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Nuclear Factor-κB Decoy Oligodeoxynucleotides Prevent Acute Lung Injury in Mice with Cecal Ligation and Puncture-Induced Sepsis

Naoyuki Matsuda, Yuichi Hattori, Subrina Jesmin, and Satoshi Gando

Departments of Pharmacology (Y.H.) and Anesthesiology and Critical Care Medicine (N.M., S.J., S.G.), Hokkaido University Graduate School of Medicine, Sapporo, Japan

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ABSTRACT

The transcription factor nuclear factor-κB (NF-κB) plays a key role in expression of many inflammatory genes responsible for the pathophysiology of sepsis-induced acute lung injury. We investigated whether the introduction of synthetic double-stranded oligodeoxynucleotides (ODNs) with consensus NF-κB sequence as transcription factor decoy can prevent acute lung injury with suppression of pulmonary expression of multiple genes involved in its pathological process in a cecal ligation and puncture septic mouse model. NF-κB decoy ODNs were introduced with the aid of the hemagglutinating virus of Japan-envelope vector method. Northern blot analysis indicated that transfection of NF-κB decoy ODN, but not of its scrambled form, resulted in a significant inhibition of sepsis-induced gene overexpression of inducible nitric-oxide synthase (iNOS), cyclooxygenase-2, histamine H_1-receptor, platelet-activating factor receptor, and bradykinin B_1 and B_2 receptors in lung tissues. Histological damage in lungs (wall thickening, inflammatory infiltrate, and hemorrhage), increased pulmonary vascular permeability, and blood gas exchange impairment were clearly documented in mice after cecal ligation and puncture. These changes were strongly eliminated by the introduction of NF-κB decoy but not of scrambled ODN. The effects of the iNOS inhibitor FR260330 on these histological and functional derangements compared unfavorably with those of NF-κB decoy ODN transfection. Our results suggest that ODN decoy, acting as in vivo competitor for the transcription factor's ability to bind to cognate recognition sequence, may represent an effective strategy in the treatment of septic acute lung injury.

One of the common lethal complications of endotoxemia is ARDS. ARDS is the most severe form of acute lung injury that is clinically characterized by refractory hypoxemia, diffuse pulmonary infiltrates, and high permeability pulmonary edema (Lechin and Varon, 1994). Despite significant advances in the treatment of ARDS, the mortality from this syndrome remains high (Nuckton et al., 2002). The current treatment of ARDS is predominately supportive. Current research is being directed to prevention or amelioration of the progression of ARDS by exploiting effective treatment strategies based on the thorough understanding of the molecular pathogenesis of lung injury.

Inflammatory states, such as endotoxemia, lead to overexpression of iNOS, which produces excessive amounts of NO for sustained time periods (Moncada and Higgs, 1995). High levels of NO from iNOS have been assumed to be a key event in the pathogenesis of several inflammatory lung diseases, including sepsis-induced lung injury (Kristof et al., 1998; Hinder et al., 1999). Excessive NO generation increases pulmonary vascular permeation (Worral et al., 1997) and may contribute to lung edema formation (Heremans et al., 2000). In addition, NO from iNOS may cause inappropriate vasodilation, which is a key feature in septic lung failure (Ullrich et al., 1999). NO synthase inhibitors could prevent acute lung injury observed in animals during endotoxin-induced sepsis (Heremans et al., 2000). Furthermore, studies in mice deficient in iNOS gene activity have shown that they are more resistant to endotoxin-induced acute lung injury than wild-type animals (Kristof et al., 1998; Weimann et al., 1999). Thus, iNOS-dependent NO overproduction has been principally implicated in the pathophysiologic mechanisms of ARDS.

The promoter region of iNOS gene contains at least one putative NF-κB consensus sequence that has been shown to act as positive regulatory element for iNOS transcription in

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ABBREVIATIONS: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BK, bradykinin; CLP, cecal ligation and puncture; COX, cyclooxygenase; iNOS, inducible nitric-oxide synthase; NF-κB, nuclear factor-κB; NO, nitric oxide; ODN, oligodeoxynucleotide; PAF, platelet-activating factor; kb, kilobase pair(s).

murine macrophages (Xie et al., 1994). NF-κB is a member of the *Rel* family proteins and is typically a heterodimer of p50 and p65 subunit (Bäurle and Baltimore, 1996). In quiescent cells, NF-κB resides as a latent cytoplasmic complex bound to its inhibitory protein IκB. Through nonvalent association, IκB masks the nuclear localization signal of NF-κB, thereby preventing NF-κB nuclear translocation. Stimulation of cells with endotoxin triggers a series of signaling events that ultimately converge to activation of one or more redox-sensitive kinases that specifically phosphorylate IκB, resulting in IκB polyubiquitination and subsequent degradation, followed by liberation of NF-κB (Bäurle and Henkel, 1994). NF-κB then translocates to the nucleus and regulates synthesis of multiple molecules involved in inflammatory responses, including iNOS (Bäurle and Baltimore, 1996). Several studies on this activation pathway of NF-κB led to the discovery of new agents capable of blocking transcriptional activity of this transcriptional factor (Nathens et al., 1997; Pierce et al., 1997; Sheehan et al., 2002). The decoy strategy has recently been developed (Bielins et al., 1990) and is considered a useful tool for analyzing the blockade of expression of a wide variety of NF-κB-dependent proinflammatory mediators. Indeed, recent evidence has shown that synthetic double-stranded ODNs as decoy *cis* elements block the binding of NF-κB to promoter regions of its targeted genes, resulting in the inhibition of gene transcription in vivo (Morishita et al., 1997).

We very recently demonstrated that in vivo ODN decoy to NF-κB reduced lipopolysaccharide-induced lung injury in mice (Matsuda et al., 2004a). In the present study, we used a more clinically relevant mouse model of sepsis. The CLP model, which causes peritonitis, leads to a polymicrobial sepsis and represents an indirect insult similar to the pathogenesis of ARDS (Villar et al., 1994). Furthermore, for in vivo gene transfer, the hemagglutinating virus of Japan envelope vector system was employed in this study. The hemagglutinating virus of Japan envelope vector has been shown to be more effective for in vivo gene transfer compared with other commercially available transfer kits (Kaneda et al., 2002). We examined in the CLP model whether double-stranded ODN decoy to NF-κB can suppress the enhanced pulmonary expression levels of iNOS gene and a number of genes for target molecules that may play a role in the development of lung edema and/or infiltrates. Our final goal was to assess the potential of ODN decoy to NF-κB to mitigate septic lung injury in this in vivo rodent model.

Materials and Methods

Animal Preparation. This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Care and handling of the animals were in accord with National Institutes of Health guidelines. The previously described model of CLP-induced sepsis (Baker et al., 1983) was used with minor modifications. In brief, male ICR mice (25–30 g) were lightly anesthetized with gaseous diethyl ether under aseptic conditions. After shaving the abdominal fur and application of a topical disinfectant, a 4-mm paramedian incision was made through the skin and peritoneum of the left abdomen to exposure the cecum. The cecal appendage was filled with stool, ligated at 3 mm from the tip, and then perforated in two locations with a 21-gauge needle distal to the point of ligation. After this, a small amount of stool was extruded through both puncture holes to ensure patency. Finally, the bowel was repositioned, and the abdomen was closed with sterile suture. Sham-operated animals underwent the same procedure except for ligation and puncture of the cecum.

