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Inhibition of c-Jun NH$_2$-Terminal Kinase Activity Improves Ischemia/Reperfusion Injury in Rat Lungs

Makoto Ishii,* Yukio Suzuki,† Kei Takeshita,† Naoki Miyao,* Hiroyasu Kudo,* Rika Hiraoka,* Kazumi Nishio,* Negato Sato,* Katsuhiko Naoki,* Takuya Aoki,* and Kazuhiro Yamaguchi†*

Although c-Jun NH$_2$-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-$k$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-$k$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-$k$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 injury. 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 NH$_2$-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 caused by I/R during 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-$k$B, and degradation of Ix$B$-α, an inhibitory protein of NF-$k$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 (anil[1H-]imidazole-6[2H]-one) (29, 30), on the activities of JNK, p38, and ERK, and the DNA binding activities of AP-1 and NF-$k$B; 4) the effects of JNK inhibition on lung injury evaluated by...

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2 Abbreviations used in this paper: I/R, ischemia/reperfusion; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH$_2$-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; LDH, lactate dehydrogenase; BAF1, bronchoalveolar lavage fluid; L-JNKI1, JNK peptide inhibitor 1 (L-stereoisomer); EC, extracorporeal membrane oxygenator; BCA, bicinchoninic acid.
PROTECTION OF I/R LUNG INJURY BY JNK INHIBITION

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 150 N; Taitec, Tokyo, Japan) for up to 3 h. The hematocrit was adjusted to 10.5 ± 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, 5% and 5% CO2 flowed into the ECMO, allowing adjustment of the perfusate PO2, PCO2, and pH to 140/40 mm Hg, 35 ± 5 mm Hg, 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 ± 0.2°C and to avoid desiccation of the lung surface.

Experimental protocols

The whole lungs 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 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, and those in the perfusion solution were kept to 5 and 10 µM. Lung tissues in which JNK was inhibited by L-JNKI1 were classified 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 SF-IR group, we simply examined the inhibitory effects of these agents on JNK activity in the lung tissue and their advantages over preventing I/R-elicted 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) centrifuged at 15,000 g for 30 s at 4°C, and the supernatants were collected and centrifuged for 5 min at 5,000 rpm. The pellet nuclei were resuspended at 4°C in 300 µl solution B (25% glycercol, 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 benzamidine, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 5 µg/ml aprotinin) and incubated on ice for 20 min. Samples were centrifuged at 15,000 rpm for 1 min, and the supernatants were collected as nuclear protein contained at −30°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′-CGGTGATGAGTCAAGGCGAA-3′ and 5′-AGTTGAGGACTTCCCGAGC-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). 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 oligonucleotide for an additional 20 min (n = 4 for either AP-1 or NF-κB).

Western blotting analysis for JNK, p38, ERK, and IκB-α

Whole lung tissues were homogenized using a Dounce tissue homogenizer on ice in 1 × 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 µg/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 mixture were mixed with 2× SDS sample buffer containing 20% glycerol, 4% SDS, 0.16 M Tris-HCl (pH 6.8), 4% 2-ME, and 0.5% bromphenol 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 re-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 IkB-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 1×B-α, lung homogenate samples were treated with a primary Ab against phospho-1×B-α (n = 3 for each; Cell Signaling), IkB-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; Amerham 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 μl cell lysate containing 250 μg 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 μl cell lysate containing 200 μg total protein using the mAb for phospho-p38 or phospho-ERK, respectively. After that, each pellet was suspended in the kinase buffer containing both ATP and fusion protein of either AT-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× 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 by this method was centrifuged at 2000 g for 10 min, and then used for assaying LDH, total protein, and TNF-α concentrations.

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-μm 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 1×R-induced apoptosis in the lung tissue was evaluated by the methods of TUNEL and DNA ladder detection. Paraffin sections with 3-μm thickness were used for assay with the TUNEL method (ApopTag 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 diamobenzidine 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 by ×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 DIA Na (M&K Instruments Trading, Osaka, Japan).

