

# Lung Injury after In Vivo Reperfusion

## Outcome at 27 Hours after Reperfusion

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**Background:** Although short-term findings after lung reperfusion have been extensively reported, *in vivo* animal studies have not described outcome beyond the immediate time period. Therefore, the authors evaluated lung injury 27 h after reperfusion. They also investigated whether attenuation of lung injury with the A<sub>3</sub> adenosine receptor agonist MRS3558 was sustained beyond the immediate time period.

**Methods:** In intact-chest, spontaneously breathing cats in which the left lower lung lobe was isolated and subjected to 2 h of ischemia and 3 h of reperfusion, MRS3558 was administered before reperfusion. Animals were killed 3 or 27 h after reperfusion.

**Results:** When compared with 3 h of reperfusion, at 27 h the left lower lobe showed reduced apoptosis and no change in inflammation, but increased edema. Increased edema of the nonischemic right lung and hypoxemia were observed at 27 h after left lower lobe reperfusion. Increases in phosphorylated p38 levels were found at 3 h of reperfusion compared with control lung, with further increases at 27 h. The attenuation of injury observed with MRS3558 treatment at 3 h of reperfusion was sustained at 27 h.

**Conclusions:** Lung edema may worsen hours after the immediate postreperfusion period, even though lung apoptosis and inflammation are reduced or show no change, respectively. This was associated with further increases in phosphorylated p38 levels. The nonischemic lung may also be affected, suggesting a systemic response to reperfusion. In addition, early attenuation of injury is beneficial beyond the immediate period after reperfusion. Treatment aimed at inhibiting p38 activation, such as A<sub>3</sub> receptor activation, should be further studied to explore its potential long-term beneficial effect.

PULMONARY ischemia-reperfusion (IR) may contribute to the magnitude and duration of pulmonary dysfunction

seen after cardiopulmonary bypass, pulmonary thrombolysis, and lung transplantation.<sup>1,2</sup> The pathophysiology of IR injury in the lung involves alveolar-capillary barrier leakage, interstitial and alveolar edema, neutrophil migration, tissue inflammation, and cell injury and death.<sup>1</sup>

Mechanisms of reperfusion lung injury have been extensively studied. Using different models, the events and pathways that lead to injury within the first few seconds to hours after reperfusion have been described. Studies that look beyond the first few hours, however, are scant. In human lungs after transplantation, Fischer *et al.*<sup>3</sup> found that apoptosis only became evident after reperfusion. Furthermore, the number of apoptotic cells increased with longer periods of reperfusion.<sup>3</sup> In a model of lung transplantation in rats, the time course of apoptosis after reperfusion was followed for 12 h.<sup>4</sup> The peak of apoptotic pneumocytes occurred 2 h after reperfusion, with a rapid decline thereafter. Other parameters or pathways of lung injury were not evaluated.

Eukaryotic cells possess multiple mitogen-activated protein kinase (MAPK) pathways, which can be grouped into three categories<sup>5</sup>: extracellular signal-regulated protein kinase (ERK), p38 MAPK (or p38), and c-Jun NH<sub>2</sub>-terminal protein kinases (JNK). Activation of MAPK has been postulated to be involved in mediating IR-induced lung dysfunction.<sup>6</sup> The degree of activation and types of MAPK being activated after the acute phase of reperfusion are largely unknown.

Most investigations of reperfusion injury of the lung have been performed in isolated lungs perfused with physiologic solution or in mechanically ventilated animals. In an attempt to reproduce physiologic conditions, experiments in this study were performed in closed-chest, spontaneously breathing animals. Using this *in vivo* animal model, we have recently reported that reperfusion for 3 h induced significant inflammation, edema, and apoptosis.<sup>7</sup> This was accompanied by significant increases in the expression of phosphorylated JNK and p38. In addition, the potent and highly selective A<sub>3</sub> adenosine receptor agonist MRS3558, administered before reperfusion, attenuated reperfusion lung injury and apoptosis, a protective effect associated with activation of ERK during reperfusion.

Given the important role of MAPK in reperfusion injury<sup>8,9</sup> and the lack of data regarding the fate of lung injury beyond the acute phase, the aim of the current study was to explore the extent of injury, apoptosis, and MAPK activation 27 h after lung reperfusion. In addition, we wished to explore whether attenuation of injury with

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the A<sub>3</sub> adenosine receptor agonist MRS3558 was sustained beyond the immediate period.

## Materials and Methods

### Animals

Adult cats weighing 2.9–4.3 kg were used in this investigation. All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Hebrew University–Hadassah School of Medicine (Jerusalem, Israel) and with the approval of the Institutional Animal Care and Use Committee.

### Materials

All chemicals were obtained from sigma (Sigma-Aldrich Israel, Ltd., Rehovot, Israel), unless specified. MRS3558 ((1'R,2'R,3'S,4'R,5'S)-4-[2-chloro-6-(3-chlorophenylmethyl)amino]purin-9-yl)-1-(methylaminocarbonyl)bicyclo[3.1.0]-hexane-2,3-diol) was prepared as described.<sup>7</sup>

### In Vivo Animal Model

A standard reperfusion lung model of injury in intact-chest, spontaneously breathing cat was used, as described previously in detail.<sup>10–12</sup> Briefly, in barbitol-anesthetized cats (20 mg/kg intravenously), with the aid of fluoroscopy, a specially designed 6-French triple-lumen catheter was advanced from the left external jugular vein into the lobar artery of the left lower lobe (LLL). Also, with the use of fluoroscopy, a 4-French bronchial blocker was inserted into the LLL bronchus. After heparinization, the LLL was perfused at 35 ml/min with blood withdrawn from the aorta through a catheter in the femoral artery, using a Harvard peristaltic pump. The LLL was isolated by distending a balloon with contrast dye on the LLL arterial catheter. After a 1-h period of stabilization, ischemia of the LLL was induced by discontinuing the Harvard peristaltic pump for 2 h (ischemia period), and the perfusion circuit was then attached to a femoral vein catheter. The balloon on the tip of the bronchial blocker was distended with contrast dye to block ventilation to the lobe. After 2 h of ischemia, the balloon on the bronchial blocker was deflated, the perfusion circuit was reattached to the arterial catheter in the LLL, and the LLL was reperfused (reperfusion period) for 3 or 27 h at a rate of 35 ml/min, using a Harvard peristaltic pump, as described above. Hemodynamic measurements and arterial blood gases were obtained before ischemia, after 2 h of ischemia, and after 10 min, 3 h, and 27 h of reperfusion. Anesthesia was maintained with pentobarbital given as an intravenous continuous infusion of 0.1 mg · kg<sup>-1</sup> · min<sup>-1</sup>, and animals received 2 ml · kg<sup>-1</sup> · h<sup>-1</sup> lactated Ringer's solution intravenously. All animals lay on a warming blanket throughout the experiment, and their temperature was kept in the range of 36.6–37.3. At the end of the experiment, animals received an overdose of pentobarbital sodium (30 mg/kg).