Synthesis of ODN and Selection of Sequence Targets. The previously reported sequences of phosphorothioate double-stranded ODN against NF-κB binding site and of scrambled ODN (Morishita et al., 1997) were used in this study. NF-κB decoy ODN (consensus sequences are underlined): 5′-CCTTGAAGGGATTTCCCTCC-3′ and 3′-GGAACTTCCCTAAAGGGAGG-5′; scrambled decoy ODN: 5′-TTGCCGTACCTGACTTAGCC-3′ and 3′-AACGGCATGGACTGAATCGG-5′.

In Vivo Transfer of ODN. For in vivo gene transfer, the hemagglutinating virus of Japan envelope vector system (HVJ Envelope Vector Kit GenomeONE-Neo; Ishihara Sangyo, Osaka, Japan) was used. This hemagglutinating virus of Japan envelope vector has been proven to be an effective ODN delivery system both in vitro and in vivo (Kaneda et al., 2002). Sterile distilled water (200 μl) containing synthetic double-stranded ODN (80 μg/animal) was infused into the tail vein over 20 s at room temperature 60 min after sepsis induction. The dose of NF-κB decoy ODN was chosen because our preliminary study showed that it was a minimum dose to produce a constant reduction (>70%) in sepsis-induced NF-κB activation in lung tissues. At the times indicated in the text, mice were anesthetized with gaseous diethyl ether, the blood samples were collected by cardiac puncture for blood gas analysis, and lungs were harvested, frozen immediately in liquid nitrogen, and stored at −80°C.

Gel Mobility Shift Assay. The nuclear extract was prepared from lung tissues according to a method reported previously (Morishita et al., 1997). NF-κB decoy ODN was labeled as a probe at the 3′ end by means of a 3′ end-labeling kit (PerkinElmer Life and Analytical Sciences, Boston, MA). After end-labeling, ^{32}P-labeled ODN was separated from unbound ATP using a P-20 column. Nuclear proteins were incubated with the ^{32}P-labeled probe (10,000–15,000 cpm) in the mixtures including polydeoxyinosinic acid for 30 min at room temperature. Nuclear protein-ODN complexes were resolved by electrophoresis on 5% polyacrylamide gel. The gel was dried and autoradiographed with an intensifying screen. As a control, samples were incubated with excessive doses of unlabeled NF-κB ODN, which resulted in the disappearance of signals.

Poly(A)$^+$ RNA Purification and Northern Blot Analysis. Total RNA was extracted from lung tissues by the guanidinium thiocyanate-phenol-chloroform method with Isogen (Nippon Gene, Toyama, Japan) used routinely in our laboratory (Matsuda et al., 1999). Then, Poly(A)$^+$ RNA was purified from total RNA using an Oligotex-dT30 <Super> mRNA Purification kit (Takara, Ohtsu, Japan), and the amount of extracted Poly(A)$^+$ RNA was determined by UV absorption. Northern blot was performed as described previously (Matsuda et al., 2000) with slight modifications. In brief, samples (4 μg) were electrophoresed on agarose/formaldehyde gels and transferred to a Hybond-N$^+$ nylon membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The filter was baked, prehybridized, and hybridized with ^{32}P-labeled oligonucleotides or full-length cDNA for target molecules. The intensity of hybridization was visualized by autoradiography. Expression of mRNA was quantitated by counting the radioactivity using a Bioimaging Analyzer (Fujix BAS 2000; Fuji Photo Film, Tokyo, Japan). To control for differences in RNA content, the membranes were sequentially probed for β-actin after stripping. Thus, the amount of target molecule mRNA was normalized to the mRNA of the constitutively expressed protein β-actin on the same filter.

Histological Examination. At the time of kill, the trachea was cannulated with a catheter, and the lungs were inflated with neutral buffered 4% formalin and were subsequently paraffin embedded. The tissues were then sectioned at a thickness of 5 μm and stained with hematoxylin and eosin using standard methods. Light microscopic analysis of lungs was performed by blinded observers to evaluate the presence of significant lung injury. Several sections from each lung from various probes were examined, and the results were documented by photographs.

Immunohistochemistry. Sections of fixed lung tissue were deparaffinized and treated with 3% hydrogen peroxide for 15 min to block the endogenous peroxidase activity. Antigen retrieval was performed by microwave pretreatment. Citric acid buffer (10 mM citric acid, pH 6.0) was preheated by incubation in a microwave oven at 750 W for 30 min according to the manufacturer's protocols with modification. After cooling, nonspecific binding of antibody was blocked by nonimmune serum (1% bovine albumin in Tris) for 30 min at room temperature. Then, the primary antibody, Cl:A3-1, which recognizes murine F4/80 antigen expressed by monocytes/macrophages (BMA Biomedicals AG, Augst, Switzerland), was incubated in a moist chamber overnight at 4°C, followed by a suitable secondary antibody coupled to horseradish peroxidase. Immunostains were visualized by light microscopy with diaminobenzidine. The samples processed without the primary antibody served as negative controls.

Lung Vascular Leak Assessment. The lungs were weighed in preweighed aluminum dishes immediately after dissection (wet weight), and again after overnight at 80°C in a drying oven (dry weight) for determination of the lung wet-to-dry ratio.

In another series of experiments to assess lung vascular permeation, mice received intravenous injection of 10 μCi of ^{125}I-labeled albumin 30 min before euthanasia. At the end of the experimental protocol, mice were anesthetized with pentobarbital at a dose that provided general anesthesia without depression of respiration. Then, a polyethylene catheter (22-gauge; JELCO; Critikon, Tampa, FL) was inserted into the trachea, BAL was performed by repeatedly infusing and removing 2 ml of prewarmed saline, and the third drainage of effluent was kept as BAL fluid. Thirty min after ^{125}I-albumin injection, blood samples were obtained from the atrium in the presence of heparin. The radioactivity of the samples was counted in a γ counter. Permeability index was defined as counts per minute (cpm) of ^{125}I in microliters of BAL fluid divided by cpm in microliters of blood.

Statistics. Data are expressed as means ± S.E. Data were analyzed using the StatView II program (Abacus Concepts, Berkeley, CA). Statistical analysis was performed using Student's t test or a repeated-measures one-way analysis of variance followed by Bonferroni's multiple comparison test when appropriate; a P value less than 0.05 was considered significant.

