Therapeutic effect of in vivo transfection of transcription factor decoy to NF-κB on septic lung in mice

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Histamine is well known to increase pulmonary microvascular permeability (9). Endotoxemia in animals and patients elevates circulating histamine levels (13, 25, 31, 36). In our recent work using endotoxemic rabbits (20, 21), the sustained elevation of plasma histamine has been shown to be associated with the time-dependent increases in protein expression of histidine decarboxylase (HDC), by which histamine is synthesized from L-histidine in tissues (20, 21). Moreover, we have found that endotoxin administration causes superinduction of histamine H1 receptors in cardiovascular tissues of rabbits (20, 21). Earlier studies showed that blockade of histamine H1 receptors with diphenhydramine was effective in the increase in lung vascular permeability in animal models of sepsis (10, 34). Thus the hypothesis that histamine may, at least in part, mediate increased lung vascular permeability caused by endotoxemia is plausible.

Recent evidence suggests that many of the effects of septic mediators, including endotoxin, are mediated by increased production of nitric oxide (NO) (23). NO is synthesized from L-arginine by NO synthase (NOS). Although endothelial NOS (eNOS) is constitutively expressed and generates small amounts of NO in response to physical and receptor stimuli, inducible NOS (iNOS) produces much larger amounts of NO for sustained time periods and is principally implicated in the pathophysiological actions of NO. An important role for NO in septic shock has been suggested by observations that iNOS expression and increased NO production occurs during septic shock (37). NO has been reported to contribute to increased vascular permeation in response to inflammatory agonists, including endotoxin (6).

The mechanism by which the synthesis of multiple proteins contributed to inflammatory responses is triggered during gram-negative sepsis involves the activation of the transcriptional factor nuclear factor-κB (NF-κB). In unstimulated cells, NF-κB is present as a latent cytoplasmic complex bound to its inhibitor protein I-κB (2, 4). However, during sepsis, NF-κB can be activated by bacterial lipopolysaccharide (LPS) and inflammatory cytokines. Once activated, NF-κB dissociates from its inhibitor and translocates to the nucleus where it leads to the regulation of inflammatory molecule production (2, 4). The importance of the therapeutic strategy associated with prevention of NF-κB activation in septic shock has been emphasized in a few studies. Pyrrolidine dithiocarbamate, a potent inhibitor of NF-κB, has been shown to attenuate endo-

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toxin-induced acute lung injury in rats (26). Furthermore, the anti-inflammatory agent parthenolide could protect against endotoxic shock in vivo by a specific inhibition of the NF-κB pathway (33).

Recently, Morishita et al. (24) demonstrated in rats that inhibition of NF-κB activation with the use of a decoy oligodeoxyribonucleotide (ODN) prevented myocardial infarction after coronary ligation. In the present study, we examined whether in vivo transfer of NF-κB decoy ODN could inhibit overexpression of the molecules involved in vascular leak and prevent increased lung vascular permeability in mice after LPS challenge. Thus we sought to evaluate the potential for improving lung failure after induction of sepsis with endotoxin when synthetic double-stranded DNA with high affinity for the NF-κB binding site was introduced.

MATERIALS AND METHODS

Animal care and housing. All animals received humane care in compliance with the guidelines of the Hokkaido University School of Medicine Animal Care and Use Committee (Sapporo, Japan). Male ICR mice weighing 25–30 g were quarantined in quiet, humidified, light-cycled rooms for 2–3 wk before use. Mice were allowed ad libitum access to food and water throughout quarantine.

Synthesis of ODN and selection of sequence targets. The following sequences of phosphorothioate double-stranded ODN against NF-κB binding site and of scrambled ODN were utilized in this study, which were the same as reported previously (24): 5′-CCCTGAAGGGAGTTCTCCCGGA-3′ and 5′-GGGAATTCCTGACCTAGACCC-3′ for NF-κB decoy ODN (consensus sequences are underlined); 5′-TTGCCATGACCTAGACCC-3′ and 5′-AAGGGCATGACCTAGATCGG-5′ for scrambled ODN.