### Experimental Protocol

After a 1-h period of stabilization, cats were randomly assigned to six groups (all studies were new and not part of previously published studies):

I and II, nonischemic groups (n = 11/group): The LLL was perfused for 3 or 27 h, respectively (no ischemia). III and IV, IR groups (n = 11/group): Animals were subjected to ischemia and reperfusion of the LLL. Reperfusion times were 3 and 27 h, respectively. V and VI, MRS3558 groups (n = 6/group): The selective A<sub>3</sub> adenosine receptor agonist MRS3558, 100 μg/kg, was administered systemically as an intravenous bolus 1 h after the beginning of the ischemic period. The dose of the A<sub>3</sub> adenosine receptor agonist MRS3558 and its pretreatment time were selected based on previous *in vivo* studies in cats.<sup>7</sup>

At the end of the experiment, lung tissue samples were snap frozen in liquid nitrogen (for terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling [TUNEL] and Western blot assays, lung myeloperoxidase activity) or embedded in paraffin (for histologic examination), whereas the remainder was used for determination of lung wet:dry weight ratio (see next paragraphs for full description of assays used to evaluate these parameters).

### Assessment of Injury and Apoptosis

For light microscopy, samples of lung tissue were embedded in paraffin, cut into 4-μm slices, and stained with hematoxylin and eosin. The slides were coded and examined in a blinded manner by a single examiner. Fifty microscopic fields at ×40 magnification were examined in each section, and the total number of alveoli in the 50 microscopic fields was scored. The severity of alveolar injury was assessed according to the percentage of injured alveoli as defined previously.<sup>7,10–12</sup> Samples of excised lung tissue were also snap frozen in liquid nitrogen and stored at –70°C for determination of lung myeloperoxidase.<sup>7</sup> The remainder of the LLL was used for determination of lung wet:dry weight ratio, after sequential weighing demonstrated maximal dehydration at 80°C.

Apoptosis was assessed through a TUNEL assay as described previously.<sup>7,11,12</sup> This was performed on formaldehyde-fixed lung sections using the Deadend Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. Lungs were also tested after the reperfusion period for activated caspase-3 expression, as outlined previously in detail.<sup>7,11,12</sup> To allow comparison between groups, data were shown as percent density of bands *versus* nonischemic (group I) lungs.

### Western Blotting for JNK, p38, and ERK

Lung tissue samples were analyzed for levels of the activated, phosphorylated forms of JNK, p38, and ERK1/2 by Western blotting. Kits were purchased from

Cell Signaling Technology (Beverly, MA) and used per the manufacturer's protocol. In brief, lung specimens were snap frozen in liquid nitrogen. Protein (50  $\mu$ g) from the 10,000g supernatant of lung specimens was resolved on 12% sodium dodecylsulfate polyacrylamide gels and transferred to nitrocellulose membranes (Osmotics Inc., Westborough, MA). The membrane was blocked with 5% nonfat dry milk and immunoblotted with specific antibodies for each of the phosphorylated forms of MAPK (1:200) or actin C-11 (Santa Cruz Biotechnology, Santa Cruz, CA) for 8 h at 4°C and then incubated with a 1/1,000 dilution of horseradish peroxidase-conjugated secondary antibody (Sigma, Rehovot, Israel) for 2 h at room temperature. Quantitative analysis of the band density was performed using simultaneously blotted actin density to correct for protein content. Relative band intensities, expressed in arbitrary units of phospho-JNK, phospho-p38, and phospho-ERK to control (group I, no ischemia), were assessed by densitometry using a ChemImager 4000 Imaging System (Alpha Innotech Corp., San Leandro, CA).

#### Measurement of Serum TNF- $\alpha$

Blood samples were spun at 1,000g for 10 min, and the serum was decanted and stored at -80°C. Levels of tumor necrosis factor (TNF)- $\alpha$  were measured with a TNF- $\alpha$  enzyme-linked immunosorbent assay kit according to the manufacturer's instruction (Chemicon International, Temecula, CA) before ischemia, before reperfusion, and every 3 h thereafter.

#### Cell Counts and Biochemical Studies of BAL Fluid

At the end of reperfusion, bronchoalveolar lavage (BAL) was obtained by instilling 3 ml saline (three times, 9 ml total) through the LLL bronchial blocker. Recovery was approximately 90% of instilled volume and did not differ significantly between groups. BAL was centrifuged (150g, 10 min, 4°C), the cell pellet was resuspended in Eagle minimal essential medium (GIBCO, Grand Island, NY), and total and differential cell counts obtained were quantified using a hemocytometer-trypan blue solution and Wright Giemsa-stained cytocentrifuge preparations, respectively. Cell-free BAL was frozen at -80°C for subsequent analysis. Total protein determinations were made by Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL). The supernatant of the first lavage was used for interleukin (IL)-1 $\beta$  protein measurement. IL-1 $\beta$  protein levels in BAL were measured in BAL supernatant of all groups, using a commercially available sandwich enzyme-linked immunosorbent assay kit as specified by the manufacturer (Biosource International, Nivelles, Belgium). The sensitivity of the cytokine enzyme-linked immunosorbent assay is 7 pg/ml.