Results

NF-κB Activation in Lungs. Fig. 1A shows gel mobility shift assay for NF-κB activity in nuclear extracts from lung tissues. The NF-κB activity was greatly increased in lungs from mice 10 h after the onset of CLP-induced sepsis compared with those from sham-operated control mice. This NF-κB binding was eliminated by preincubation of nuclear extracts with an excess amount of unlabeled NF-κB decoy ODN but not with excess unlabeled scrambled decoy ODN. Moreover, transfection of NF-κB decoy ODN, but not of scrambled decoy ODN, via intravenous injection resulted in a marked reduction in the binding of NF-κB nearly to the control level in lungs from CLP-induced septic mice. To confirm the identity of the sepsis-induced NF-κB binding complexes, antibody supershift analysis was also performed on nuclear extracts from CLP-induced septic mouse lungs. Thus, the presence of the antibodies to NF-κB subunits of p50 and p65 supershifted the binding complex (Fig. 1B), although the antibody against p65 used in this study (TransCruz antibody; Santa Cruz Biotechnology, Santa Cruz, CA) seems to cross-react with p50 because the band, which could be referred to as p50, was supershifted not only by the p50 antibody but also by the p65 antibody. These results encouraged us to study the potential activity of NF-κB decoy strategy for the treatment of sepsis-induced lung injury by in vivo transfection into lung tissues via intravenous injection.

Pulmonary Expression of κB-Associated Genes. Northern blot analysis using the iNOS probe revealed a major mRNA species of 4.4 kb in mouse lung tissues (Fig. 2A). Because the bioimaging analysis of Northern blots indicated that β-actin mRNA was unaffected by sepsis, this mRNA was used as an internal standard. When normalized to β-actin mRNA levels, 8- and 14-fold increases in iNOS mRNA were found in lungs from mice 5 and 10 h after CLP, respectively, compared with sham-operated mouse lungs (Fig. 4A). Transfection of NF-κB decoy ODN (Figs. 2A and 4A), but not of its scrambled form (Figs. 3A and 4A), greatly inhibited the increase in gene expression of iNOS in lungs from mice after 10 h of CLP.

As illustrated in Fig. 2B, COX-2, an inducible isoform of COX, mRNA was detected as a single band of 4.7 kb in mouse lung tissues. Sepsis induction with CLP caused an increase in COX-2 mRNA in a time-dependent manner (3.6-fold at 5 h and 5.4-fold at 10 h) (Fig. 4B). This increase was strongly suppressed by treatment with wild-type ODN decoy (Figs. 2B and 4B). However, the scrambled ODN decoy failed to inhibit sepsis-induced COX-2 mRNA expression (Figs. 3B and 4B).

Expression of mRNA of the histamine H_1-receptor, which migrated at 3.3 and 3.9 kb, was detected by Northern blot analysis (Fig. 2C), as demonstrated in detail in our recent

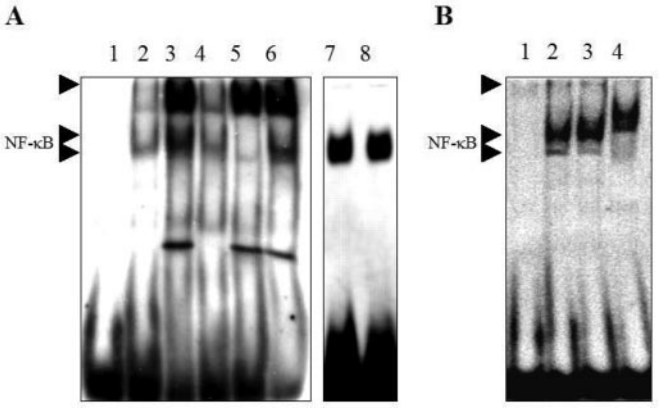

Fig. 1. A, gel mobility shift assay for NF-κB activity in mouse lung tissues. Nuclear extracts from lung tissues under different conditions were used for gel mobility shift assay. Lane 1, free probe showed no detection of NF-κB binding activity; lane 2, nuclear extracts from sham-operated mouse lung tissues were incubated with a ^{32}P-labeled NF-κB probe; lane 3, nuclear extracts from lung tissues of the mouse 10 h after the onset of CLP-induced sepsis were incubated with a ^{32}P-labeled NF-κB probe; lane 4, nuclear extracts from lung tissues of the mouse that was transfected with NF-κB decoy ODN 60 min after CLP were incubated with a ^{32}P-labeled NF-κB probe; lane 5, nuclear extracts from lung tissues of the septic mouse were incubated with a ^{32}P-labeled NF-κB probe in the presence of a 100-fold excess of unlabeled NF-κB ODN; lane 6, detection of NF-κB in nuclear extracts from lung tissues of the septic mouse was unaffected by a 100-fold excess of scrambled sequence ODN. Lanes 6 and 7 show the comparison of NF-κB binding activity in nuclear extracts from lung tissues of the mice 10 h after the onset of CLP-induced sepsis and of the mice that was transfected with scrambled decoy ODN 60 min after CLP. Note that sepsis-induced NF-κB binding activity was unaffected by scrambled decoy ODN transfection. B, nuclear extracts obtained from the lungs of mice subjected to CLP for 10 h were incubated with the antibody to p50 or p65 on ice for 30 min before being processed for electrophoretic mobility shift assay by using the above ^{32}P-labeled probe. Lane 1 shows the probe without nuclear extract. Strong NF-κB DNA binding activity was observed in nuclear extract from the CLP-induced septic mouse lung (lane 2). Specificity of NF-κB was verified by supershift assay in the presence of antibodies specific to p50 (lane 3) and p65 (lane 4).

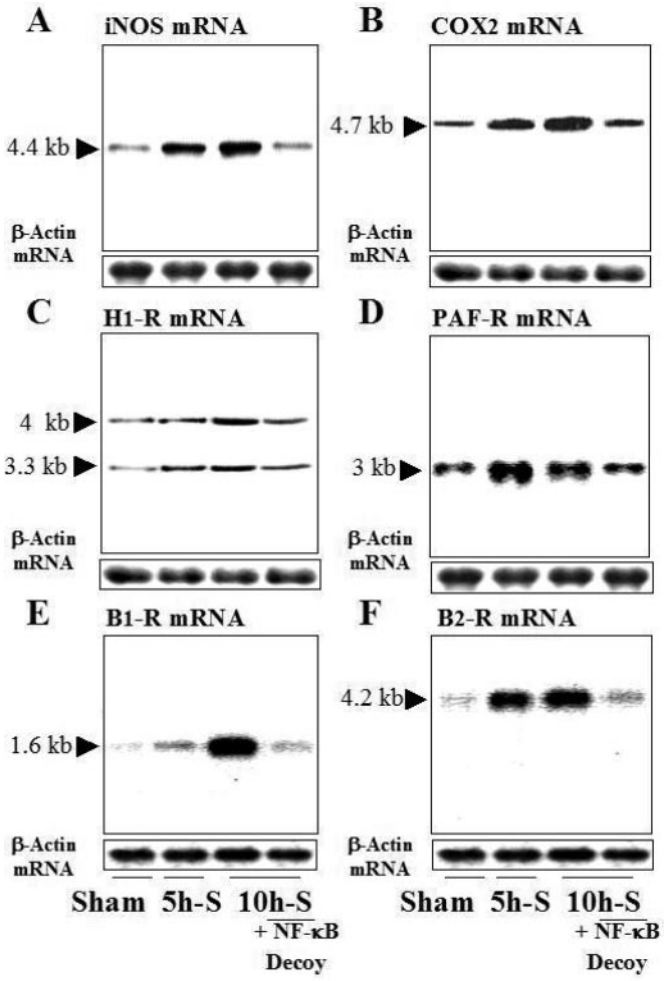

Fig. 2. Autoradiograms of Northern blot analysis of iNOS (A), COX-2 (B), histamine H_1-receptor (C), PAF receptor (D), BK B_1 receptor (E), and BK B_2 receptor (F) mRNAs from lung tissues of mice that were subjected to sham-operation (control), sepsis (5 and 10 h after CLP), and sepsis with transfection of NF-κB decoy ODN (10 h after CLP). β-Actin mRNA served as internal control.