Localization of cells with activated JNK

First, 3-μm 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 ENVIEN+®HRP kit (DakoCytomation, Carpinteria, CA). After deparaffinization and dehydration, the sections were autochloled 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 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 and incubated with anti-mouse secondary Ab for 1 h at room temperature, and washed again with TBS. Subsequently, the samples were stained with diamobenzidine 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 ± 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 Scheffe’s test (StatView II; Abacus Concepts, Inc., 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-κB 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-κB 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-κB 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-κB-DNA binding was confirmed by competition assay (Fig. 1F).

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

The Journal of Immunology
Transition changes in phosphorylation of p38 in both groups. See text for further details.

(19.3-fold) after the start of reperfusion, and the phosphorylation was maintained throughout the period of reperfusion (Fig. 2C). Phosphorylation of ERK was marked enhanced in the early phase (11.1-fold at 30-min ischemia), but gradually decreased in the late phase, during ischemia in the DMSO group (Fig. 2G). The phosphorylated protein level of ERK was markedly enhanced at 30 min (19.3-fold) after the start of reperfusion, and the phosphorylation was sustained or further increased as reperfusion continued. Phosphorylation of ERK was little influenced by JNK inhibition during both ischemia and reperfusion in the SP-IR group (Fig. 2H).

**Kinase activities of JNK, p38, and ERK in absence and presence of JNK inhibitor**

JNK activity detected by the in vitro kinase assay in the DMSO group showed two peaks; at 30 min during ischemia and again after the start of reperfusion, the latter being sustained throughout the period of reperfusion (Fig. 3A). This tendency was qualitatively the same as that of phosphorylated JNK expression (Fig. 2C). The increased activity of JNK under the conditions of ischemia and reperfusion investigated in the DMSO group was clearly inhibited in the SP-IR group (Fig. 3B).

The increase in p38 activity in the DMSO group was mild in the ischemic period but was significant during reperfusion (Fig. 3C), increased as reperfusion continued (Fig. 2C). Phosphorylation of JNK during both ischemia and reperfusion was slightly depressed by a JNK inhibitor in the SP-IR group (Fig. 2D). Although the transitional changes in AP-1-DNA binding activity during ischemia and reperfusion in the absence of a specific JNK inhibitor roughly paralleled those in phosphorylated JNK (Figs. 1A and 2C), they appeared to be not entirely explained by the changes in JNK phosphorylation alone, requiring additional factors such as p38 and ERK.

The transitional changes in phosphorylated p38 appeared to qualitatively differ from those in phosphorylated JNK in the DMSO group (Fig. 2E); i.e., phosphorylation of p38 was augmented at 30 min (1.8-fold) after the start of ischemia and this augmentation was sustained until the end of ischemia. Phosphorylation of p38 was again enhanced at 30 min (5.2-fold) after the start of reperfusion, and this enhancement continued during the reperfusion phase. In addition, the relative increments of p38 phosphorylation seemed to be much lower than those of JNK in both periods of ischemia and reperfusion. A JNK inhibitor exerted little influence on phosphorylation of p38 in the SP-IR group (Fig. 2F).

**Protein level of phosphorylated proteins**

In comparison with the control, the protein level of phosphorylated JNK in the DMSO group was markedly increased at 30 min (8.9-fold) followed by a gradual decrease, but was slightly increased again at 4 h (6.5-fold) under ischemic conditions (Fig. 2B). Phosphorylated JNK expression was markedly enhanced in the early phase of reperfusion (25.2-fold at 30-min reperfusion), but it decreased as reperfusion continued (Fig. 2C). Phosphorylation of p38 was again enhanced at 30 min (5.2-fold) after the start of reperfusion, and this enhancement continued during the reperfusion phase. In addition, the relative increments of p38 phosphorylation seemed to be much lower than those of JNK in both periods of ischemia and reperfusion. A JNK inhibitor exerted little influence on phosphorylation of p38 in the SP-IR group (Fig. 2F).

