

Inhibition of c-Jun NH₂-Terminal Kinase Activity Improves Ischemia/Reperfusion Injury in Rat Lungs

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Although c-Jun NH₂-terminal kinase (JNK) has been implicated in the pathogenesis of transplantation-induced ischemia/reperfusion (I/R) injury in various organs, its significance in lung transplantation has not been conclusively elucidated. We therefore attempted to measure the transitional changes in JNK and AP-1 activities in I/R-injured lungs. Subsequently, we assessed the effects of JNK inhibition by the three agents including SP600125 on the degree of lung injury assessed by means of various biological markers in bronchoalveolar lavage fluid and histological examination including detection of apoptosis. In addition, we evaluated the changes in p38, extracellular signal-regulated kinase, and NF- κ B-DNA binding activity. I/R injury was established in the isolated rat lung preserved in modified Euro-Collins solution at 4°C for 4 h followed by reperfusion at 37°C for 3 h. We found that AP-1 was transiently activated during ischemia but showed sustained activation during reperfusion, leading to significant lung injury and apoptosis. The change in AP-1 was generally in parallel with that of JNK, which was activated in epithelial cells (bronchial and alveolar), alveolar macrophages, and smooth muscle cells (bronchial and vascular) on immunohistochemical examination. The change in NF- κ B qualitatively differed from that of AP-1. Protein leakage, release of lactate dehydrogenase and TNF- α into bronchoalveolar lavage fluid, and lung injury were improved, and apoptosis was suppressed by JNK inhibition. In conclusion, JNK plays a pivotal role in mediating lung injury caused by I/R. Therefore, inhibition of JNK activity has potential as an effective therapeutic strategy for preventing I/R injury during lung transplantation. *The Journal of Immunology*, 2004, 172: 2569–2577.

Lung transplantation has been established as an important therapeutic strategy for various end-stage lung diseases. To date, over 14,000 patients have received lung transplantation (1). Despite recent refinements of surgical techniques and medical therapy, the mortality is still high and primary graft failure is considered to be its principal cause, especially in the early phase of lung transplantation (1). Since primary graft failure is a syndrome of acute allograft dysfunction that is presumed to be mainly caused by ischemia/reperfusion (I/R)² injury (2), it is important to elucidate the molecular mechanism of I/R injury in the lung and to develop effective therapeutic intervention for successful lung transplantation. The signaling pathways related to NF- κ B, a member of inflammatory transcription factors, have been demonstrated to play a pivotal role in inducing I/R injury in the transplantation of various organs including the lung (3–6). In contrast, conflicting findings have been reported concerning the significance of AP-1 (7–12), another important transcription factor mediating acute inflammation, and its associated signaling pathways such as p38 mitogen-activated protein kinase (MAPK) (13–15) in I/R in-

jury. Mocanu et al. (14) showed that activated p38, which is one of the superfamilies of mammalian MAPKs enhancing AP-1-DNA binding activity (16), inhibits myocardial injury especially during the ischemic period, while Barancik et al. (15) demonstrated the opposite. In the lung, activated p38 has been reported to promote injury in a warm I/R model (17) and in lung transplantation-related I/R (18). Unfortunately, however, in these studies, close attention was not paid to transitional changes in p38 and/or AP-1 activities during the ischemia and reperfusion phases. Evidence for I/R-induced up-regulation of c-Jun NH₂-terminal kinase (JNK), another MAPK member, activated by environmental stress and inflammatory cytokines, and called stress-activated protein kinase (SAPK) (19, 20) has accumulated (21–27). However, the absolute significance of JNK and its downstream transcription factor, AP-1, in promoting or inhibiting lung injury caused by I/R has not been adequately addressed. This may be partially attributed to the fact that there has been no reliable JNK inhibitor until quite recently. Furthermore, there have been few studies demonstrating the responsible cells in which JNK activity is actually augmented in I/R injury including lung transplantation. Based on these facts, we attempted, in the present study, to systematically measure the following events during both periods of ischemia and reperfusion in an isolated rat lung model mimicking I/R injury during lung transplantation: 1) transitional changes in DNA binding activities of AP-1 and NF- κ B, and degradation of I κ B- α , an inhibitory protein of NF- κ B activation; 2) transitional changes in the activities of three major members of MAPK including JNK, p38, and extracellular signal-regulated kinase (ERK), the third superfamily of MAPK having the effect of cell proliferation and survival (28); 3) transitional effects of a specific JNK inhibitor, SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) (29, 30), on the activities of JNK, p38, and ERK, and the DNA binding activities of AP-1 and NF- κ B; 4) the effects of JNK inhibition on lung injury evaluated by

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Received for publication April 28, 2003. Accepted for publication October 20, 2003.

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² Abbreviations used in this paper: I/R, ischemia/reperfusion; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; LDH, lactate dehydrogenase; BALF, bronchoalveolar lavage fluid; L-JNK11, JNK peptide inhibitor 1 (L-stereoisomer); EGCG, epigallocatechin gallate; EC, Euro-Collins; ECMO, extracorporeal membrane oxygenator; BCA, bicinchoninic acid.

histological examination, degree of apoptosis, and protein leakage and release of lactate dehydrogenase (LDH) as well as TNF- α into the bronchoalveolar lavage fluid (BALF) at the end of reperfusion; 5) immunohistochemical determination of what kinds of cells are responsible for JNK up-regulation; and 6) further certification concerning the significance of JNK for the pathogenesis of I/R lung injury by applying other JNK inhibitors of JNK peptide inhibitor 1, L-stereoisomer (L-JNKI1) (31, 32) and epigallocatechin gallate (EGCG) (33). Based on these findings, we examined whether JNK inhibition could act as an important new therapeutic strategy for preventing I/R injury during lung transplantation.