report (Matsuda et al., 2004b). Five and 10 h after induction of sepsis with CLP, the transcript levels of the H_1-receptor was increased 1.8~2.6-fold and 2.9~3.4-fold in lungs, respectively, compared with sham-operated control mice (Fig. 3C). Transfection of NF-κB decoy ODN (Figs. 2C and 4C), but not of its scrambled form (Figs. 3C and 4C), led to a significant decrease in the H_1-receptor gene in lungs from septic mice.

The hybridization with the PAF receptor probe revealed single mRNA for the PAF receptor at 3 kb in mouse lung tissues (Fig. 2D). Its expression level was increased 2.4-fold from the sham-operated control group at 5 h after the onset of CLP (Fig. 4D). Although it declined thereafter, gene expression of the PAF receptor remained at significantly higher levels than the control level 10 h after CLP (1.7-fold). Transfection of NF-κB decoy (Figs. 2D and 4D), but not of scrambled decoy ODN (Figs. 3D and 4D), sharply lowered the increased PAF receptor mRNA expression in septic lungs after 10 h of CLP to the same level as that obtained in the sham-operated control group.

On Northern blots, lungs from sham-operated control mice showed a very low level of two mRNAs of BK receptors, B_1 and B_2, which were detected as single bands of 1.6 and 4.2 kb, respectively (Fig. 2, E and F). However, pulmonary expression levels of B_1 and B_2 receptor mRNAs became awfully abundant from 10 and 5 h after induction of sepsis with CLP, respectively (Fig. 4, E and F). The dramatic increases in B_1 and B_2 receptor genes were strongly prevented by treatment with NF-κB decoy ODN (Figs. 2, E and F and 4, E and F) but not with scrambled decoy ODN (Figs. 3, E and F and 4, E and F).

Histopathology of Lungs. Fig. 5A represents the normal lung architecture observed in sham-operated control mice. Figure 5B represents the histopathological findings in lungs from mice subjected to CLP. At 10 h after CLP-induced sepsis, diffuse pulmonary edema was indicated by alveolar wall thickening. Inflammatory infiltrate and red blood cell extravasation into the interstitium were consistently apparent. Lungs from septic mice that received transfection of scrambled NF-κB decoy showed essentially the same histopathological changes as those from septic mice without treatment (Fig. 5D). NF-κB decoy treatment of septic mice resulted in minimal lung injury, characterized by less distortion of alveolar architecture, scattered interstitial infiltrates, and rare areas of focal hemorrhage (Fig. 5C). Lungs from septic mice treated with the selective iNOS inhibitor

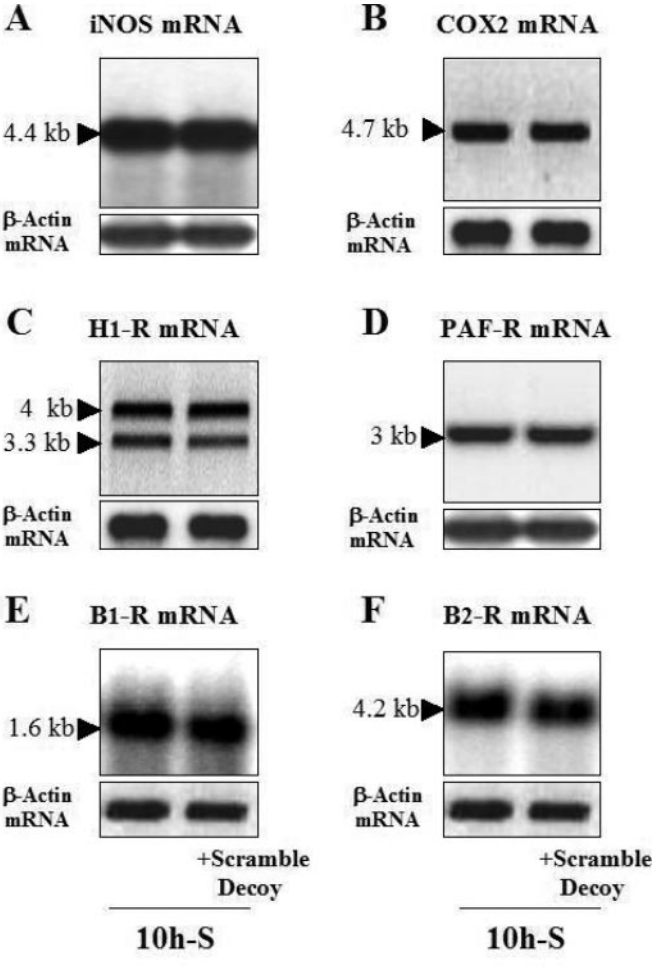

Fig. 3. Lack of effect of scrambled decoy ODN transfection on iNOS (A), COX-2 (B), histamine H_1-receptor (C), PAF receptor (D), BK B_1 receptor (E), and BK B_2 receptor (F) mRNAs from lung tissues of mice that were subjected to sepsis (10 h after CLP). β-Actin mRNA served as internal control.