Phosphorylation of ERK was markedly enhanced in the early phase (11.1-fold at 30-min ischemia), but gradually decreased in the late phase, during ischemia in the DMSO group (Fig. 2G). The phosphorylated protein level of ERK was markedly enhanced at 30 min (19.3-fold) after the start of reperfusion, and the phosphorylation was sustained or further increased as reperfusion continued. Phosphorylation of ERK was little influenced by JNK inhibition during both ischemia and reperfusion in the SP-IR group (Fig. 2H).

**FIGURE 1.** Transitional changes in DNA binding activity of AP-1 and NF-κB in the absence (DMSO group) and presence (SP-IR group) of JNK inhibitor SP600125 during ischemia and reperfusion. A and B, AP-1-DNA binding activity in the absence of JNK inhibitor (DMSO group) and in the presence of JNK inhibitor (SP-IR group), respectively. C and D, NF-κB-DNA binding activity in DMSO and SP-IR groups, respectively. E, Competition assay for AP-1-DNA binding. F, Competition assay for NF-κB-DNA binding. See text for further details.

**FIGURE 2.** Transitional changes in phosphorylation of IκB-α, JNK, p38, and ERK during ischemia and reperfusion. A and B, Phosphorylated and total IκB-α in the absence (DMSO group) and presence (SP-IR group) of JNK inhibitor SP600125, respectively. C and D, Phosphorylation of JNK in DMSO and SP-IR groups, respectively. E and F, Phosphorylation of p38 in both groups. G and H, Phosphorylation of ERK in both groups. See text for further explanation.
in accordance qualitatively with the tendency observed for phosphorylated p38 (Fig. 2E). The augmentation of p38 activity was not influenced by JNK inhibition in the SP-IR group (Fig. 3D).

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

**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 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. 4C). The increased level of TNF-α was restored to the baseline in the SP-IR group but not in the SP-I group (Fig. 4C).

**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. 6A), 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 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.

**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.

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

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. 7E). Phosphorylated JNK was weakly positive in bronchial and vascular smooth muscle cells (Fig. 7, D and F).

Effects of L-JNKI1 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. 8A). The degree of 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 obtained in the control and DMSO groups.

FIGURE 6. Decrease in apoptosis by JNK inhibition. A, Control lung section stained with TUNEL method (magnification: ×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: ×200). C, The same lung tissue section as presented in B but with ×1000 magnification. D, Lung tissue section obtained at the end of reperfusion in the presence of JNK inhibitor SP600125 (SP-IR group) (magnification: ×200). E, Relative frequency of TUNEL-positive cells in three fields of lung tissue section (×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.

FIGURE 7. Immunohistochemical staining of cells with activated JNK. A, Control lung tissue (×200). B, Control lung tissue (×1000). C–F, Lung tissue sections in DMSO group (C, ×200; D, ×1000; E, ×1000; F, ×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.

FIGURE 8. Effects of L-JNKI1 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-JNKI1 (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-κB-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 ERK (Figs. 2 and 3). 5) Lung injury and apoptosis caused by I/R was degree of activation upon I/R stimulation seemed to be lowest for ERK (Figs. 2 and 3). 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-κB 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-κB-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-κB, both of which inhibit JNK-induced apoptosis (20, 36). Since we found that ERK and NF-κB 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-κB 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, Ilesalniks 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 summed activities of these MAPKs should be taken into account 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-κB-DNA binding activity

We found that NF-κB was differentially activated in comparison with AP-1; i.e., NF-κB-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 IκB-α degradation (Fig. 2). These results suggest that NF-κB may play an important role especially in the reperfusion period. Proinflammatory signaling pathways associated with NF-κB 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 EGGCG, 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.

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References