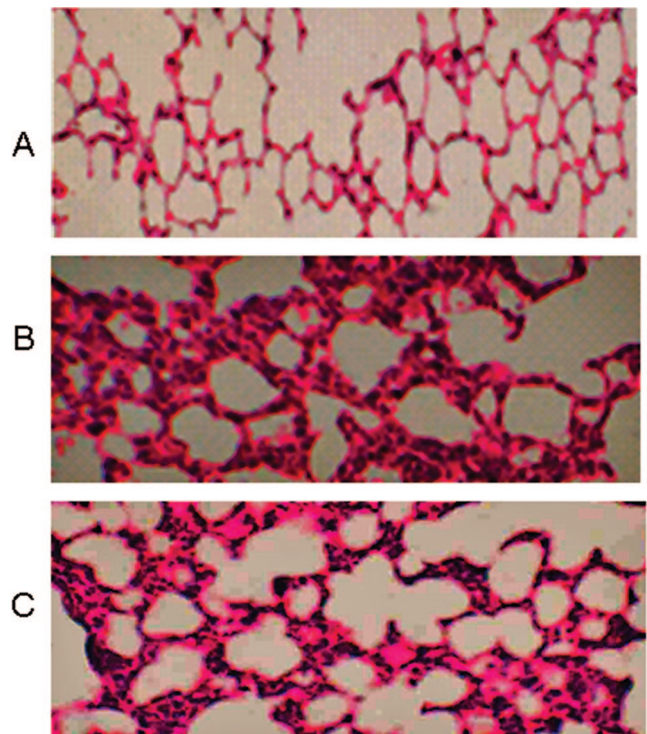


Fig. 1. Photomicrographs of histologic findings in the animal lungs at 27 h of perfusion (group II; A). Normal alveolar structure; 3 h of reperfusion (group III; B). Severe inflammation; 27 h of reperfusion (C). Severe inflammation and edema. Hematoxylin and eosin staining; original magnification  $\times$ 100.

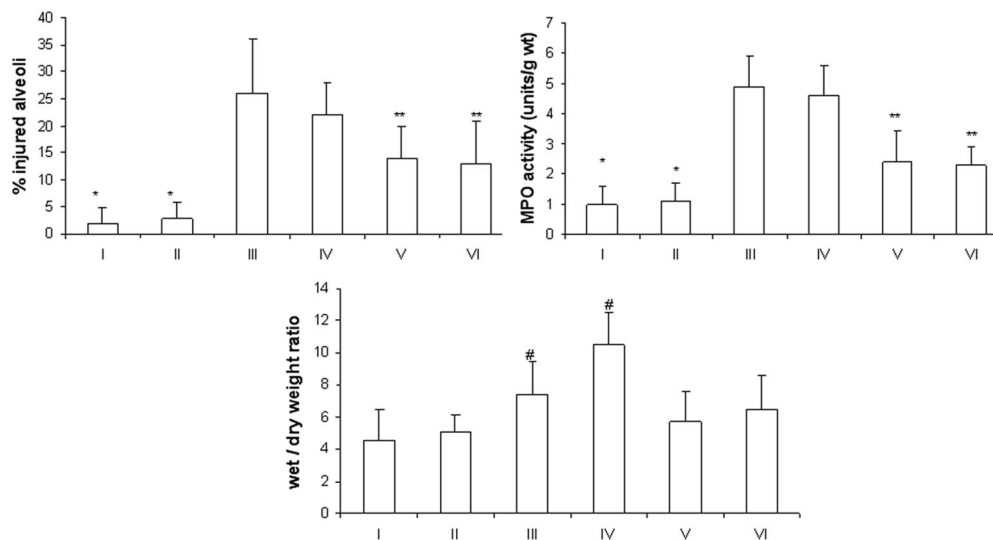
#### Statistical Analysis

Data were analyzed with one-way analysis of variance with Bonferroni correction for multiple comparisons between groups. Differences were considered significant at  $P < 0.05$ . Results are presented as mean  $\pm$  SD. Data were analyzed using SigmaStat (Jandel, San Rafael, CA).

## Results

#### IR Lung Injury and Apoptosis

*In vivo* perfusion of the LLL for 27 h was well tolerated (group II). Lung injury and apoptosis parameters (figs. 1-3) as well as arterial blood gas (table 1) did not change significantly over time and did not differ between the two non-ischemic groups (I and II). As in previous studies,<sup>7,11</sup> animals had increased inflammation (as assessed by percentage of injured alveoli and myeloperoxidase activity) and apoptosis (as assessed by percentage of TUNEL-positive cells and expression levels of activated caspase-3 protein) after 3 h of reperfusion (group III). After 27 h of reperfusion (group IV), no significant changes were found in lung inflammation when compared with 3 h of reperfusion (group III), whereas markers of apoptosis decreased by approximately 40% ( $P < 0.05$ ) of the corresponding 3-h reperfusion values (fig. 3). Significant lung edema developed at 27 h of reperfusion as evidenced by lung histology (fig. 1C) and wet-to-dry weight ratio of the LLL (fig. 2),



**Fig. 2.** Percentage of injured alveoli, tissue myeloperoxidase (MPO) activity, and lung tissue wet:dry weight ratio, in the left lower lobe of the different groups. Values shown are mean  $\pm$  SD. The groups were as follows: I, nonischemic group, 3 h perfusion,  $n = 11$ ; II, nonischemic group, 27 h perfusion,  $n = 11$ ; III, ischemia–reperfusion, 3 h reperfusion,  $n = 11$ ; IV, ischemia–reperfusion, 27 h reperfusion,  $n = 11$ ; V, MRS3558 administered before reperfusion, 3 h of reperfusion,  $n = 6$ ; and VI, MRS3558 administered before reperfusion, 27 h of reperfusion,  $n = 6$ . \* $P < 0.01$  versus groups III–VI. \*\* $P < 0.05$  versus groups I–IV. # $P < 0.05$  versus the other groups.

which more than doubled its value when compared with control (group IV *vs.* group II). This was accompanied by a significant decrease in partial pressure of oxygen (to  $64 \pm 14$  mmHg in group IV compared with  $97 \pm 16$  mmHg in group II;  $P < 0.05$ ) and acidosis (table 1).

We also examined the right lung after 3 and 27 h of LLL control perfusion (groups I and II) and reperfusion (groups III and IV). Three and 27 h of control perfusion of the LLL did not result in any right lung injury or edema

(fig. 4, groups I and II). With LLL reperfusion, no inflammation or edema of the right lung was evidenced after 3 h (as assessed by percentage of injured alveoli, myeloperoxidase activity, and wet:dry weight ratio). Significant inflammation developed at 27 h (fig. 4, group IV). However, it was significantly lower than that found in the reperfused LLL after either 3 or 27 h. In addition, after 27 h, significant edema developed (fig. 4). Examination of the right lung did not reveal apoptotic cell death in any of the groups (data not shown).