NF-κB decoy ODN has been shown to bind the NF-κB transcriptional factor (24). On the other hand, scrambled decoy ODN is a mutated form of wild-type NF-κB consensus sequence (27). Synthetic ODNs were initially dissolved in sterile Tris-EDTA buffer [10 mM Tris (pH 8.0), 1 mM EDTA], and the ODN concentration was quantitated by spectrophotometry. Then, after addition of 3 M CH3COONa and 99.8% ethanol, the mixture was centrifuged at 15,000 g, and the resulting pellet was washed in 70% ethanol. Ethanol was dried immediately before use.

Experimental protocol. Mice were intravenously injected with sterile saline or 10 mg/kg LPS (E. coli 055:B5; List Biological Laboratories, Campbell, CA) into the tail vein. For in vivo transfer of ODN, a gene transfer kit (In Vivo GeneSHUTTLE: GDS0090, Obiogene, Carlsbad, CA) was used. In Vivo GeneSHUTTLE enables us to prepare an improved liposome-DNA complex structure responsible for 100-fold enhanced gene delivery in vivo. The preparation of lipoplexes was performed under aseptic conditions according to the manufacturer’s instructions. Briefly, in a microcentrifuge tube, 20 μl of 20 mM liposome were mixed with 30 μl of 5% dextrose in water (liquid 1). In a second microcentrifuge tube, 50 μg of ODN were mixed with 5% dextrose in water to produce a final volume of 50 μl (liquid 2). ODN (liquid 2) was mixed with liquid 1. This gives a final ODN concentration of 50 μg/100 μl with liposomes at 4 mM. Notably, it has been established that DNA, RNA, ribozymes, and oligonucleotides can all be delivered successfully with the liposomes (35). Finally, 200 μl of sterile distilled water containing synthetic double-stranded ODN (5 μg/g wt) were infused into the tail vein over 60 s at room temperature 60 min after LPS administration. This dose of NF-κB decoy ODN was chosen because our preliminary study showed that it was the minimum dose to cause constantly a >70% reduction in LPS-induced NF-κB activation in lung tissues. At the indicated time, 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. Nuclear extracts were prepared from lung tissues of untreated and LPS-treated mice using methods described previously (24). In brief, mouse lung tissues were homogenized with a Polytron in 4 vol of ice-cold homogenization buffer [10 mM HEPES (pH 7.5), 0.5 M sucrose, 0.5 mM spermidine, 0.15 mM spermin, 5 mM EDTA, 0.25 M EGTA, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride]. After centrifugation at 12,000 g for 30 min at 4°C, the pellet was lysed and homogenized in 1 vol of ice-cold homogenization buffer containing 0.1% Nonidet P-40. It was then centrifuged at 12,000 g for 30 min at 4°C, and the pelleted nuclei were washed twice with ice-cold buffer containing 0.35 M sucrose. The nuclei were preextracted with 1 vol of ice-cold homogenization buffer containing 0.05 M NaCl and 10% glycerol for 60 min at 4°C, and the concentration of DNA was adjusted to 1 mg/ml. After the nuclear extract was pelleted at 12,000 g for 30 min at 4°C, the supernatant was brought to 45% (NH4)2SO4 and stirred for 30 min at 4°C. The precipitated protein was collected after 17,000 g for 30 min, resuspended in homogenization buffer containing 0.35 M sucrose, and stored in aliquots at −80°C.

NF-κB decoy ODN was labeled as a probe at the 3′ end by means of a 3′ end-labeling kit (Perkin Elmer Life Sciences, Boston, MA). After end-labeling was completed, 32P-labeled ODN was purified by application of a P-20 column. Binding reactions (10 μl) including the 32P-labeled probe (0.5–1 ng, 10,000–15,000 cpm) and 1 μg of polydeoxyinosinic acid (Sigma, St. Louis, MO) were incubated with nuclear extract for 30 min at room temperature and then loaded onto 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and preincubated with parallel samples 10 min before the addition of the labeled probe. As a control, samples were incubated with excessive doses of cold NF-κB ODN, which resulted in the disappearance of signals. Gels were analyzed by autoradiography.