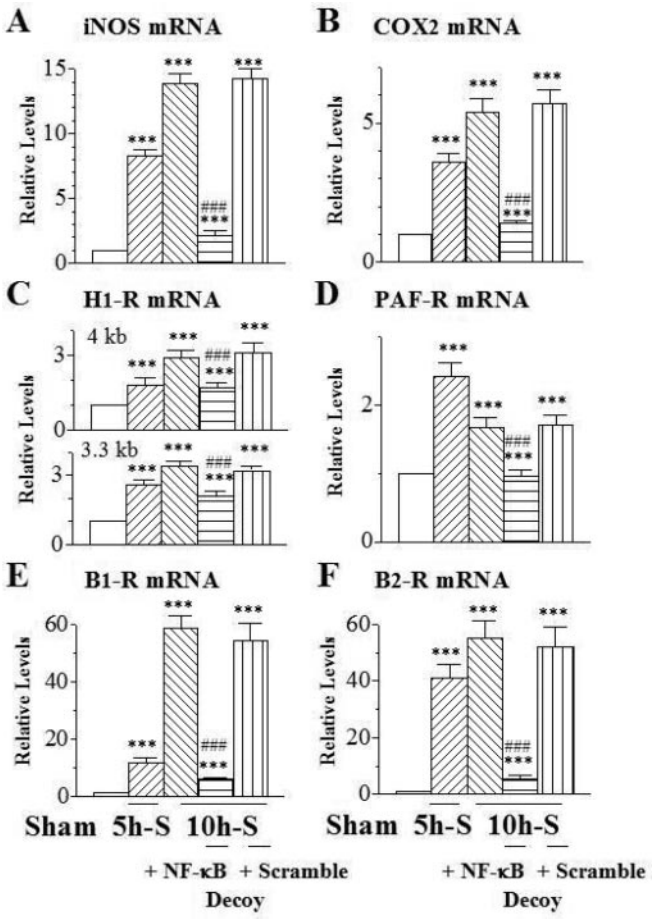

Fig. 4. Relative mRNA levels of iNOS (A), COX-2 (B), histamine H_1-receptor (C), PAF receptor (D), BK B_1 receptor (E), and BK B_2 receptor (F) in lungs as quantified by densitometry and normalized to the signal for β-actin mRNA. Lungs were taken from mice that were subjected to sham-operation (control), sepsis (5 and 10 h after CLP), and sepsis with transfection of NF-κB decoy ODN or its scrambled form (10 h after CLP). To standardize between experiments, an arbitrary density of 1 was assigned to be the band obtained from lungs of sham-operated control mice. Because H_1-receptor mRNA was detected as major two bands of 3.3 and 3.9 kb, the results of densitometric measurement of each transcript are shown. Values are expressed as means ± S.E. ($n = 5$). ***, $P < 0.001$ compared with the sham-operated control group. ###, $P < 0.001$ compared with the 10-h CLP group without treatment.

FR260330 (100 mg/kg, i.p.) exhibited diverse histopathology. Thus, because of the variability from preparation to preparation, the histopathological alterations were detected as minimal, mild, or severe injury (data not shown).

Figure 6 shows the presence of numerous cells, which were identified as monocytes/macrophages based upon immunostaining of the sections for their antigen F4/80, in lungs from mice at 10 h after CLP-induced sepsis but not in those of sham-operated control mice. NF-κB decoy ODN greatly inhibited the migration of these cells into lungs after sepsis.

Pulmonary Edema and Blood Gas Exchange Impairment. As a measure of pulmonary edema, we used the wet-to-dry lung ratios for each group after 10 h of CLP. The CLP group without treatment (5.62 ± 0.12, $n = 5$) showed a significantly higher lung water content than the sham-operated group (4.08 ± 0.06, $n = 5$, $P < 0.001$). This increase in lung water content by sepsis induction was strongly prevented by transfection of NF-κB decoy ODN 60 min after CLP (4.42 ± 0.11, $n = 5$, $P < 0.001$). Transfection of mutated NF-κB decoy ODN had no effect on the increased lung water content in septic mice (5.55 ± 0.15, $n = 5$). Treatment with FR260330 (100 mg/kg, i.p.) 60 min after CLP attenuated partially but significantly the lung water content increase caused by sepsis induction (5.08 ± 0.09, $n = 5$, $P < 0.001$).

For assessment of changes in pulmonary vascular permeability, we also measured the transpulmonary flux of radiolabeled albumin. Compared with the finding in sham-operated mice, a 22-fold increase in lung vascular permeability occurred 10 h after the onset of CLP-induced sepsis (Fig. 7). Transfection of NF-κB decoy ODN, but not of its scrambled form, resulted in a reduction in lung vascular permeability nearly to that in the sham-operated group. Although administration of FR260330 also conferred significant protection against increased lung permeability, the FR260330-treated CLP group still had an 11-fold increase in lung permeability.

To determine whether transfection of NF-κB decoy ODN can mitigate impaired pulmonary blood gas exchange during sepsis, blood gases were measured in arterial blood samples from mice 10 h after the onset of CLP-induced sepsis. As summarized in Table 1, arterial PO_2 showed profound deterioration in the CLP group. This hypoxemia after sepsis induction was strongly blocked when NF-κB decoy ODN was introduced in mice 60 min after CLP. Such an effect was not mimicked by transfection of scrambled decoy ODN. Arterial PCO_2 was also reduced after induction of sepsis as compared with the sham-operated group. Furthermore, septic mice had significantly lowered arterial pH and base excess. Transfection of NF-κB decoy ODN, but not of its scrambled form, led to a significant improvement of the falls in arterial PCO_2, pH, and base excess noticed in septic mice. Treatment with FR260330 (100 mg/kg, i.p.) 60 min after CLP revealed a significant attenuation of the sepsis-induced changes in the values for blood gases, but this effect was less pronounced than that of NF-κB decoy ODN.

Discussion

We showed a novel experimental approach to reduce CLP-induced septic lung injury in mice by using in vivo adminis-

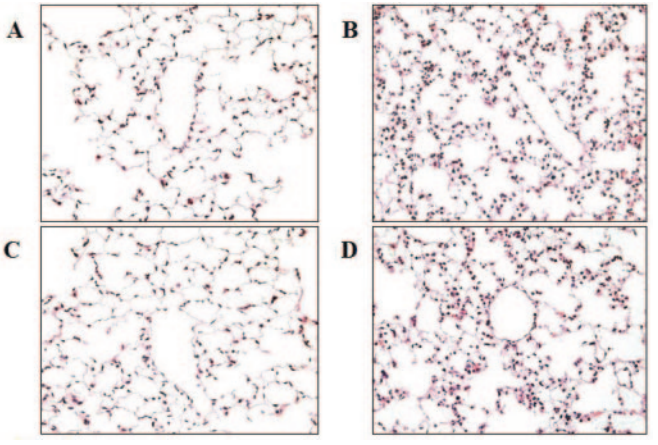

Fig. 5. Representative photomicrographs of lung sections stained with hematoxylin and eosin. This figure shows the comparison between sham-operated control (A), 10-h CLP-induced septic (B), NF-κB decoy transfected septic (C), and scrambled decoy transfected septic animals (D). Sepsis caused severe lung injury (wall thickening, inflammatory infiltrate, and hemorrhage). Such histopathological changes were strongly prevented by transfection of NF-κB decoy ODN but not by transfection of scrambled decoy ODN. Original magnification, 100×.