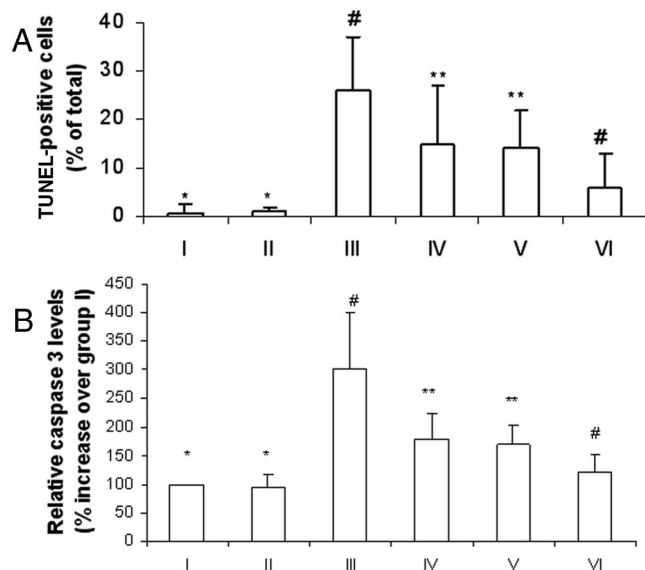
Serum levels of TNF- $\alpha$  increased after 3 h of reperfusion and peaked at 9 h (fig. 5). Thereafter, there was no significant change in serum TNF- $\alpha$  levels. Treatment with MRS3558 attenuated the increase in TNF- $\alpha$  levels with reperfusion, but it did not reach control values.

#### Activation of MAPK after IR

The temporal profile of the expression of the three MAPK pathways is shown in figure 6. Perfusion of the LLL for 27 h did not result in any changes in expression of the three MAPKs assessed. Moderate but significant increases in expression of phospho-JNK and phospho-p38 were detected 3 h after reperfusion. No increase was observed in phospho-ERK protein levels. A longer reperfusion period (27 h) resulted in a marked increase in phosphorylated p38 levels, with no change in the levels of the two other MAPK members.

#### BAL Results

Lung reperfusion resulted in significant increases in BAL total protein concentration compared with controls (groups I and II). In general, the increases tended to be significantly larger after 27 h than 3 h of reperfusion ( $P <$



**Fig. 3.** Terminal deoxynucleotidyl transferase–mediated dUTP–biotin end labeling (TUNEL)–positive cells (A), changes in activated caspase-3 expression as analyzed by Western blotting (B) in the left lower lobe of the different groups, and representative Western blot analysis of activated caspase 3 (C). Values shown are mean  $\pm$  SD. Group details are in figure 2. \* $P < 0.05$  versus groups III–VI. \*\* $P < 0.05$  versus groups I, II, III, and VI. # $P < 0.05$  versus other groups.

**Table 1. Arterial Blood Gases**

	Group					
	I	II	III	IV	V	VI
Po <sub>2</sub> , mmHg						
Baseline	99 ± 11	98 ± 13	93 ± 11	92 ± 12	99 ± 11	93 ± 9
Ischemia, 2 h			91 ± 9	95 ± 13	101 ± 13	98 ± 12
Reperfusion, 3 h	94 ± 13*	96 ± 15*	91 ± 14	94 ± 14	93 ± 17	87 ± 12
Reperfusion, 27 h		97 ± 16†		64 ± 14‡		95 ± 9
Pco <sub>2</sub> , mmHg						
Baseline	38 ± 10	38 ± 8	35 ± 4	39 ± 7	39 ± 9	40 ± 9
Ischemia, 2 h			38 ± 6	36 ± 7	36 ± 8	36 ± 6
Reperfusion, 3 h	36 ± 8*	35 ± 7	34 ± 7	36 ± 6	35 ± 7	37 ± 6
Reperfusion, 27 h		38 ± 9†		40 ± 7		39 ± 8
pH						
Baseline	7.39 ± 0.03	7.37 ± 0.02	7.38 ± 0.05	7.38 ± 0.05	7.36 ± 0.06	7.35 ± 0.06
Ischemia, 2 h			7.37 ± 0.05	7.39 ± 0.05	7.37 ± 0.03	7.39 ± 0.05
Reperfusion, 3 h	7.37 ± 0.06*	7.41 ± 0.06	7.37 ± 0.05	7.37 ± 0.05	7.41 ± 0.06	7.39 ± 0.05
Reperfusion, 27 h		7.37 ± 0.05†		7.31 ± 0.05‡		7.38 ± 0.06

Values shown are mean ± SD; n = 6–11 cats/group. The groups were as follows: I, nonischemic group, 3 h of perfusion; II, nonischemic group, 27 h of perfusion; III, ischemia–reperfusion, 3 h of reperfusion; IV, ischemia–reperfusion, 27 h of reperfusion; V, MRS3558 administered before reperfusion, 3 h of reperfusion; VI, MRS3558 administered before reperfusion, 27 h of reperfusion.

The value obtained in this group after \* 3 h of perfusion or † 27 h of perfusion in nonischemic control. ‡ Significantly (*P* < 0.05) different from previous measurements in the same group and from measurements obtained in other groups after 27 h of reperfusion.

Pco<sub>2</sub> = partial pressure of carbon dioxide; Po<sub>2</sub> = partial pressure of oxygen.

0.01). There was no difference between the two control groups (fig. 7A).

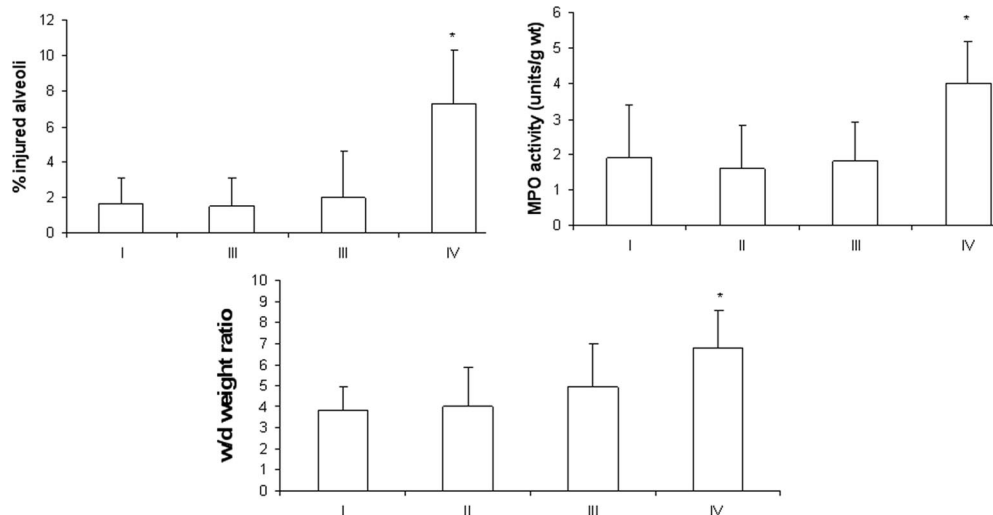
Interleukin-1β protein levels in BAL for the different groups are presented in figure 7B. IL-1β levels in BAL increased progressively with longer reperfusion period, becoming significant at 27 h compared with control groups and 3 h of reperfusion (*P* < 0.001). No significant difference in IL-1β levels was observed between 3 and 27 h of reperfusion.