Total RNA extraction and Northern blot analysis. Total RNA was extracted from lung tissues by the guanidinium thiocyanate-phenol-chloroform method with Isogen (Nippon Gene, Toyama, Japan), which has been used routinely in our laboratory (18). RNA purity was determined by the ratio of optical density measured at 260 and 280 nm (OD260/OD280), and RNA quantity was estimated at OD260.

For Northern blot analysis, RNA, 20 μg/lane, was subjected to electrophoresis on agarose-formaldehyde gels and then transferred to a Hybond-N+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK). The membrane was prehybridized in prewarmed rate-enhanced hybridization buffer (Amersham) at 65°C for 60 min. Hybridization was done with a [32P]dCTP-labeled random-primed murine HDC, histamine H1 receptor, or iNOS or eNOS cDNA probe (19, 21, 22). We quantitated the expression of mRNA by counting the radioactivity using a bioimaging analyzer (Fujix BAS 2000; Fuji Photo Film, Tokyo, Japan), as described previously (18); this was standardized to the mRNA of the constitutively expressed protein β-actin on the same filter to correct for any variability in gel loading.

Western blot analysis. Samples of tissue homogenate (5–10 μg) were run on SDS-PAGE (12.5% polyacrylamide gel) and electrotransferred to a polyvinylidene difluoride filter membrane. To reduce nonspecific binding, the membrane was preincubated for 60 min at room temperature in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.5 mM KH2PO4) containing 1% BSA. The membrane was then incubated for 60 min at 4°C with primary antibody recognizing HDC, H1 receptor, iNOS, eNOS, intracellular adhesion molecule-1 (ICAM-1), or actin. For HDC recognition, we used a mouse monoclonal antibody that was kindly provided by Dr. S. Tanaka (Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan). For recognizing H1 receptors, iNOS, eNOS, ICAM-1, and actin, we used the following commercially available antibodies: anti-human H1 receptor rabbit polyclonal antibody (Chemicon International, Temecula, CA), anti-rabbit iNOS mouse monoclonal antibody (Affinity BioReagents, Inc., Cincinnati, OH), and antihuman H1 receptor antibody (Wako, Osaka, Japan). After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG, respectively, and visualized with enhanced chemiluminescence.
We initially ascertained whether in vivo transfection of NF-κB decoy ODN can effectively prevent the DNA binding of NF-κB in mouse lungs. As shown in Fig. 1, the binding activity of NF-κB, as assessed by gel mobility shift assay, was markedly increased in lung tissues from 10-h endotoxemic animals compared with controls. Transfection of NF-κB decoy ODN, but not of scrambled decoy ODN, via intravenous injection resulted in a decrease in the binding of NF-κB nearly to the control level in lung tissues from LPS-treated mice. These results encouraged us to study the potential utility of NF-κB decoy ODN strategy for the treatment of septic lung injury in vivo transfection into lung tissues via intravenous injection.

Table 1 summarizes the values for blood gases at 10 h after mice were given LPS or its vehicle (sterile saline). The arterial PO$_2$ value was significantly reduced from the baseline at 10 h after LPS administration. Also, a marked fall in arterial PCO$_2$ was observed at 10 h. There was a drastic decrease from baseline detected for base excess in LPS-treated mice. Arterial blood pH became essentially acidosis after injection of endotoxin. NF-κB decoy ODN, introduced in mice in vivo 60 min after LPS administration, resulted in an evident improvement of all of these reduced blood-gas parameters. This effect was not seen in the case of transfection of scrambled decoy ODN.

The basal concentration of histamine in the plasma was 75 ± 57 nM (n = 5). Administration of LPS to the mouse profoundly elevated the concentration of histamine in the plasma (516 ± 200 nM at 10 h, n = 5). The LPS-induced increase in the plasma histamine concentration was strongly inhibited by in vivo transfection of NF-κB decoy ODN (147 ± 48 nM, n = 5) but not of scrambled decoy ODN.