Materials and Methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Preparation of isolated perfused rat lungs

Male pathogen-free Sprague Dawley rats (8 wk old) weighing 250–300 g were anesthetized by i.p. administration of 50 mg/kg pentobarbital sodium ($n = 230$). Tracheotomy was performed and the lungs were ventilated with room air, adjusting the tidal volume to 10 ml/kg and the respiratory rate to 60 breaths/min. Median sternotomy was performed and the chest was opened widely. A few minutes after administration of 1000 U/kg heparin into the left ventricle, blood was expelled by cardiac puncture, leading to rapid exsanguination and death. Thereafter, cannulas were inserted into the left atrium and main pulmonary artery. Subsequently, the lungs were flushed with modified Euro-Collins (EC) solution to discharge the blood remaining in the lungs. The trachea was ligated at the end-inspiratory position, and the heart and lungs were excised en bloc and preserved in EC solution at 4°C for up to 4 h. The isolated heart-lung block was perfused with Krebs-Henseleit solution with 3% BSA at a constant flow rate of 12 ml/min and recirculated with a roller pump (rotor 1500 N; Taitec, Tokyo, Japan) for up to 3 h. The hematocrit was adjusted to $10.5 \pm 0.5\%$ by adding fresh blood obtained from donor rats. Gas exchange was maintained with an extracorporeal membrane oxygenator (ECMO) (Merasilox-S; Senko, Tokyo, Japan) connected to the isolated lungs. A gas mixture containing 21% O₂ and 5% CO₂ flowed into the ECMO, allowing adjustment of the perfusate PO₂, PCO₂, and pH to 140 ± 5 mmHg, 36 ± 5 mmHg, and 7.41 ± 0.03 , respectively. A warmed and humidified gas mixture containing the same composition of gases as used for the ECMO was continuously supplied to the lung surface to maintain a temperature of $37.0 \pm 0.2^\circ\text{C}$ and to avoid desiccation of the lung surface.

Experimental protocols

The whole lung tissues were harvested at 0, 0.5, 1, 2, and 4 h under cold ischemic conditions maintained at 4°C, and at 0.5, 1, and 3 h after the initiation of warm reperfusion adjusted to 37°C in the absence or presence of a specific JNK inhibitor. JNK inhibition was generally achieved by administering SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one; BIOMOL Research Laboratories, Plymouth, PA) into both the preservation and perfusion solutions. In another series of experiments, SP600125 was administered only into the preservation solution but not into the perfusion solution, allowing evaluation of the possible role of JNK activation in the ischemic period on the final lung injury after reperfusion. SP600125 has been shown to selectively inhibit the kinase activity of JNK through a mechanism of ATP-competitive inhibition of JNK, leading to inhibition of the phosphorylation of c-Jun to which JNK is bound, with >20-fold specificity in comparison with its inhibitory effects on kinase activities of other MAPKs including p38 and ERK (29, 30). However, SP600125 has also been demonstrated to inhibit JNK phosphorylation per se in a dose-dependent manner (29). SP600125 was dissolved in DMSO and its concentration was adjusted to 1 mM in the preservation solution and 25 μM in the perfusion solution. These concentrations of SP600125 were determined on the basis of preliminary experiments in which we examined what concentrations of SP600125 were appropriate to sufficiently inhibit the kinase activity of JNK in lung tissues harvested under conditions of ischemia at 4°C followed by reperfusion at 37°C. Lung tissues in which JNK was inhibited by SP600125 during both ischemia and reperfusion were defined as the SP-IR group ($n = 78$), while lung tissues in which JNK was inhibited by SP600125 during ischemia only were assigned to the SP-I group ($n = 12$). Lung tissues obtained without JNK inhibition were defined as the DMSO group, in which the same amount of DMSO as used for the SP-IR and SP-I groups was administered ($n = 110$). Isolated lungs that were simply flushed with EC solution and were not exposed to ischemia and reperfusion were

used as the control under each experimental condition. The lung tissues thus obtained were used for further analysis to examine the transitional changes in activities of the two transcription factors and the three MAPK members.

To confirm a vital role of JNK in developing I/R lung injury, we additionally tested the other two agents having the capability of inhibiting JNK activity, including L-JNKI1 (Alexis Biochemicals, San Diego, CA) and EGCG (BIOMOL Research Laboratories). L-JNKI1 has been shown to selectively inhibit the JNK activity by competing the binding between c-Jun and JNK (31, 32), while EGCG has been considered to be the agent that specifically inhibits the signaling pathway inducing JNK activation (33). These agents were administered into both the preservation and perfusion solutions. The concentrations of L-JNKI1 and EGCG in the preservation solution were, respectively, adjusted to 100 and 200 μM , while those in the perfusion solution were kept to 5 and 10 μM . Lung tissues in which JNK was inhibited by L-JNKI1 were defined as the LJ-IR group ($n = 15$), while lung tissues in which JNK was inhibited by EGCG were classified as the EG-IR group ($n = 15$). The lung tissue samples were harvested at the end of reperfusion in the presence of either L-JNKI1 or EGCG. Differing from the SP-IR group, we simply examined the inhibitory effects of these agents on JNK activity in the lung tissue and their advantage over preventing I/R-elicited lung injury.

Nuclear protein extraction

Nuclear protein extracts were prepared according to the method previously described (34). Briefly, frozen lung tissue weighing 300 mg 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 PMSF). 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 PMSF, 2 mM benzamide, 5 $\mu\text{g/ml}$ pepstatin A, 5 $\mu\text{g/ml}$ leupeptin, 5 $\mu\text{g/ml}$ aprotinin) and incubated on ice for 20 min. Samples were centrifuged at 15,000 rpm for 1 min, and the supernatants containing nuclear protein were stored at -80°C until use. The total protein concentration in the extract was determined with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

EMSA for AP-1 and NF- κB

EMSA was performed using a kit purchased from Promega (Madison, WI). Double-stranded AP-1 and NF- κB consensus oligonucleotide probes were 5'-CGCTTGATGAGTCAGCCGAA-3' and 5'-AGTTGAGGGGACTT TCCCAGGC-3', respectively (underline: binding sites). The oligonucleotides were 5' end-labeled by T4 polynucleotide kinase in the presence of [γ -³²P]ATP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The labeled oligonucleotides were purified on push columns (Stratagene, La Jolla, CA). For the binding reaction, 5 μg nuclear protein was incubated with 35 fmol ³²P-labeled double-stranded oligonucleotide in gel shift binding buffer for 20 min at room temperature. DNA-protein complexes were then analyzed on 6% DNA retardation gels (Novex; Invitrogen, Carlsbad, CA) run at 100 V for 1 h in 0.5 \times Tris-borate-EDTA buffer. The gels were then dried and visualized by autoradiography ($n = 4$ for either AP-1 or NF- κB in DMSO and SP-IR groups).