Total cell counts in all study groups are shown in figure 8. No differences in total cell counts were observed between nonischemic groups (group I *vs.* group II). Total cell number in BAL increased significantly with reperfusion compared with the two nonischemic groups (I and II; *P* < 0.001). However, the cell count in BAL was

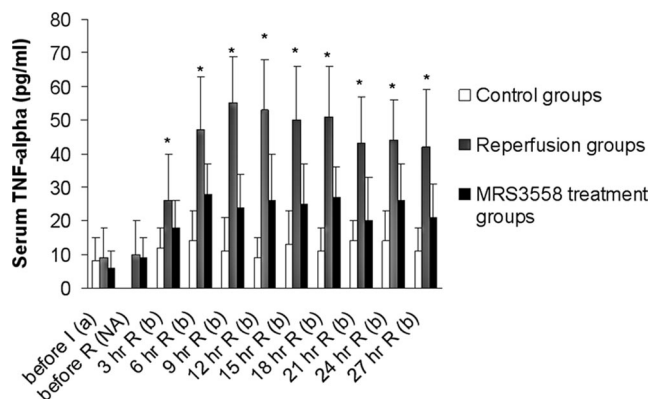
similar at 3 and 27 h of reperfusion. BAL demonstrated a significant increase in the numbers of recruited macrophages and neutrophils after reperfusion. Despite the lack of consistent change in total cells in BAL at the two time points or reperfusion, differential cell counts revealed a significant increase in the percentage of macrophages at 3 h, with subsequent decrease at 27 h of reperfusion. The decrease in macrophage cell count at 27 h of reperfusion was mirrored by a significant increase in neutrophils at 27 h of reperfusion.

*Hemodynamics and Hemoglobin/Hematocrit Data*

Mean arterial blood pressure and mean lobar arterial pressures at baseline were similar in all groups. Three



**Fig. 4. Right lobe of the different groups: percentage of injured alveoli, tissue myeloperoxidase (MPO) activity, and lung tissue wet:dry (w/d) weight ratio. Values shown are mean ± SD. Group details are in figure 2. \* *P* < 0.05 versus groups I, II, and III.**



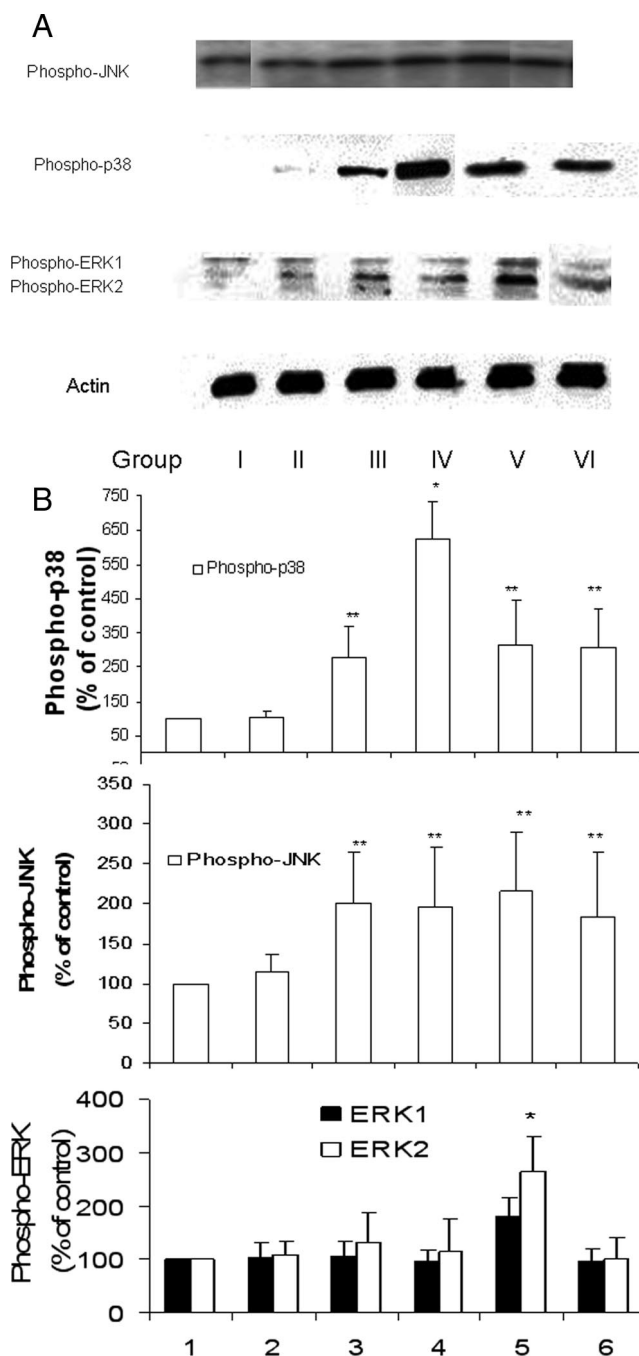
**Fig. 5.** Tumor necrosis factor (TNF)- $\alpha$  serum concentrations before ischemia (I) and reperfusion (R) and every 3 h after the beginning of reperfusion in reperfusion and MRS3558 treatment groups. Corresponding numbers for the control groups: a = after 1 h of stabilization; b = number of hours of perfusion; NA = not applicable. \*  $P < 0.05$  versus control (I and II) and treatment (V and VI) groups. With reperfusion, levels increased significantly after 3 h of reperfusion and reached a peak within 9 h. Treatment with MRS3558 attenuated the increase in TNF- $\alpha$  levels with reperfusion, but it did not reach control values. Results are expressed as absolute values and represent mean  $\pm$  SD. Group details are in figure 2. One-way analysis of variance followed by the pairwise multiple comparison Tukey test was used.

or 27 h of left lower lung lobe perfusion did not affect systemic (table 2) or lobar arterial pressures (table 3). Ten minutes after reperfusion, the lobar arterial pressure increased significantly in all groups compared with the pressures present in the same lung at baseline and after 2 h of ischemia (table 3). The increase in lobar arterial pressure observed during the reperfusion period was significantly smaller in the groups in which MRS3558 was administered (groups V and VI). This increase in lobar arterial pressure was followed by a gradual decline toward the baseline value with time. At the end of 3 and 27 h of reperfusion, lobar arterial pressure was still significantly elevated (compared with baseline) in all groups. The mean lobar arterial pressure, however, was not significantly different among the reperfusion groups at the end of either 3 or 27 h of reperfusion.