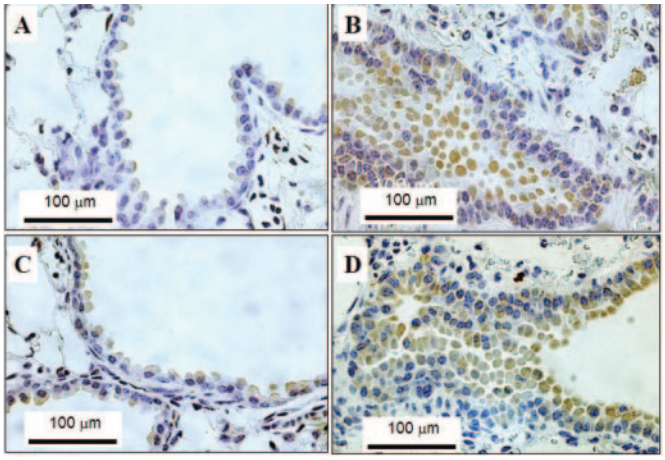

Fig. 6. Immunohistochemical localization of monocytes/macrophages in lung tissues. Tissues were obtained from sham-operated control (A), 10-h CLP-induced septic (B), NF-κB decoy transfected septic (C), and scrambled decoy transfected septic mice (D). The sections of deparaffinized tissues were immunostained for the monocyte/macrophage marker F4/80 as described under *Materials and Methods*. In the septic lung tissue, numerous cells that stain as monocytes/macrophages can be found. Scale bar, 100 μm.

tration of a decoy *cis* element to bind the transcription factor NF-κB. To deliver the transcription factor decoy into lung tissues, we used the hemagglutinating virus of Japan envelope vector method of gene transfer. This method has been shown to enhance the efficiency of transcription of ODNs (Kaneda et al., 2002). The successful in vivo transfer of a sufficient quantity of NF-κB decoy ODN into lungs was demonstrated by the gel shift assay. Thus, transfection of NF-κB decoy ODN via intravenous injection resulted in a great reduction in the increased NF-κB activity in septic lung tissues. In addition, our gel shift assay showed that NF-κB decoy ODN exhibited competitive and specific binding for NF-κB. The specificity of the inhibitory effect of the decoy ODN against NF-κB in lungs could be further supported by

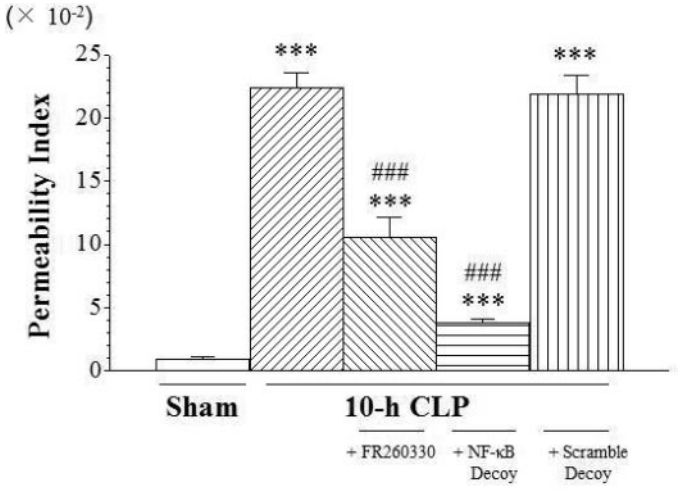

Fig. 7. Changes in lung vascular permeability in mice. The animals were subjected to sham-operation (control), sepsis (10 h after CLP), sepsis with FR260330 treatment (100 mg/kg, i.p.), and sepsis with transfection of NF-κB decoy ODN or its scrambled form. Transpulmonary flux of radiolabeled albumin was used to assess changes in lung permeability. Values are expressed as means ± S.E. ($n = 5$). ***, $P < 0.001$ compared with the sham-operated control group. ###, $P < 0.001$ compared with the CLP group without treatment.

the finding that the use of scrambled decoy ODN was without effect.

Many genes elicited in septic lungs can be regulated by κB sites in the DNA, which bind NF-κB proteins (Li and Verma, 2002). Excessive expression of κB-associated genes may be responsible for acute lung injury during sepsis. Thus, septic lung injury may be mediated by overwhelming inflammatory dysregulation caused by overexpression of not one or several but many κB-regulated genes. We assumed that the inhibition of stimulated NF-κB activity during sepsis by NF-κB decoy ODN would result in suppression of dangerous genes that may play a pivotal role in the pathogenesis of septic lung injury. Based on this assumption, we evaluated pulmonary expression levels of genes for iNOS, COX-2, histamine H_1-receptor, PAF receptor, and BK receptors.

We showed profound increases in gene expression of iNOS and COX-2 in lungs from CLP-induced septic mice. Excessive production of NO via iNOS can result in inappropriate vasodilation and peroxynitrite-related cellular injury (Beckman and Koppenol, 1996) and is thought to be a key mechanism in sepsis-associated pulmonary edema via cytotoxic peroxynitrite formation (Kristof et al., 1998; Hinder et al., 1999). Because of such tissue damage by NO overproduction, iNOS is suggested to play a harmful role in acute lung injury (Beckman and Koppenol, 1996). The elevated production of prostanoids after COX-2 induction may also be important in the pathogenesis of septic lung injury, because prostanoids are released during inflammatory reactions, causing an increase in microvascular hydrostatic pressure, which is a primary cause of edema and a contributory factor in hypovolemia (Griffin et al., 1991). COX-2 is the primary target of various nonsteroidal anti-inflammatory drugs to diminish inflammation (Laneuville et al., 1994). Moreover, many of the clinically important anti-inflammatory agents share the ability to inhibit NF-κB activation and COX-2 gene expression (Kopp and Ghosh, 1994; Fernandez de Arriba, 1999). The promoter regions of COX-2 and iNOS genes in murine cell lines contain at least one putative NF-κB consensus sequence to act as positive regulatory element for their transcription (Xie et al., 1994; Yamamoto et al., 1995). In this study, we demonstrated that in vivo ODN decoy to NF-κB reduced the gene expression levels of iNOS and COX-2 in CLP-induced septic mice. This would result in suppression of excessive production of NO and prostanoids, both of which seem to play a harmful role in septic lungs.

As fully demonstrated in our recent report (Matsuda et al., 2002), circulating levels of histamine are greatly elevated in endotoxemic animals. This elevation is thought to be associated with a marked increase in tissue expression of histidine decarboxylase, which catalyzes decarboxylation of L-histidine and is the only enzyme that forms histamine in mammals (Matsuda et al., 2002). Furthermore, we provided evidence of up-regulation of histamine H_1-receptors in cardiovascular tissues during endotoxemia (Matsuda et al., 2002). In agreement with this, the current study showed a significant increase in H_1-receptor mRNA in lungs from CLP-induced septic mice. Histamine can affect pulmonary vascular response, lung mechanics, and pulmonary vascular permeability, effects that may be blocked by H_1-receptor antagonism (Brigham et al., 1980). Thus, histamine may be a potentially important mediator in the pathogenesis of septic lung injury. In this study, NF-κB decoy ODN treatment significantly

TABLE 1

Blood gases in mice

The values are expressed as the means ± S.E. of five animals. Data were obtained from mice 10 h after sham-operation or CLP. FR26330 (10 mg/kg, i.p.) was administered or NF-κB decoy ODN or its scrambled form was transfected 60 min after CLP.

	PaO_2	$PaCO_2$	pH	Base Excess
	mm Hg	*mm Hg*		*mM*
Sham-operated	123.0 ± 1.4	34.0 ± 0.9	7.43 ± 0.02	0.6 ± 0.5
10-h CLP	70.7 ± 3.5*	20.5 ± 0.6*	7.17 ± 0.02*	−11.2 ± 0.6*
+ FR260330	87.6 ± 3.0*†	28.4 ± 1.5*†	7.25 ± 0.02*†	−6.9 ± 0.6*†
+NF-kB decoy	109.7 ± 3.8†	32.6 ± 0.9†	7.35 ± 0.02†	−9.7 ± 0.6†
+Scrambled decoy	67.7 ± 4.7*	21.0 ± 1.2*	7.17 ± 0.03*	−11.7 ± 1.0*

* Significant difference from sham-operated control values.
† Significant difference from values obtained in CLP-induced septic mice without treatment.

attenuated the sepsis-induced increase in pulmonary expression of the H_1-receptor gene, but an increase of ~40% above control was still observed even after this gene therapy. This is because transcription factors other than NF-κB, such as activator protein-1 or CCAAT/enhancer binding protein-β, may participate in this gene expression. Therefore, only the blockage of NF-κB activation may be insufficient for suppression of the induction of H_1-receptors.