For competition assay, nuclear extracts were preincubated with a 100-fold excess of unlabeled double-stranded oligonucleotides for 10 min at room temperature and then incubated with labeled AP-1 or NF- κB oligonucleotides for an additional 20 min ($n = 4$ for either AP-1 or NF- κB).

Western blotting analysis for JNK, p38, ERK, and I κB - α

Whole lung tissues were homogenized using a Dounce tissue homogenizer on ice in 1 \times cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 4 $\mu\text{g/ml}$ leupeptin). The homogenates were centrifuged at 15,000 rpm at 4°C for 20 min, and the supernatants were collected. Protein concentration was determined by BCA protein assay. Samples with equal amounts of protein were mixed with 2 \times SDS sample buffer consisting of 20% glycerol, 4% SDS, 0.16 M Tris-HCl (pH 6.8), 4% 2-ME, and 0.5% bromophenol blue, and then boiled for 3 min.

Thirty micrograms of each sample were fractionated on a 7–15% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). To estimate the protein level that actually has kinase activity, the phosphorylated form of each MAPK was examined using a specific Ab. After immunoblotting of the

phosphorylated form of each MAPK, the membrane was stripped and probed with the primary Ab against each MAPK to measure the total protein level of each MAPK including phosphorylated and nonphosphorylated forms. The total protein of each MAPK thus estimated was used as the loading control. Concerning I κ B- α , both the phosphorylated form and total protein were examined as well. The expression of β -actin was used as the loading control. Thus, the samples were incubated overnight at 4°C with the primary Ab against phospho-SAPK/JNK ($n = 3$ for each of DMSO and SP-IR groups), phospho-p38 MAPK ($n = 3$ for each), phospho-ERK ($n = 3$ for each), SAPK/JNK ($n = 3$ for each), p38 MAPK ($n = 3$ for each), and ERK ($n = 3$ for each) (all Abs were purchased from Cell Signaling, Beverly, MA). For analysis of I κ B- α , lung homogenate samples were treated with a primary Ab against phospho-I κ B- α ($n = 3$ for each; Cell Signaling), I κ B- α ($n = 3$ for each; Santa Cruz Biotechnology, Santa Cruz, CA), or β -actin ($n = 3$ for each). The primary Ab was counterstained with HRP-conjugated rabbit or mouse IgG Ab (Santa Cruz Biotechnology), visualized with ECL detection reagents (Amersham Biosciences, Buckinghamshire, U.K.), and finally exposed to photographic film (Hyperfilm ECL; Amersham Biosciences). The images were analyzed using NIH image version 1.62 (National Institutes of Health, Bethesda, MD).

In vitro kinase assay for JNK, p38, and ERK

Cell lysates were prepared by applying the same method as described in the Western blotting protocol. Kinase assay for JNK, p38, and ERK was conducted using *in vitro* kinase assay kits (Cell Signaling). The pellet for JNK (containing both phosphorylated and nonphosphorylated forms) was harvested by pulling down 250 μ l cell lysate containing 250 μ g total protein by c-Jun fusion protein, and was suspended in the kinase buffer supplemented with ATP, thus evoking phosphorylation of c-Jun (phospho-c-Jun) through the kinase reaction induced by activated, phosphorylated JNK. In contrast, the pellet of phosphorylated p38 or phosphorylated ERK was, respectively, prepared by immunoprecipitating 200 μ l cell lysate containing 200 μ g total protein using the mAb for phospho-p38 or phospho-ERK. Thereafter, each pellet was suspended in the kinase buffer containing both ATP and fusion protein of either ATF-2 (for phospho-p38) or Elk-1 (for phospho-ERK), making each fusion protein phosphorylated through the kinase reaction in the presence of activated, phosphorylated p38 or ERK. The suspended solution was then mixed with the 3 \times sample buffer to stop the kinase reaction. Finally, the phosphorylated form of fusion protein including phospho-c-Jun, phospho-ATF-2, or phospho-Elk-1 was quantified by the Western blotting as the representative of the kinase activity of JNK, p38, or ERK in the original sample. The final solution was loaded on 7–15% SDS-PAGE gel, and was immunoblotted by using anti-phospho-c-Jun Ab for JNK assay ($n = 3$ for either DMSO, SP-IR, LJ-IR, or EG-IR group), anti-phospho-ATF-2 Ab for p38 assay ($n = 3$ for DMSO or SP-IR group), or anti-phospho-Elk-1 Ab for ERK assay ($n = 3$ for DMSO or SP-IR group).

Bronchoalveolar lavage

At the end of reperfusion, bronchoalveolar lavage was performed by intratracheal instillation of 3 ml normal saline twice in each of the DMSO, SP-I, SP-IR, LJ-IR, and EG-IR groups ($n = 7$ for each group). BALF obtained from the isolated lung simply flushed with EC solution without exposure to ischemia and reperfusion was designated as the control ($n = 7$). BALF was centrifuged at 1,200 rpm for 5 min and the supernatant was collected for measurement of LDH, total protein, and TNF- α concentrations. LDH in BALF was assayed with an LDH assay kit, while protein concentration was estimated by the BCA protein assay. TNF- α in BALF was measured by sandwich ELISA (Biosource International, Camarillo, CA).