Hemoglobin and hematocrit were measured after 1 h of stabilization, after 2 h of ischemia, and after 10 min, 3 h, and 27 h of reperfusion or perfusion in control groups and did not change significantly with time; there were no differences between groups (data not shown).

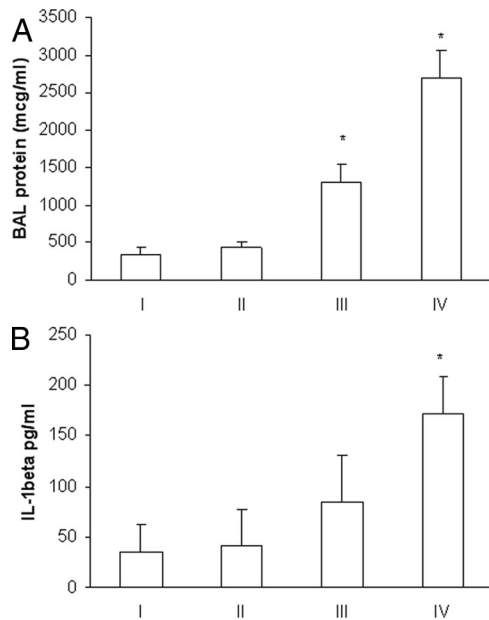
#### Long-term Effect of Systemic Administration of the $A_3$ Adenosine Receptor Agonist MRS3558

The lung-protective effects observed after a single dose administration of the  $A_3$  adenosine receptor agonist MRS3558 (given 1 h before reperfusion) were sustained after 27 h. Lung inflammation and edema were significantly attenuated as well as apoptosis (figs. 2 and 3). Arterial oxygen and carbon dioxide partial pressure were within normal range (table 1). Phosphorylated ERK



**Fig. 6.** Western blot analyses of phospho-p38, phospho-c-Jun N-terminal kinase (JNK), and phospho-extracellular signal-regulated kinase 1 and 2 (ERK1/2). (A) Representative Western blot; (B) densitometric analysis of five to six blots (mean  $\pm$  SD) expressed as a percentage of values for corresponding control tissue samples (no ischemia or reperfusion, 3 h perfusion, group I). Group details are in figure 2. Densitometry data presented are normalized to the intensity of actin bands. \*  $P < 0.01$  compared with the other groups. \*\*  $P < 0.05$  compared with the two control groups (I and II).

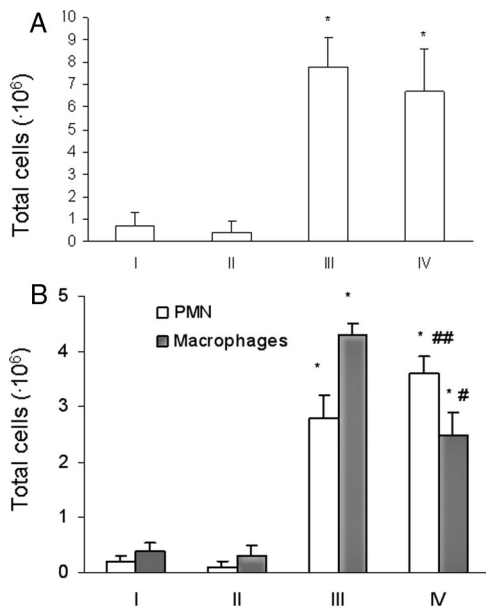
levels increased significantly with MRS3558 treatment at 3 h of reperfusion but returned to basal level after 27 h (fig. 6). In addition, phospho-p38 levels at 27 h were attenuated with MRS3558 when compared with reperfusion without treatment (group VI vs. group IV).



**Fig. 7.** Effect of reperfusion on total protein content (A) and on interleukin (IL)-1 $\beta$  protein levels (B) in bronchoalveolar lavage (BAL) from the left lower lung lobe. Amounts of total protein and IL-1 $\beta$  protein levels were quantified after 3 and 27 h of lung reperfusion (groups I and II, respectively, control groups) and lung reperfusion (groups III and IV, respectively). Values shown are mean  $\pm$  SD; n = 6/group. (A) Reperfusion caused an increase in BAL total protein levels with time of reperfusion. \* $P < 0.01$  versus the other three groups. (B) Reperfusion of 27 h (but not 3 h) led to a significant increase in IL-1 $\beta$  protein levels in BAL (\* $P < 0.05$ ).

**Discussion**

Short-term findings after lung reperfusion have been extensively reported. This is the first *in vivo* animal



**Fig. 8.** Bronchoalveolar lavage fluid analyses from the left lower lung lobe for total cell count (A) and total polymorphonuclear (PMN) and macrophage counts (B). \* $P < 0.05$  versus groups I and II. # $P < 0.05$  versus group III macrophages. ## $P < 0.05$  versus group III PMNs. Values shown are mean  $\pm$  SD; n = 6/group.

study to investigate the time course of inflammation, apoptosis, and edema of the lung till 27 h after reperfusion and to evaluate whether an intervention shown to be effective in attenuating the immediate response to reperfusion has a sustained effect. In an *in vivo* model of warm IR of a lung, we found that the development of significant apoptosis, inflammation, lung edema, and hypoxemia do not occur simultaneously. The immediate response to reperfusion is significant apoptosis, inflammation, and edema of the reperfused lung, with no effect on the nonischemic lung. With prolongation of the reperfusion period to 27 h, edema and inflammation develops in the remote lung, whereas the reperfused lung becomes more edematous with an ensuing hypoxemia. In the meantime, inflammation and apoptosis in the reperfused lung are sustained or decrease, respectively. This is associated with significant p38 activation, elevated IL-1 $\beta$  levels in BAL, and increases in BAL total protein and cell count. At 27 h after reperfusion, the attenuation of injury and apoptosis observed with MRS3558 was sustained. Our results, showing increased hypoxemia associated with increased injury with time after reperfusion, are in agreement with a previous study in a lung transplantation model in rats<sup>13</sup> in which the degree of injury was found to be dramatically aggravated by the duration of reperfusion (till 24 h).