PAF is much more potent than histamine in increasing vascular permeability (Humphrey et al., 1984), and PAF-induced hyperpermeability may be relevant for the development of inflammatory reaction. Despite variable results obtained in the studies of the therapeutic efficacy of PAF antagonists in experimental models of sepsis or endotoxemia, PAF has been considered a potential candidate for mediation of lung complications of septic shock (Montrucchio et al., 2000). We observed that pulmonary expression of the PAF receptor gene was markedly increased at 5 h after CLP-induced sepsis and declined thereafter, although it did remain significantly elevated throughout the 10-h study period. It is thus possible that PAF may partially contribute to the early event in pulmonary derangements that can be initiated by sepsis induction. The exhaustive inhibition by NF-κB decoy of overexpression of PAF receptor mRNA was found in septic lungs, assuming that in the promoter region of the PAF receptor gene, NF-κB may play a key role in transcriptional induction by inflammatory insults.

The proinflammatory effects of BK include vasodilation, increased vascular permeability, generation of microthrombi, and organ failure, suggesting a role in certain aspects of the septic syndrome. BK exerts its effects by selective activation of two distinct receptors termed B_1 and B_2. Although B_2 receptors are constitutively expressed and mediate the majority of the acute vascular actions of BK, B_1 receptors are generally absent from healthy tissues; after inflammatory insults, however, including endotoxin, receptor expression is rapidly induced in a variety of cell types (Regoli et al., 1993). However, the present study showed that both B_1 and B_2 receptor genes were poorly expressed in normal mouse lungs. When mice were subjected to CLP-induced sepsis, gene expression levels of both B_1 and B_2 receptors were dramatically increased in lungs. Besides, the B_2 receptor gene appeared more rapidly and sharply than the B_1 receptor gene. This suggests that sepsis causes superinduction of not only B_1 receptor genes but also B_2 receptor genes in mouse lungs. NF-κB binding domains have been identified in the promoter region of the B_1 receptor (Ni et al., 1998). Although there is no evidence that B_2 receptors are regulated during inflammation, interleukin-1β can increase B_2 as well as B_1 receptor mRNA and protein in human lung fibroblasts (Phagoo et al., 2000), and interleukin-1β-induced up-regulation of B_1 receptors occurs through activation of NF-κB (Ni et al., 1998). Because NF-κB decoy ODN equally suppressed overexpression of B_1 and B_2 receptor genes in septic lungs, superinduction of the B_2 receptor gene promoted by sepsis may be transcriptionally controlled by NF-κB.

In addition to the above-mentioned enzymes and receptors, numerous proinflammatory cytokines and adhesion molecules are regulated by κB sites (Bäurle and Henkel, 1994; Collins et al., 1995; Beckman and Koppenol, 1996) and may play a critical role in inflammatory lung injury during sepsis. Elevated levels of proinflammatory cytokines correlate with life-threatening inflammatory injuries, particularly septic shock and ARDS (Pittet et al., 1997). Exogenous proinflammatory cytokines can mimic the pathophysiology of these disease processes, and inhibition of endogenous cytokines can prevent lung injury in septic animal models (Russell et al., 1995). Accordingly, NF-κB activation of these proinflammatory gene products seems to play an important role as central features of the pathogenesis of septic shock and multiorgan failure, although these cytokines may be required for the host-defense response.

Herein, we clearly demonstrated that in vivo transfection of NF-κB decoy ODN resulted in a dramatic improvement of histological damage in lungs, increased pulmonary vascular permeability, and blood gas exchange impairment in mice with CLP-induced sepsis. This beneficial effect would be the result of suppression of overexpression of κB-associated dangerous genes involved in inflammatory lung injury during sepsis. The selective iNOS inhibitor FR260330 was only partially effective in the sepsis-induced pulmonary derangements. Thus, the results with FR260330 support the idea that septic lung injury involves multiple mediators, often with overlapping dysfunctions, and therapeutic strategies inhibiting only actions of one mediator are insufficient.

The inability to inhibit multiple inflammatory molecules involved in the pathophysiology of sepsis-induced acute lung injury represents a major hurdle in this treatment. We demonstrated that in vivo transfection of NF-κB decoy ODN suppressed the transactivation of many κB-associated genes and prevented acute lung injury during CLP-induced sepsis. Although a number of important issues, such as safety and side-effects, have to be addressed in further study, the present results suggest that transcription decoy strategy, by blocking NF-κB activation and expression of κB-associated dangerous genes, may provide a new approach for the treatment of acute lung injury resulting from sepsis.

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References

Baker CC, Chaudry IH, Gaines HO, and Baue AE (1983) Evaluation of factors affecting mortality rate after sepsis in murine cecal ligation and puncture model. *Surgery* **94:**331–335.

Bäurle PA and Baltimore D (1996) NF-κB: ten years after. *Cell* **87:**13–20.

Bäurle PA and Henkel T (1994) Function and activation of NF-κB in the immune system. *Annu Rev Immunol* **12:**141–179.

Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271:**C1424–C1437.

Bielins A, Schivdasani RA, Zhang I, and Nabel GJ (1990) Regulation of gene expression with double stranded phosphorothioate oligonucleotides. *Science (Wash DC)* **250:**997–1000.

Brigham KL, Padove SJ, Bryant D, McKeen CR, and Bowers RE (1980) Diphenhydramine reduces endotoxin effects on lung vascular permeability in sheep. *J Appl Physiol* **49:**516–520.

Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, and Maniatis T (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* **9:**899–909.

Fernandez de Arriba A, Cavalcanti F, Miralles A, Bayon Y, Alonso A, Merlos M, Garcia-Rafanell J, and Forn J (1999) Inhibition of cyclooxygenase-2 expression by 4-trifluoromethyl derivatives of salicylate, triflusal, and its deacetylated metabolite, 2-hydroxy-4-trifluoromethylbenzoic acid. *Mol Pharmacol* **55:**753–760.

Griffin MP, Gore DC, Lobe TE, Flynn JF, Traber DL, and Herndon DN (1991) Ibuprofen therapy in experimental porcine Gram-negative septic shock. *Resuscitation* **22:**75–83.

Heremans H, Dillen C, Groenen M, Matthys P, and Billiau A (2000) Role of interferon-gamma and nitric oxide in pulmonary edema and death induced by lipopolysaccharide. *Am J Respir Crit Care Med* **161:**110–117.