Histopathological examination

For histopathological examination, lung specimens harvested at the end of reperfusion were fixed in 10% formalin. After dehydration and embedding in paraffin, 3- μ m sections were stained with H&E ($n = 5$ for each of DMSO, SP-I, SP-IR, LJ-IR, and EG-IR groups).

Degree of apoptosis

The degree of I/R-induced apoptosis in the lung tissue was evaluated by the methods of TUNEL and DNA ladder detection. Paraffin sections with 3- μ m thickness were used for assay with the TUNEL method (Apoptosis In Situ Detection kit; Wako, Osaka, Japan) ($n = 5$ for either DMSO or SP-IR group). The sections were deparaffinized in xylene, dehydrated in ethanol, and rinsed with distilled water. Thereafter, the tissues were digested by protein digestion enzyme at 37°C for 5 min, washed with PBS, and then incubated with TdT. After washing with PBS, these sections were treated with 3% hydrogen peroxide, and washed with PBS again. Finally,

the sections were exposed to anti-peroxidase conjugated Ab at 37°C for 10 min, rinsed with PBS, and stained with diaminobenzidine solution. After washing with PBS, the sections were counterstained with methyl green, and dipped in *n*-butanol and xylene. For each paraffin section, three fields were randomly selected and the frequency of TUNEL-positive cells was estimated at $\times 400$ magnification.

DNA ladder formation was examined with an Apoptosis Ladder Detection kit (Wako) ($n = 5$ for either DMSO or SP-IR group). Genomic DNA harvested from the lung tissue was extracted according to the manufacturer's instructions. One microgram of total DNA was electrophoresed on 1.5% agarose gel. Subsequently, the gel was stained with SYBR Green I and the band was visualized with a Chemiluminescence Imaging System DIANA III (M&S Instruments Trading, Osaka, Japan).

Localization of cells with activated JNK

First, 3- μ m paraffin sections were prepared from lung tissue obtained at 30 min after the start of reperfusion and used for immunostaining of activated JNK with a DAKO ENVISION+/HRP kit (DakoCytomation, Carpinteria, CA). After deparaffinization and dehydration, the sections were autoclaved at 121°C for 10 min in 10 mM sodium citrate buffer (pH 6.0) for Ag unmasking, and rinsed with distilled water and TBS. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 15 min. The sections were incubated overnight at 4°C, with anti-p-JNK mAb (G-7; Santa Cruz Biotechnology) diluted in 1% BSA/TBS. The samples were then rinsed with TBS, incubated with anti-mouse secondary Ab for 1 h at room temperature, and washed again with TBS. Subsequently, the samples were stained with diaminobenzidine solution for 10 min at room temperature and washed with distilled water. After counterstaining with hematoxylin for 10 s, the samples were washed in distilled water, dehydrated in ethanol, cleared in xylene, and mounted ($n = 3$ for either DMSO or SP-IR group). Normal mouse IgG1 (DakoCytomation) was used as a negative control.

Statistical analysis

Values are presented as mean \pm SEM. The significance of differences in concentrations of LDH, total protein, and TNF- α in BALF between the experimental groups at the end of reperfusion was determined by one-way ANOVA followed by multiple comparisons by Scheffé's test (StatView II; Abacus Concepts, Berkeley, CA). Differences in the number of apoptotic cells between the groups were assessed by unpaired *t* test. A *p* value of <0.05 was deemed to be statistically significant.

Results

Transitional changes in DNA binding activities of AP-1 and NF- κ B with and without JNK inhibition

In the DMSO group, the DNA binding activity of AP-1 was increased at 30 min after the start of ischemia and gradually decreased thereafter under ischemic conditions. The AP-1-DNA binding activity in this group was again augmented at 1 h and was sustained up to 3 h after the start of reperfusion (Fig. 1A). The augmented DNA binding activity of AP-1 observed in both the ischemia and reperfusion phases in the DMSO group was markedly inhibited in the presence of a JNK inhibitor, i.e., in the SP-IR group (Fig. 1B). The specificity of AP-1-DNA binding was confirmed by the almost complete displacement of AP-1-DNA complex in the presence of a 100-fold molar excess of unlabeled AP-1 (Fig. 1E).

In contrast to the AP-1-DNA binding, the DNA binding activity of NF- κ B in the DMSO group remained at the baseline level during the ischemic phase, but was markedly enhanced at 30 min after the start of reperfusion (Fig. 1C). The increased binding between NF- κ B and DNA was sustained throughout the period of reperfusion in the DMSO group, and this increase was not modified by JNK inhibition in the SP-IR group (Fig. 1, C and D). The specificity of NF- κ B-DNA binding was confirmed by competition assay (Fig. 1F).

Since NF- κ B-DNA binding activity is mainly regulated by cytoplasmic I κ B- α , the protein expression of phosphorylated I κ B- α and total I κ B- α was also investigated. Phosphorylated I κ B- α gradually increased upon exposure to ischemia in the DMSO group

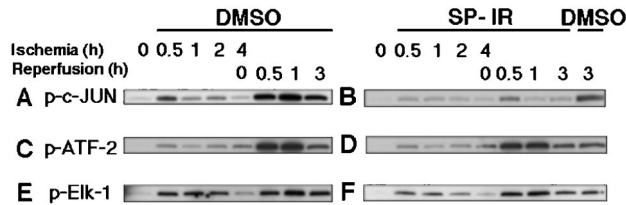


FIGURE 3. Kinase assay for JNK, p38, and ERK. *A*, Kinase activity of JNK in the absence of JNK inhibitor (DMSO group). *B*, Inhibition of kinase activity of JNK in the presence of JNK inhibitor SP600125 (SP-IR group). *C* and *D*, Kinase activity of p38 in DMSO and SP-IR groups, respectively. *E* and *F*, Kinase activity of ERK in both groups. See text for further details.

in accordance qualitatively with the tendency observed for phosphorylated p38 (Fig. 2*E*). The augmentation of p38 activity was not influenced by JNK inhibition in the SP-IR group (Fig. 3*D*).