Northern blot analysis showed that mouse lungs expressed a 2.7-kb transcript corresponding to HDC mRNA (Fig. 2). The transcript level of HDC was increased 10.2-fold at 10 h after LPS administration. Immunoblot analysis indicated the presence of two forms of HDC with different molecular sizes,

<table>
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<th>pH</th>
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<th>Paco$_2$, Torr</th>
<th>Base Excess, mM</th>
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<tr>
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<tr>
<td>10-h LPS-treated</td>
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<tr>
<td>+ NF-κB decoy</td>
<td>7.346 ± 0.010†</td>
<td>34.9 ± 0.9†</td>
<td>105.3 ± 1.7†</td>
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Values are means ± SE of 5 animals. Animals were transfected with NF-κB decoy oligodeoxynucleotide (ODN) or its scrambled form at 60 min after lipopolysaccharide (LPS) challenge and killed 10 h later. *P < 0.05 compared with respective control. †P < 0.05 compared with respective LPS treatment alone.

Results

We initially ascertained whether in vivo transfection of NF-κB decoy ODN can effectively prevent the DNA binding of NF-κB in mouse lungs. As shown in Fig. 1, the binding activity of NF-κB, as assessed by gel mobility shift assay, was markedly increased in lung tissues from 10-h endotoxemic animals compared with controls. Transfection of NF-κB decoy ODN, but not of scrambled decoy ODN, via intravenous injection resulted in a decrease in the binding of NF-κB nearly to the control level in lung tissues from LPS-treated mice. These results encouraged us to study the potential utility of NF-κB decoy ODN strategy for the treatment of septic lung injury in vivo transfection into lung tissues via intravenous injection.

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Northern blot analysis showed that mouse lungs expressed a 2.7-kb transcript corresponding to HDC mRNA (Fig. 2). The transcript level of HDC was increased 10.2-fold at 10 h after LPS administration. Immunoblot analysis indicated the presence of two forms of HDC with different molecular sizes,
53 and 74 kDa, in mouse lungs (see Fig. 6). It is believed that the 74-kDa form of HDC is the cytosolic enzyme and the 53-kDa form is the particulate enzyme recognized as an active form (21). Densitometric analysis revealed an 8.3-fold increase in the active form of the HDC protein in lungs from 10-h endotoxemic animals compared with controls ($n = 5$). NF-$\kappa B$ decoy ODN, but not of its scrambled form, strongly inhibited the increases in gene and protein expressions of HDC in lung tissues from 10-h endotoxemic mice (Figs. 2 and 6).

Expression of the transgene of the histamine $H_1$ receptor, which migrated at 3.3 kb and 3.9 kb, was detected by Northern blot analysis (Fig. 3), as demonstrated in our previous reports (20, 22). After induction of sepsis by LPS injection, the transcript level of the $H_1$ receptor was increased $\sim$2.7- to 3.8-fold in lungs compared with that shown in controls. NF-$\kappa B$ decoy ODN treatment significantly lowered the increased $H_1$-receptor mRNA level in the lung tissue from LPS-treated mice. On Western blots (see Fig. 6), the protein level of the $H_1$ receptor was determined by the density of a single band migrating at 57 kDa as previously reported (22). The $H_1$-receptor protein level was increased 3.2-fold by LPS challenge ($n = 4$), which was partially suppressed by NF-$\kappa B$ decoy ODN transfection (2.2-fold from control, $n = 4$). Transfection of mutated NF-$\kappa B$ decoy ODN had no effect on the LPS-induced increases in $H_1$-receptor mRNA and protein (Figs. 3 and 6).

When LPS was given to mice, the mRNA level of iNOS was elevated in lungs in a time-dependent manner, as detected by Northern blot analysis (Fig. 4). However, in vivo transfection of NF-$\kappa B$ decoy ODN, but not of its scrambled form, resulted in a marked decrease in iNOS mRNA to the level almost similar to that obtained in control lungs. On Western blots (see Fig. 6), the 130-kDa iNOS protein was detectable in control lungs, which was increased 12.6-fold by LPS. This LPS-induced increase in iNOS protein expression was suppressed by treatment with NF-$\kappa B$ decoy ODN (3-fold from control) but not with scrambled decoy ODN (12.1-fold from control, $n = 4$ for each).

In contrast to iNOS, mRNA and protein levels of eNOS showed a time-dependent decrease after LPS injection (Figs. 5 and 6).
and 6). At 10 h after LPS administration, the relative amounts of mRNA and 140-kDa protein for eNOS were decreased to 53 ± 5% (n = 5) and 63 ± 4% (n = 4) of control, respectively. The decreases in eNOS mRNA and protein were significantly prevented by treatment with NF-κB decoy ODN but not with scrambled decoy ODN (Figs. 5 and 6).