Hinder F, Stubbe HD, van Aken H, Waurick R, Booke M, and Meyer J (1999) Role of nitric oxide in sepsis-associated pulmonary edema. *Am J Respir Crit Care Med* **159:**252–257.

Humphrey DM, McManus LM, Hanahan DJ, and Pinckard RN (1984) Morphologic basis of increased vascular permeability induced by acetyl glyceryl ethyl phosphorylcholine. *Lab Invest* **50:**16–25.

Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, Suzuki N, Nakamura H, Morishita R, and Kotani H (2002) Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther* **6:**219–226.

Kopp E and Ghosh S (1994) Inhibition of NF-κB by sodium salicylate and aspirin. *Science (Wash DC)* **265:**956–959.

Kristof AS, Goldberg P, Laubach V, and Hussain SNA (1998) Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* **158:**1883–1889.

Laneuville O, Breuer DK, CeWitt DL, Hla T, Funk CD, and Smith WL (1994) Differential inhibition of human prostaglandin endoperoxide H synthase-1 and −2 by nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther* **271:**927–934.

Lechin AE and Varon J (1994) Adult respiratory syndrome (ARDS): the basics. *J Emerg Med* **12:**63–68.

Li Q and Verma IM (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2:**725–734.

Matsuda N, Hattori Y, Akaishi Y, Suzuki Y, Kemmotsu O, and Gando S (2000) Impairment of cardiac β-adrenoceptor cellular signaling by decreased expression of $G_{s\alpha}$ in septic rabbits. *Anesthesiology* **93:**1465–1473.

Matsuda N, Hattori Y, Gando S, Akaishi Y, Kemmotsu O, and Kanno M (1999) Diabetes-induced down-regulation of β_1-adrenoceptor mRNA expression in rat heart. *Biochem Pharmacol* **58:**881–885.

Matsuda N, Hattori Y, Sakuraya F, Kobayashi M, Zhang X-H, Kemmotsu O, and Gando S (2002) Hemodynamic significance of histamine synthesis and histamine H_1- and H_2-receptor gene expression during endotoxemia. *Naunyn-Schmiedeberg's Arch Pharmacol* **366:**513–521.

Matsuda N, Hattori Y, Takahashi Y, Nishihira J, Jesmin S, Kobayashi M, and Gando S (2004a) Therapeutic effect of in vivo transfection of transcription factor decoy to NF-κB on septic lung in mice. *Am J Physiol* **287:**L1248–L1255.

Matsuda N, Jesmin S, Takahashi Y, Hatta E, Kobayashi M, Matsuyama K, Kawakami N, Sakuma I, Gando S, Fukui H, et al. (2004b) Histamine H_1 and H_2 receptor gene and protein levels are differentially expressed in the hearts of rodents and humans. *J Pharmacol Exp Ther* **309:**786–795.

Moncada S and Higgs EA (1995) Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J* **9:**1319–1330.

Montrucchio G, Alloatti G, and Camussi G (2000) Role of platelet-activating factor in cardiovascular pathophysiology. *Physiol Rev* **80:**1669–1699.

Morishita R, Sugimoto T, Aoki M, Kita I, Tomita N, Moriguchi A, Maeda K, Sawa Y, Kaneda Y, Higaki T, et al. (1997) *In vivo* transfection of *cis* element "decoy" against nuclear factor-κB binding site prevents myocardial infarction. *Nature (Lond) Med* **8:**894–899.

Nathens AB, Bitar R, Davreux C, Bujard M, Marshall JC, Dackiw APB, Watson RW, and Rotstein OD (1997) Pyrrolidine dithiocarbamate attenuates endotoxin-induced acute lung injury. *Am J Respir Cell Mol Biol* **17:**608–616.

Ni A, Chao L, and Chao J (1998) Transcription factor nuclear factor κB regulates the inducible expression of the human B_1 receptor gene in inflammation. *J Biol Chem* **273:**2784–2791.

Nuckton TJ, Alonso JA, Kallet RH, Daniel BM, Pittet JF, Eisner MD, and Matthay MA (2002) Pulmonary dead-space fractions as a risk factor for death in the acute respiratory distress syndrome. *N Engl J Med* **346:**1281–1286.

Phagoo SB, Yaqoob M, Herrera-Martinez E, McIntyre P, Jones C, and Burgess GM (2000) Regulation of bradykinin receptor gene expression in human lung fibroblasts. *Eur J Pharmacol* **397:**237–246.

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, and Gerritsen ME (1997) Novel inhibitor of cytokine-induced IκBα phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. *J Biol Chem* **272:**21096–21103.

Pittet JF, Mackersie RC, Martin TR, and Matthay MA (1997) Biological markers of acute lung injury: prognostic and pathogenetic significance. *Am J Respir Crit Care Med* **155:**1187–1205.

Regoli D, Jukic D, Gobeil F, and Rhaleb N-E (1993) Receptors for bradykinin and related kinins: a critical analysis. *Can J Physiol Pharmacol* **71:**56–567.

Russell DA, Tucker KK, Chinookoswong N, Thompson RC, and Kohno T (1995) Combined inhibition of interleukin-1 and tumor necrosis factor in rodent endotoxemia: improved survival and organ function. *J Infect Dis* **171:**1528–1538.

Sheehan M, Wong HR, Hake PW, Malhotra V, O'Connor M, and Zingarelli B (2002) Parthenolide, an inhibitor of the nuclear factor-κB pathway, ameliorates cardiovascular derangement and outcome in endotoxic shock in rodents. *Mol Pharmacol* **61:**953–963.

Ullrich R, Bloch KD, Ichinose F, Steudel W, and Zapol WM (1999) Hypoxic pulmonary blood flow distribution and arterial oxygenation in endotoxin-challenged NOS2-deficient mice. *J Clin Investig* **104:**1421–1429.

Villar J, Ribeiro SP, Mullen JB, Kuliszewski M, Post M, and Slutsky AS (1994) Induction of the heat shock response reduces mortality rate and organ damage in a sepsis-induced acute lung injury model. *Crit Care Med* **22:**914–921.

Weimann J, Bloch KD, Takata M, Steudel W, and Zapol WM (1999) Congenital NOS2 deficiency protects mice from LPS-induced hyporesponsiveness to inhaled nitric oxide. *Anesthesiology* **91:**1744–1753.

Worrall NK, Chang K, LeJeune WS, Misko TP, Sullivan PM, Ferguson TB Jr, and Williamson JR (1997) TNF-α causes reversible in vivo systemic vascular barrier dysfunction via NO-dependent and -independent mechanisms. *Am J Physiol* **273:**H2565–H2574.

Xie Q, Kashiwabara Y, and Nathan C (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide. *J Biol Chem* **269:**4705–4708.

Yamamoto K, Arakawa T, Ueda N, and Yamamoto S (1995) Transcriptional roles of nuclear factor κB and nuclear factor-interleukin-6 in the tumor necrosis factor α-dependent induction of cyclooxygenase-2 in MC3T3–E1 cells. *J Biol Chem* **270:**31315–31320.

Address correspondence to: Dr. Yuichi Hattori, Department of Pharmacology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan. E-mail: yhattori@med.hokudai.ac.jp