The activity of ERK was markedly increased in the early phase but was decreased at the end of ischemia in the DMSO group (Fig. 3*E*). The activity of ERK was again enhanced during reperfusion (Fig. 3*E*). These findings were qualitatively consistent with those obtained for phosphorylated ERK (Fig. 2*H*). The enhanced ERK activity was not affected by JNK inhibition in the SP-IR group, as well (Fig. 3*F*).

Effects of JNK inhibition on LDH, protein, and TNF-α release into BALF

In the DMSO group, the concentrations of LDH and total protein in BALF at the end of reperfusion were much higher than those in the controls without exposure to ischemia and reperfusion (Fig. 4, *A* and *B*). The I/R-induced increase in LDH and protein leakage

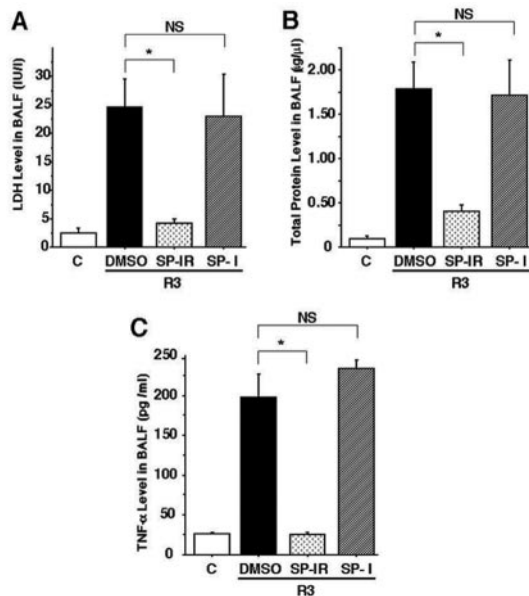


FIGURE 4. Improvement of LDH, protein, and TNF-α leakage into BALF by JNK inhibition. R3, 3 h after initiation of reperfusion. *C*, Control groups in which samples were obtained without exposure to ischemia and reperfusion. DMSO: groups with no addition of JNK inhibitor and samples were obtained at R3 time point. SP-IR: groups with addition of JNK inhibitor SP600125 during both periods of ischemia and reperfusion (samples: at R3 time point). SP-I, Groups with addition of JNK inhibitor SP600125 only during ischemic period (samples: at R3 time point). *A*, LDH release. *B*, Protein leakage. *C*, TNF-α production. *, $p < 0.05$ vs DMSO groups.

was markedly attenuated in the SP-IR group in which JNK activity was inhibited during both ischemia and reperfusion. In contrast, LDH release and protein leakage into BALF were not reduced in the SP-I group in which JNK activity was solely inhibited under ischemic conditions (Fig. 4, *A* and *B*).

In the DMSO group, the BALF TNF-α level at the end of reperfusion was also significantly increased in comparison with that in the control (Fig. 4*C*). The increased level of TNF-α was restored to the baseline in the SP-IR group but not in the SP-I group (Fig. 4*C*).

Effects of JNK inhibition on pathological changes in lung tissue

Although there was little abnormal change in the lung tissue harvested from the control preparation (Fig. 5, *A* and *B*), alveolar and interstitial edema was evident in the lung tissue obtained after completing reperfusion in the DMSO group (Fig. 5, *C* and *D*). In contrast, JNK inhibition significantly attenuated the alveolar and interstitial edema in the SP-IR group (Fig. 5, *E* and *F*), but not in the SP-I group (data not shown).

Effects of JNK inhibition on lung tissue apoptosis

Compared with the control group (Fig. 6*A*), the number of apoptotic cells examined by the TUNEL method was significantly increased at the end of reperfusion in the DMSO group (Fig. 6, *B*, *C*, and *E*). JNK inhibition appreciably reduced the number of TUNEL-positive cells in the SP-IR group (Fig. 6, *D* and *E*). Apoptosis was predominantly observed in alveolar epithelial cells (Fig. 6, *B* and *C*).

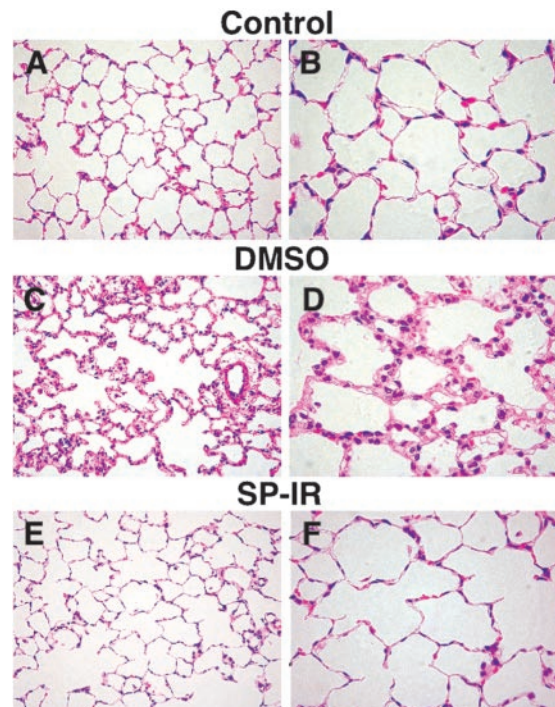


FIGURE 5. Improvement of lung injury by JNK inhibition. Lung tissue sections were stained with H&E. *A*, Control lung tissue section without exposure to ischemia and reperfusion (magnification: ×200). *B*, The same lung tissue as presented in *A* but with ×400 magnification. *C*, Lung tissue section obtained at the end of reperfusion in the absence of JNK inhibitor (DMSO group) (magnification: ×200). *D*, The same lung tissue as presented in *C* but with ×400 magnification. *E*, Lung tissue section obtained at end of reperfusion in the presence of JNK inhibitor SP600125 (SP-IR group) (magnification: ×200). *F*, The same lung tissue as presented in *E* but with ×400 magnification.

