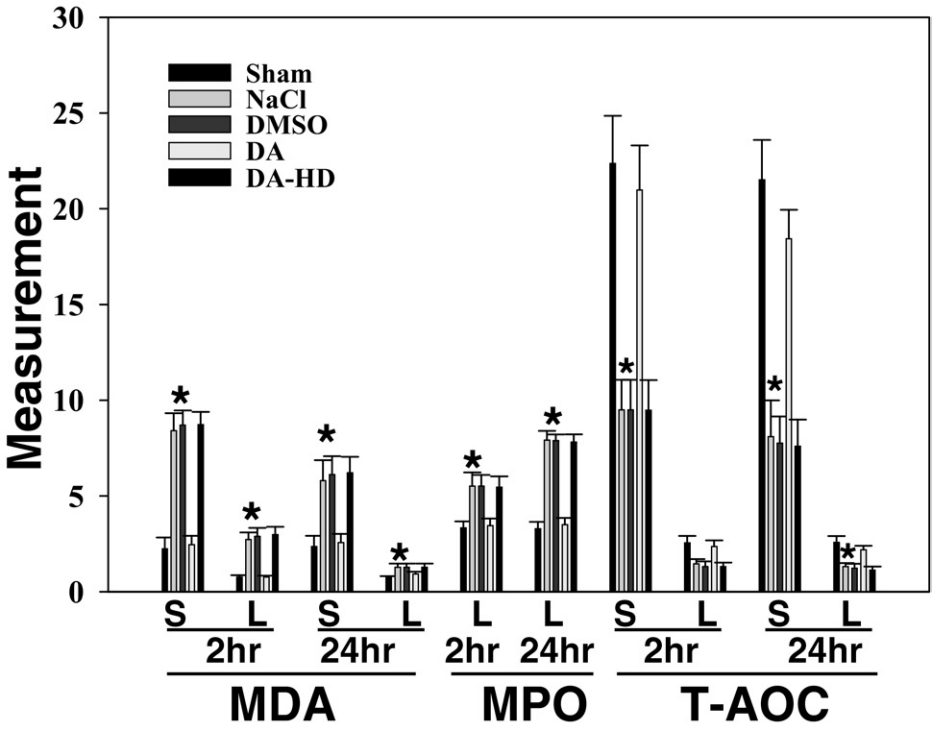

Fig 4. Dynamic changes of the abundance of MDA, MPO, and T-AOC. Values are expressed as mean ± SD (n = 6). The control groups (NaCl and DMSO) had significantly increased (MDA and MPO) or decreased (T-AOC) measurements in comparison with the sham group (*, $P < .05$). These alterations were minimized upon the administration of DA ($P < .05$). Abbreviations: S, serum; L, left lung.