Intravenous LPS challenge caused an 18-fold increase in lung microvascular permeability, as assessed by the transpulmonary flux of radiolabeled albumin (Fig. 7). Treatment with the histamine H1-receptor antagonist diphenhydramine (5 mg/kg iv) or the NOS inhibitor \(N^G\)-nitro-L-arginine (L-NNA; 5 mg/kg iv) 60 min after LPS challenge significantly but partially attenuated lung permeability compared with that shown in mice receiving LPS. Even when the two drugs were given together, the effect was not complete. However, transfection of NF-κB decoy ODN, but not of its scrambled form, 60 min after LPS administration resulted in much stronger protection against increased lung permeability than treatment with both diphenhydramine and L-NNA.

Furthermore, as a quantitative measure of fluid clearance in lungs, wet-to-dry weight ratios were evaluated in lungs removed from mice killed at 10 h after LPS administration (Fig. 8). Although a combination of diphenhydramine and \(N^G\)-nitro-L-arginine significantly but incompletely prevented the increase in the ratio induced by LPS, NF-κB decoy ODN, but not its scrambled form, reduced the ratio nearly to the level of nonendotoxemic controls.

Light microscopy findings showed that massive infiltration of inflammatory cells and thickening of the alveolar septum were observed in lungs from mice at 10 h after LPS administration compared with that shown in control lungs (Fig. 9). There were no significant light microscopic differences between lungs from septic and scrambled decoy ODN-transfected septic animals. In lungs from septic animals that had been transfected with NF-κB decoy ODN, architecture of alveoli was preserved and infiltration of inflammatory cells was strongly prevented.

Finally, we assessed the effect of NF-κB decoy ODN transfection on LPS-induced lung inflammation. When leukocytes in BAL fluid were counted with a hemacytometer, cell counts increased dramatically in the animals at 10 h after LPS administration (73 ± 6 × 10^5 cells/ml, n = 3) compared with those seen in the control animals (7 ± 1 × 10^5 cells/ml, n = 3). This LPS-induced increase in BAL leukocyte counts was strongly inhibited by transfection of NF-κB decoy ODN (14 ± 2 × 10^5 cells/ml) but not of its scrambled form (74 ± 9 × 10^5 cells/ml).
n = 3). Moreover, the 90-kDa ICAM-1 protein, which plays a central role in cell-cell contact-mediated immune responses (7) and the adherence of leukocytes to epithelial cells (17), was markedly increased by LPS challenge in the lung (Fig. 6). Densitometric quantification of the signal showed that the expression level of ICAM-1 was 8.7-fold higher in 10-h endotoxemic than in control lungs (n = 4). LPS-induced expression of ICAM-1 was unchanged with scrambled decoy ODN transfection (8.8-fold from control, n = 4) but was greatly suppressed by NF-κB decoy ODN transfection (2.1-fold from control, n = 4).

**DISCUSSION**

In the present study, our NF-κB decoy strategy showed a dramatic improvement of blood-gas derangements, pulmonary vascular hyperpermeability, and lung histopathology, including inflammation, in vivo animal models of endotoxic shock. It has been shown that many of the genes (i.e., cytokines, ICAM-1, and iNOS) that have been implicated in endotoxic shock contain NF-κB binding sites in the promoter/enhancer region (3, 14). With particular clinical relevance, NF-κB binding activity has been found to be increased in patients with acute inflammation and sepsis and to be correlated with clinical severity and mortality (1, 5, 29). Under the experimental conditions established in our laboratory, NF-κB activity was markedly increased in lung tissues from mice after induction of sepsis with LPS as shown by the gel shift assay, and in vivo transfer of NF-κB decoy ODN greatly reduced the stimulation of NF-κB activity in septic lungs. This clearly indicates the validity of NF-κB decoy strategy by in vivo transfection of decoy ODN containing the NF-κB cis-element. Therefore, the results of the present study led us to propose that the inhibition of stimulated NF-κB activity in lungs by NF-κB decoy ODN results in the suppression of expression of key molecules that may play a pivotal role in the pathogenesis of septic lung.