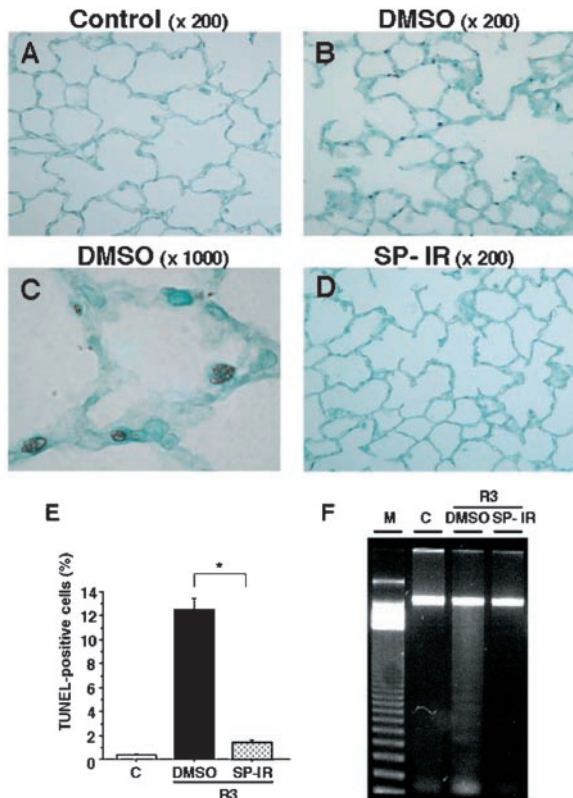


FIGURE 6. Decrease in apoptosis by JNK inhibition. *A*, Control lung section stained with TUNEL method (magnification: $\times 200$). *B*, Lung tissue section harvested at the end of reperfusion in the absence of JNK inhibitor (DMSO group) and stained with TUNEL method (magnification: $\times 200$). *C*, The same lung tissue section as presented in *B* but with $\times 1000$ magnification. *D*, Lung tissue section obtained at the end of reperfusion in the presence of JNK inhibitor SP600125 (SP-IR group) (magnification: $\times 200$). *E*, Relative frequency of TUNEL-positive cells in three fields of lung tissue section ($\times 400$ magnification). R3: 3 h after initiation of reperfusion (i.e., the end of reperfusion). *C*: control groups in which samples were harvested without exposure to ischemia and reperfusion. DMSO: groups with no addition of JNK inhibitor and samples were obtained at R3. SP-IR: groups with addition of JNK inhibitor SP600125 during both periods of ischemia and reperfusion. *, $p < 0.05$ vs DMSO groups. *F*, DNA fragmentation at the end of reperfusion. Lane *M*, DNA size marker; lane *C*, DNA obtained from control lungs; lane *DMSO*, DNA obtained from the lungs of DMSO group; lane *SP-IR*, DNA obtained from the lungs of SP-IR group.

Although there was little DNA fragmentation in the control lung, exposure to ischemia and reperfusion significantly enhanced DNA fragmentation in the DMSO group (Fig. 6*F*). This enhancement of DNA fragmentation was clearly depressed by JNK inhibition in the SP-IR group (Fig. 6*F*).

Localization of cells with activated JNK

Although positive staining of phosphorylated JNK was detected in bronchial epithelial cells in the control lung, its intensity was very weak (Fig. 7, *A* and *B*). In contrast, expression of phosphorylated JNK was distinctly enhanced in the nucleus of bronchial and alveolar epithelial cells (Fig. 7, *C* and *D*) and in both the nucleus and cytoplasm of alveolar macrophages (Fig. 7*E*). Phosphorylated JNK was weakly positive in bronchial and vascular smooth muscle cells (Fig. 7, *D* and *F*).

Effects of L-JNKII and EGCG on JNK activity and I/R lung injury

The kinase activity of JNK in the lung tissue was significantly inhibited in both LJ-IR and EG-IR groups (Fig. 8*A*). The degree of

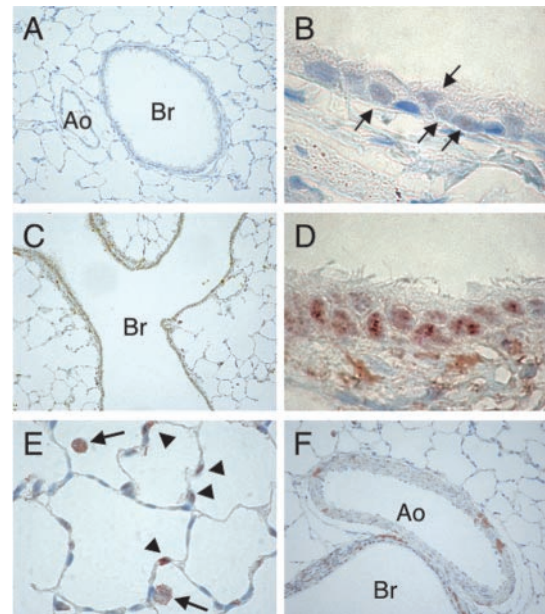


FIGURE 7. Immunohistochemical staining of cells with activated JNK. *A*, Control lung tissue ($\times 200$). *B*, Control lung tissue ($\times 1,000$). *C-F*, Lung tissue sections in DMSO group (*C*, $\times 200$; *D*, $\times 1,000$; *E*, $\times 1,000$; *F*, $\times 400$). Ao, bronchial arteriole; Br, bronchiole. Arrow in *B* indicates bronchial epithelial cells with weakly activated JNK in the control lung. Arrowhead and arrow in *E* indicate alveolar epithelial cells and alveolar macrophages with strong JNK activation in I/R-injured lung, respectively.