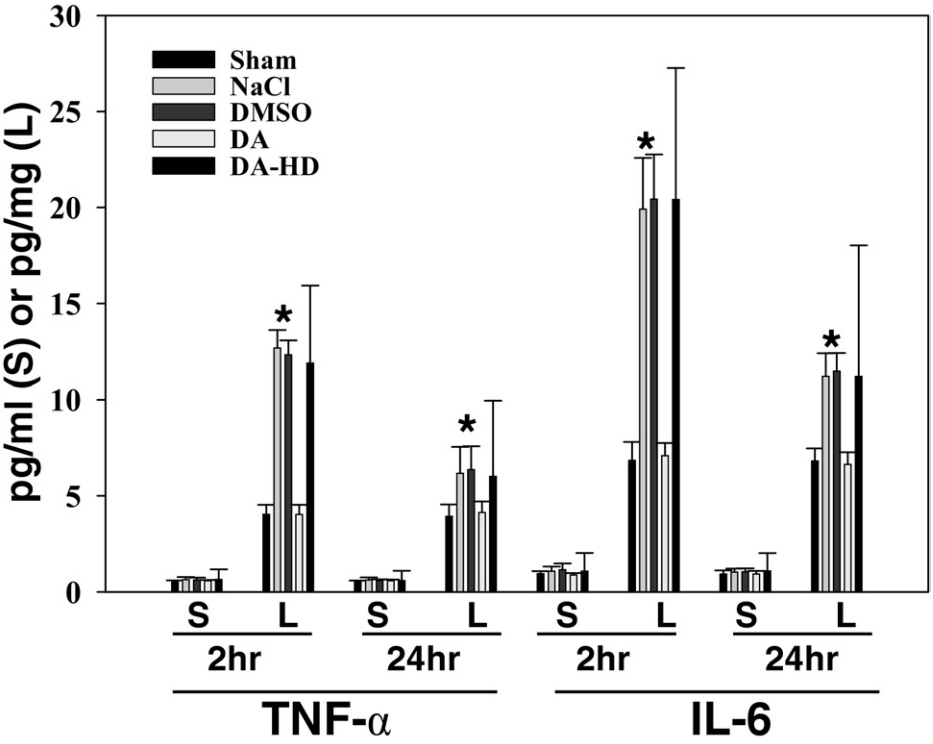

Fig 5. Cytokine expression in the experimental and control groups. Differential expression of TNF-α and IL-6 was mainly seen in left lung (L) but not in serum (S). Star indicates statistical significance ($P < .05$) when comparing the control group (NaCl and DMSO) with the sham group ($P < .05$).

superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), which bind hydroxyl radicals, the most unstable component of ROS. Therefore, the T-AOC is also an indicator of lung injury. MPO is a peroxidase, the most abundant lysosomal protein stored in azurophilic granules of the neutrophil. The amount of the MPO is closely associated with neutrophil gathering and infiltration. Finally, rapid release of pro- and anti-informmatory cytokines, such as TNF-α and IL-6 examined in this study, is a general phenomenon in the reperfusion setting after ischemia of most solid organs including the lung.[17]

Compared with the sham group, the 2 control groups showed significant changes either increasing-(morphological scores, W/D ratio, MDA, MPO, TNF-α, and IL-6) or decreasing (PaO_2/FiO2 and T-AOC) at 2 hours and 24 hours after reperfusion (Fig 1–5). Within the control groups (NaCl and DMSO), morphological examinations showed an aggravated trend at 24 hours after transplantation (Fig 3), which was accompanied by corresponding changes among other parameters except for MDA, TNF-α, and IL-6 (Fig 1, 2, 4, and 5), whose serum or tissue levels of MDA decreased at 24 hours compared to 2 hours after reperfusion. Thus MDA, an indirect marker for lung injury, does not fully reflect the dynamic pathogenic process of transplanted lungs. In contrast to lung tissues, both TNF-α and IL-6 serum levels were less different between the 2 times after the reperfusion, suggesting a local origin and retention within transplanted lungs. The autocrine and paracrine pathways of cytokines may play roles in lung IRI. Taken together, these data supported the successful construction of a rat IRI model.

The extent of the above changes in the control groups was dramatically reduced by the administration of DA (Figs 1–5). Importantly, The roles of DA in all parameters assessed in the study were completely reversed by subsequent administration of 5-HD. These results were consistent with previous reports showing protective effects of the mitoK_{ATP} in the IPC of heart and brain as well as in the IRI of various organs, including kidney, liver, and lung.[18–22] In the latter case, Fukuse et al used an ex vivo rat lung IRI model with administration of pinacidil, an opener of both mitoK_{ATP} and surfaceK_{ATP}.[22] To this point, our study represents the first report to examine the role of mitoK_{ATP} opening in an in vivo lung IRI model.

The pharmacological roles of both DA and 5-HD may not solely depend on the mitoK_{ATP}. In heart IPC models, the metabolic effects of DA and 5-HD were responsible for protective and inhibitory actions, respectively.[23] Thus, both DA and 5-HD may have alternative mechanisms contributing to both IPC and IRI models.[9,24,25] Our observations in this setting showed the protective roles of DA in an in vivo lung IRI model, providing insight for further investigation including potential clinical applications.

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