Our study extends the previous work of Stammberger *et al.*<sup>4</sup> in a model of lung transplantation in rats. These authors found that apoptosis of pneumocytes after reperfusion of the transplanted lung is a very early event that peaks at 2 h. The experiments ended after 12 h of reperfusion; by that time, the number of apoptotic cells in the lung was one fourth of that found at 2 h after reperfusion. Similarly, Fischer *et al.*<sup>3</sup> found a significant increase in apoptosis after reperfusion of the human transplanted lung that started as early as 30 min after reperfusion, with a trend toward increased levels over time during the following 120 min. The degree of apoptosis did not correlate with short-term clinical outcome measures such as partial pressure of oxygen (P<sub>O</sub><sub>2</sub>) after reperfusion, first P<sub>O</sub><sub>2</sub> in the intensive care unit, and ventilation time after surgery. The early appearance of apoptotic cells and their gradual disappearance within a few hours after the acute phase (3 h) was also reported after intestine and liver reperfusion.<sup>14,15</sup> In these studies,<sup>3,4</sup> however, other parameters of injury and their temporal relation to the time course of the apoptotic process were not evaluated. Clearly, further investigation is required to define the implications of apoptosis in the reperfusion setting and its association with the development of inflammation and edema.

In this study, we found increased edema formation and protein exudation with time. BAL cell count increased with reperfusion. Despite the fact that the elevated BAL cell count did not change with time, the proportion of cell types in BAL did show significant changes. The increased

**Table 2. Mean Systemic Arterial Pressure (mmHg)**

Group	Baseline	2 h of Ischemia	10 min of Reperfusion	3 h of Perfusion/Reperfusion	27 h of Perfusion/Reperfusion
I	117 ± 18	NA	NA	110 ± 16	NA
II	107 ± 12	NA	NA	119 ± 11	122 ± 13
III	109 ± 13	119 ± 11	117 ± 13	117 ± 8	NA
IV	111 ± 11	109 ± 17	115 ± 11	112 ± 11	122 ± 16
V	112 ± 9	119 ± 10	110 ± 12	119 ± 7	NA
VI	114 ± 13	120 ± 12	116 ± 13	113 ± 9	126 ± 13

Mean systemic arterial pressure at baseline (15 min into stabilization period), after 2 h of ischemia, 10 min of reperfusion (groups III–VI), 3 h of perfusion (group I) or reperfusion (groups III and V), and 27 h of perfusion (group II) or reperfusion (groups IV and VI). Data are presented as mean ± SD. Group details are in figure 2.

NA = not applicable.

number of macrophages observed after 3 h of reperfusion was partly replaced by a dominance of recruited neutrophils. It may be that proteins produced by macrophages, such as IL-1 $\beta$  (which was found to be elevated) or macrophage inflammatory protein 2 (which was not assessed), are in part responsible for the observed phenomena. Indeed, in a rat model of warm lung reperfusion, Eppinger *et al.*<sup>16,17</sup> noted a bimodal pattern of increased pulmonary microvascular permeability and edema formation, an early phase that developed within minutes after reperfusion and was dependent on activated pulmonary macrophages, and a late phase that was more dependent on products from activated neutrophils. Neutropenia had no protective effect against edema formation at 30 min of reperfusion, but there was a significant reduction in lung injury at later stages. This suggests two different mechanisms for lung injury that are time dependent. It is interesting that the permeability index in the study by Eppinger *et al.*<sup>16</sup> actually decreased to lower levels after 1 h of reperfusion compared with 30 min, before a subsequent increase. This is an important finding because it may indicate that some endothelial protective effect may be present early after reperfusion. Biphasic time course behavior during reperfusion of the lung was also shown for expression of the intercellular adhesion molecule 1,<sup>18</sup> with an initial decrease at 30 min of reperfusion and a late-phase increase at 3 h. Intercellular adhesion molecule 1 is known to contribute to endothelial permeability and edema formation.<sup>17</sup> In this study,<sup>18</sup> the late up-regulation of intercellular adhesion molecule 1 protein

level temporally coincided with edema formation. Because we evaluated the lungs only at 3 and 27 h of reperfusion, we cannot rule out changes in edema formation at earlier stages of reperfusion. Future studies are necessary to evaluate whether different mechanisms modulate early *versus* late edema formation or whether it represents the course of a continuous injury that overwhelms compensatory capabilities of the endothelium.

Our results confirm and extend previous studies<sup>13,19–21</sup> by showing contralateral lung injury after reperfusion; however, in contrast to the reperfused lobe, the magnitude of inflammation and edema in the nonischemic lung was significantly lower and with no evidence of significant apoptosis. Damage to the nonischemic lung has previously been reported in a variety of reperfusion models. In an experimental lung transplantation model, increased alveolar-capillary membrane permeability, increased infiltration with neutrophils, and elevated matrix metalloproteinase expression was found in the nonischemic lung after reperfusion of the transplanted lung.<sup>13</sup> Recently, in an elegant model of isolated ventilated rat lungs in which differential perfusion to the right or left lung was feasible,<sup>20</sup> the unilateral reperfused lung affected the permeability of the nonischemic lung, achieved in part by liberating TNF- $\alpha$ . In the current study, in agreement with previous studies of lung reperfusion,<sup>22,23</sup> TNF- $\alpha$  serum levels were elevated at all times during the reperfusion phase. TNF- $\alpha$  is required for the formation of endothelial damage and pulmonary edema.<sup>23</sup> Also, in models of bowel ischemia, hepatic is-

**Table 3. Mean Lobar Arterial Pressure (mmHg)**

Group	Baseline	2 h of Ischemia	10 min of Reperfusion	3 h of Perfusion/Reperfusion	27 h of Perfusion/Reperfusion
I	5.3 ± 2.3	NA	NA	4.9 ± 1.6§	NA
II	6.7 ± 2.1	NA	NA	5.2 ± 2.5§	6.4 ± 1.9§
III	4.4 ± 1.5	0.3 ± 0.3*	24.1 ± 3.2*‡	11.2 ± 1.8*	NA
IV	6.2 ± 2.0	0.6 ± 0.3*	26.5 ± 5.3*‡	10.5 ± 2.1†	9.7 ± 1.1†
V	5.9 ± 1.9	0.7 ± 0.3*	15.7 ± 2.3*	8.9 ± 2.5†	NA
VI	5.7 ± 2.8	0.4 ± 0.3*	16.7 ± 3.5*	9.8 ± 1.8†	9.4 ± 1.2†

Mean lobar arterial pressure at baseline (15 min into stabilization period), after 2 h of ischemia, 10 min of reperfusion (groups III–VI), 3 h of perfusion (group I) or reperfusion (groups III and V), and 27 h of perfusion (group II) or reperfusion (groups IV and VI). Data are presented as mean ± SD. Group details are in figure 2.