JNK inhibition by these two agents was quantitatively similar to that observed for the SP-IR group. Moreover, the BALF concentrations of LDH, protein, and TNF- α in these groups did not differ from those obtained in the SP-IR group but much lower than those

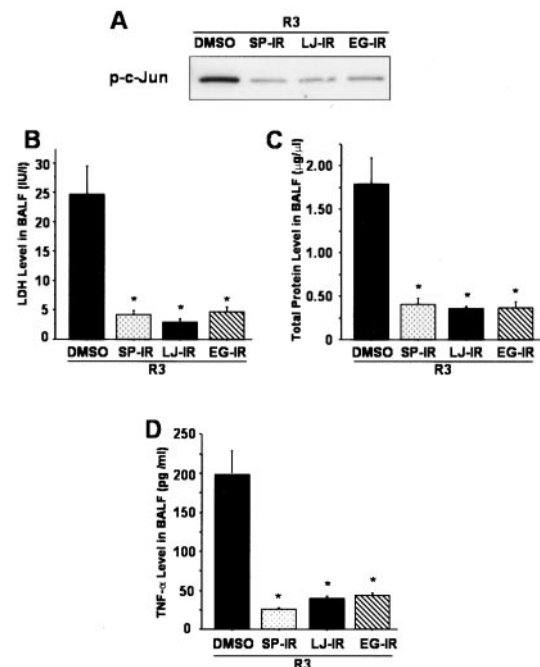


FIGURE 8. Effects of L-JNKII and EGCG on lung tissue JNK activity and LDH, protein, and TNF- α in BALF. *A*, Kinase activity of JNK in the absence (DMSO group) or presence of SP600125 (SP-IR group), L-JNKII (LJ-IR group), and EGCG (EG-IR group). *B*, LDH release. *C*, Protein leakage. *D*, TNF- α production. R3, 3 h after initiation of reperfusion (i.e., the end of reperfusion). *, $p < 0.05$ vs DMSO.

in the DMSO group (Fig. 8, *B–D*). The alveolar and interstitial edema was certainly improved in both LJ-IR and EG-IR groups (data not shown).

Discussion

The important findings obtained in the present study were as follows: 1) AP-1, a member of the redox-sensitive transcription factors, was transiently activated under ischemic conditions but it showed sustained activation under conditions with reperfusion (Fig. 1). 2) The changes of AP-1-DNA binding qualitatively differed from those of NF- κ B-DNA binding which was enhanced only during reperfusion but not during ischemia (Fig. 1). 3) The transitional changes of AP-1 activity upon exposure to I/R were generally in parallel with those of MAPKs including JNK and ERK (Figs. 2 and 3). 4) Among the three members of MAPK, the degree of activation upon I/R stimulation seemed to be lowest for p38 (Fig. 2). 5) Lung injury and apoptosis caused by I/R was attenuated by inhibiting JNK activity during both periods of ischemia and reperfusion (Figs. 4–6). However, this did not hold true when JNK activity was inhibited only during the ischemic phase (Fig. 4). 6) Expression of activated JNK was strongly up-regulated in bronchial and alveolar epithelial cells as well as alveolar macrophages, but was mildly up-regulated in bronchial and vascular smooth muscle (Fig. 7). However, we should state the fact that the isolated lung model used in the present study is somewhat artificial and may not fully represent the pulmonary milieu during I/R injury from a physiological perspective, though it allows a tight control of the pulmonary circulation.

Importance of activated JNK-related signaling pathways

The MAPK signaling pathways are composed of three-tiered cascades (28). Each MAPK is activated by various types of environmental stress and cytokines via tyrosine and threonine phosphorylation. This phosphorylation is mediated by a specific MAPK kinase (MAPKK or MKK), also known as ERK kinase (MEK): MKK3/6 for p38, MKK4/7 for JNK, and MEK1/2 for ERK. Each MAPKK, in turn, is activated by MAPKK/MEK kinase (MAPKKK or MEKK) (18, 26). There is increasing evidence that p38 is activated in response to ischemia and reperfusion (11–13). Recent reports suggest that inhibition of p38 attenuates the lung tissue injury induced by lung transplantation (18) or by warm I/R (17). JNK, one of the major upstream kinases of AP-1, has also been considered to be involved in the pathogenesis of I/R injury in various organs (21–27). However, consistent results have not been obtained concerning the significance of JNK activation in myocardial I/R injury. Fryer et al. (24) and Barancik et al. (25) suggested that JNK could act as a cardioprotective factor, whereas Sato et al. (26) reported that JNK could serve as a detrimental factor, in myocardial infarction models. To our knowledge, there has been no study addressing the issues of what is the significance of JNK activation in I/R-related lung injury and whether JNK inhibition can be adopted as a therapeutic intervention for preventing the occurrence of I/R-induced lung injury. To explore a new therapeutic strategy to provide protection against I/R-related lung injury during lung transplantation, we attempted to inhibit JNK activity in an animal model in which the lung tissue was preserved in cold EC solution at 4°C followed by exposure to warm reperfusion at 37°C. The reason why we selected JNK as a target for inhibition among MAPK members is that the degree of JNK activation from the baseline seems to be much higher than that of p38 (Fig. 2), which is another MAPK activated by various inflammatory insults in a fashion similar to JNK (19). We did not select ERK as a candidate for inhibition, simply because activation of ERK is generally associated with cell proliferation, cell survival, or inhibition

of apoptosis (28, 35), all of which could act as protective factors against tissue injury. This notion is in accord with the recent report that ERK plays a protective role in myocardial I/R injury (36).