As demonstrated in our previous study using endotoxemic rabbits (20, 21), plasma histamine levels were greatly increased in mice after induction of sepsis with LPS. This increase in the circulating level of histamine was associated with increased tissue expression of HDC, an enzyme that only forms histamine in mammals, during sepsis induction. In vitro transfection of NF-κB decoy ODN resulted in marked decreases in HDC mRNA and protein nearly to the nonseptic control level in lung tissues, leading to strong inhibition of elevated circulating histamine in septic animals. Furthermore, NF-κB decoy ODN treatment showed a significant inhibitory effect on superinduc-
tion of the histamine H1-receptor gene caused by endotoxin. These findings suggest that both HDC and H1-receptor genes are NF-κB-sensitive genes. Thus transcription of HDC and H1 receptor appears to be regulated by NF-κB in a direct or indirect way. However, because only the blockage of NF-κB activation was insufficient to suppress superinduction of the H1-receptor gene, the H1 receptor may have other transcriptional factors.

In our mouse model, endotoxin induced a tremendous increment of gene and protein expressions of iNOS in lung tissues. Because activation of the iNOS gene is dependent on binding of NF-κB to its consensus motifs in the iNOS promoter region, the iNOS gene is a target for NF-κB (41). As expected, inhibition of NF-κB nuclear translocation by NF-κB decoy ODN strongly prevented enhanced pulmonary expression of the iNOS gene. Similar to our findings obtained in endotoxin-induced septic mice, it has been demonstrated that protein expression of iNOS can be inhibited by treatment with NF-κB decoy ODN in the carrageenin-induced hind paw edema, an acute model of inflammation (12).

A contributory role for histamine and NO in increased pulmonary vascular permeation during septic shock has been documented (10, 34, 40). In this study, we found that treatment with diphenhydramine or l-NNa significantly but partially inhibited endotoxin-induced lung vascular permeability. This suggests that histamine and NO may be partly responsible for mediating increased lung vascular permeability following endotoxemia. To our surprise, the inhibition by NF-κB decoy ODN transfection of the increase in lung vascular permeability after endotoxemia was much more pronounced than that by a combination of diphenhydramine and l-NNa. We thus assume that, by activation of the NF-κB signaling pathway in lung tissues, endotoxin could induce transcription of several yet to be determined molecules that could be actually involved in pulmonary edema, in addition to HDC, H1 receptors, and iNOS.

In the present study, eNOS expression was found to be downregulated in lung tissues after endotoxemia, suggesting hindrance of physiological regulatory events with involvement of eNOS-based NO synthesis. In previous studies with cultured cells, downregulation of eNOS, triggered by increased NO production via LPS-induced iNOS expression, has been reported (11, 16, 32). Such negative feedback regulation of eNOS expression by enhanced iNOS-derived NO formation has been recently demonstrated in blood vessels taken from endotoxemic rabbits (21). The impaired expression of eNOS may be associated with endothelial histology injury (39). Because endothelial dysfunction could alter physiological regulation of blood flow distribution by interfering with metabolic vasodilation in states of limited O2 supply (38), such an endotoxin-induced endothelial disorder may well contribute to abnormalities in physiological function in septic lung failure. We observed that NF-κB decoy ODN treatment ameliorated eNOS downregulation induced by endotoxin, indicating that the blockade of NF-κB activation may offer therapeutic benefits in endothelial disorder in septic lung.

Here, we showed a novel therapeutic strategy to prevent the development of septic lung failure by transfecting decoy ODN to block the binding of the critical transcription factor NF-κB to its promoter sequence of the target genes, thereby inhibiting gene expression of key molecules necessary for increased lung vascular permeability. Because sepsis is a common cause of death and drug resistance is becoming a major medical problem, our findings may provide a useful therapeutic tool for treating septic shock. However, in vitro studies suggest that NF-κB plays a role as a survival factor, responsible in part for “turning on” genes that could block cell death by apoptosis (15). It is therefore of great importance to address a number of unavoidable issues, including safety and side effects, in ongoing studies.
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