\*  $P < 0.05$  vs. the other time points within a group. †  $P < 0.05$  vs. baseline, 2 h of ischemia, and 10 min of reperfusion. ‡ Different compared with groups V and VI. § Different compared with groups III–VI.

NA = not applicable.



chemia, hind limb ischemia, and mesenteric artery occlusion, several studies reported that visceral IR injury causes TNF- $\alpha$ -dependent lung injury.<sup>23-29</sup> The nonischemic lung in our study, however, did not show edema at the early stages of reperfusion. Studies that evaluated cytokine involvement in producing lung damage found that it usually requires 3-4 h before the endothelial barrier damage is present in measurable amounts. Even when TNF- $\alpha$  was administered to animals or placed into bathing solutions surrounding endothelial cell monolayers, several hours were required before monolayer damage occurred.<sup>30,31</sup> This could explain, in part, the absence of edema in the remote lung early after reperfusion as compared with the late phase. This could also further support the hypothesis that different mechanisms are involved in the production of the reperfusion endothelial lung damage in the LLL, in addition to TNF- $\alpha$ , in a time-dependent fashion. Early damage in the reperfused lung, which was absent in the remote lung, may be caused by injury that was actually generated during the previous ischemic insult but was only made evident by the restoration of blood flow as opposed to late injury, which may be attributed to factors generated during reperfusion. However, these arguments are only speculative, and future studies are necessary to evaluate this finding.

Given the profound implications of reperfusion lung injury to the unmanipulated contralateral, naive lung for those patients undergoing single lung transplantation, strategies developed to mitigate this additional morbidity to the unmanipulated lung could have a significant impact on outcomes. In a nice study in a rat model of lung orthotopic transplantation, Soccia *et al.*<sup>13</sup> showed the involvement of matrix metalloproteinases in reperfusion-induced lung injury. Inhibition of matrix metalloproteinases reduced significantly the alveolar-capillary leakage and the transmigration of neutrophils and improved gas exchanges after reperfusion, and the authors suggested that the positive effect of treatment with the matrix metalloproteinase inhibitor resulted, in part, from its action on the contralateral alveolar-capillary membrane. More recently, in a clinically relevant model of lung transplantation in rats, Kohmoto *et al.*<sup>21</sup> showed that exposure of donors and recipients to carbon monoxide markedly suppressed inflammatory events in the contralateral naive lung. This was associated with improved gas exchange, reduced leukocyte sequestration, preserved parenchymal and endothelial cell ultrastructure, and reduced inflammation compared with animals exposed to air. Furthermore, the beneficial effects of carbon monoxide were partially mediated by activation of p38 MAPK. Because the contralateral naive lung contributes to the hypoxemia and therefore morbidity associated with IR lung injury, these interventions to attenuate contralateral lung edema should be further investigated.

Activation of MAPK has been postulated to be involved in mediating IR-induced organ dysfunction.<sup>6,32</sup> Studies in animal models, however, reported conflicting results

depending on the species, experimental conditions, and time of evaluation.<sup>33-38</sup> Recently, we reported an up-regulation of phosphorylated JNK and p38 within 3 h of reperfusion.<sup>7</sup> Increases in phosphorylated p38 expression shortly after reperfusion were also reported in isolated perfused rat lung<sup>33</sup> and in pig lung after cardiopulmonary bypass.<sup>34</sup> p38 may therefore be an important target for the management of the IR syndrome. Indeed, in previous studies, inhibition of p38 activation attenuated injury in various organs, including the heart,<sup>21,39-41</sup> intestine,<sup>42</sup> kidney,<sup>43</sup> and liver.<sup>44</sup> Similarly, in the lung, in a rat model of warm IR<sup>45</sup> and in a canine lung transplantation-related model,<sup>46</sup> it was shown that suppression of p38 activation attenuated injury. Moreover, a recent study<sup>21</sup> showed that low concentration of exogenous (inhaled) carbon monoxide protected rats transplanted lung grafts from reperfusion injury *via* a mechanism involving p38 up-regulation. Activation of the p38-related intracellular signal pathway induces TNF- $\alpha$  and IL-1 $\beta$  production. Moreover, TNF- $\alpha$  and IL-1 $\beta$  can induce p38 MAPK expression.<sup>47</sup> Interestingly, in a warm IR injury model in rat lung, Kawashima *et al.*<sup>45</sup> revealed that the ischemic injury can also cause activation of p38 with subsequent TNF- $\alpha$  and IL-1 $\beta$  production, but without showing histologic changes in the lung. The current study extends previous knowledge by showing further significant increases in phosphorylated p38 levels and BAL levels of IL-1 $\beta$  with time in association with injury and worsened lung edema. As previously discussed, a complex interactions exist between TNF- $\alpha$ , IL-1 $\beta$ , and p38 to promote reperfusion lung injury. Further discussion of these interactions is beyond the scope of this study. In this study, we also found that the protective effects of the A<sub>3</sub> adenosine receptor agonist are sustained beyond the first few hours and that this effect is associated with suppression of phospho-p38 protein expression. Therefore, these data further stress the crucial role of p38 in the development of organ IR injury.

Our investigation has a few limitations, one of which is that this is primarily a descriptive study. Evaluation of mechanisms responsible for the observed changes with time in the course of reperfusion lung injury was incomplete. Because injury, edema, and MAPK expression levels were measured only at two time points, a more detailed time course profile is not available. Moreover, because animals became hypoxemic and tachypneic with time, studies beyond 27 h were not feasible in this model. Finally, although a lung-lung interaction was found, we did not evaluate whether reperfusion of the lung affected other organs as well.

Better understanding of the time course of reperfusion injury will help to refine the design of a therapeutic approach. The current results demonstrate that in our *in vivo* lung reperfusion model, apoptosis is an early and short-term event that occurs only in the reperfused lung. Reperfusion for longer periods is characterized by sus-

tained inflammation and worsening of edema formation, both of which occur also in the nonischemic lung, but to a significantly lower extent. These changes are associated with hypoxemia and p38 activation. The significant sustained protection by the A<sub>3</sub> adenosine receptor agonist, which was associated with sustained down-regulation of phospho-p38, provides further insight into the possible mechanisms of reperfusion lung injury and reinforces the role of A<sub>3</sub> adenosine receptor activation and suppressed p38 protein expression in lung protection.

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