As described above, we found that JNK inhibition during both ischemia and reperfusion significantly depressed AP-1 expression throughout the whole time period of observation, leading to a marked decrease in protein leakage and release of LDH and TNF- α into BALF concomitant with an improvement of lung injury after reperfusion (Figs. 1, 4, and 5). These findings strongly suggest not only that JNK is importantly involved in the pathogenesis of I/R-induced lung injury, but also that JNK inhibition has potential as an effective therapeutic strategy against I/R-induced lung injury. We believe that this is the first report demonstrating that JNK plays an aggravating role in lung injury caused by ischemia and reperfusion, and that inhibition of JNK can be adopted as a therapeutic intervention. However, the definite explanation is required for the finding that increased release of TNF- α into BALF was almost totally depressed by JNK inhibition in both ischemia and reperfusion periods, because TNF- α production is regulated not only by AP-1 pathway but also by NF- κ B pathway, the latter being shown to be enhanced in I/R-induced lung injury, as well (Fig. 1). The reliable explanation for this peculiar phenomenon is that JNK inhibition does not modify the transcription of TNF- α gene mediated by NF- κ B-DNA binding but exerts a significant influence on the expression of TNF- α at a posttranscriptional level. Supporting this idea, Bennett et al. (29) has recently demonstrated that JNK inhibition by SP600125 makes the TNF- α mRNA very unstable. We also found that AP-1-DNA binding activity was transiently enhanced in combination with transient up-regulation of JNK during cold ischemia (Figs. 1–3). These phenomena had not been reported in any organ including the lung. Therefore, we examined the effects of transient up-regulation of JNK in the ischemic period on the final severity of lung injury after reperfusion, by inhibiting JNK activity during ischemia alone. Protein leakage and the release of LDH and TNF- α into BALF at the end of reperfusion were not attenuated (Fig. 4), leading to little significant improvement in alveolar flooding and interstitial edema, when JNK was inhibited during the ischemic phase only (Fig. 4). This is in sharp contrast to the results obtained under conditions in which JNK was inhibited during both ischemia and reperfusion (Fig. 4), suggesting that activation of JNK during ischemia is not of primary importance in inducing tissue damage in a lung model exposed to ischemia and reperfusion. Further study is absolutely necessary, however, to clarify the significance of transient JNK activation during ischemia in the pathogenesis of I/R-related lung injury.

Although JNK and p38 are generally accepted as proapoptotic kinases (28), activation of JNK and its downstream AP-1 may not always induce apoptosis (16, 37, 38). This phenomenon has been explained by the fact that a given insult activating JNK simultaneously enhances other signals such as ERK and NF- κ B, both of which inhibit JNK-induced apoptosis (20, 36). Since we found that ERK and NF- κ B were markedly activated together with JNK and p38 during the time periods of ischemia and/or reperfusion (Figs. 1–3), we attempted to examine whether JNK activation would actually induce apoptosis in an I/R-induced lung injury model. We confirmed that JNK-associated apoptosis really existed in I/R lung injury, and was significantly attenuated by JNK inhibition (Fig. 6). These results are highly consistent with the idea that the proapoptotic potency of JNK exceeds the anti-apoptotic function of either ERK or NF- κ B in lung injury induced by ischemia and reperfusion.

Time course of kinase activity of p38 and ERK

Activities of MAPKs including JNK, p38, and ERK were found to be enhanced during both time periods of ischemia and reperfusion (Figs. 2 and 3). These findings are inconsistent with those reported for the liver (21, 22). Bradham et al. (21) demonstrated that JNK and p38 were not activated during cold storage in a liver transplantation model. Furthermore, Iesalnieks et al. (22) reported that p38 activity was suppressed in the same qualitative model as used by Bradham et al. Taken together, the activation of MAPKs under cold conditions with ischemia is expected to occur in an organ-specific manner.

Activation of p38 has been shown to be associated with I/R-induced lung injury, both in a warm I/R model (17) and in a lung transplantation model (18). These results appear to be qualitatively in accordance with those obtained in the present study (Figs. 2 and 3), though we did not immediately confirm that inhibition of p38 activity would improve lung injury caused by ischemia and reperfusion. In contrast, the situation seems to be quite different in I/R injury in the heart. Some researchers reported that transient activation of p38 would be cardioprotective in association with ischemic preconditioning (14), whereas others reported the opposite (15). These results suggest that a definitive comparative study in various organs would be indispensable for clarifying the issue of organ-specific up-regulation of MAPK under I/R conditions.

The MAPKs including JNK, p38, and ERK, all of which up-regulate AP-1-DNA binding activity (14), are simultaneously activated in the lung during ischemia and reperfusion (Figs. 2 and 3), suggesting that both aggravating signals (related to either JNK or p38) and protective signals (related to ERK) interact in a complicated manner at the same time in I/R-induced lung injury. Although the summated activities of these MAPKs should be taken as the factor explaining the overall transitional changes in AP-1-DNA binding activity during ischemia and reperfusion, we consider that the contribution of JNK and/or ERK may be more important than that of p38 for AP-1-DNA binding activity, because the increment of phosphorylation of JNK and/or ERK was considerably larger than that of p38 in both periods of ischemia and reperfusion (Fig. 2).

Time course of NF- κ B-DNA binding activity

We found that NF- κ B was differentially activated in comparison with AP-1; i.e., NF- κ B-DNA binding activity was enhanced in the reperfusion period but not in the ischemic period (Fig. 1). This was supported by the transitional changes in I κ B- α degradation (Fig. 2). These results suggest that NF- κ B may play an important role especially in the reperfusion period. Proinflammatory signaling pathways associated with NF- κ B and AP-1 are expected to act cooperatively to promote lung injury during reperfusion.

In conclusion, applying the three JNK inhibitors including SP600125, L-JNKI1, and EGCG, we confirmed that JNK activation would play a pivotal role in aggravating the lung injury caused by ischemia and reperfusion. The inhibition of JNK activity, in turn, led to a decrease in AP-1-DNA binding accompanied by morphological improvement as well as significant reduction in LDH release, protein leakage, and TNF- α production in the BALF after reperfusion. In addition, inhibition of the JNK/AP-1 signaling pathway decreased the tissue apoptosis after reperfusion. Based on these findings, we concluded that inhibition of JNK activity could be tried as an effective therapeutic strategy against I/R injury during lung transplantation.

Acknowledgments

We are extremely grateful to Dr. E. Ikeda, Dr. N. Watanabe, Dr. O. Takeuchi, and Dr. S. Tasaka for their helpful advice.

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