Functions of IκB Proteins in Inflammatory Responses to *Escherichia coli* LPS in Mouse Lungs

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Acute inflammation induced by intrapulmonary LPS requires nuclear factor (NF)-κB RelA. This study elucidates the effects of intrapulmonary LPS on IκB proteins, endogenous inhibitors of RelA, and the effects of deficiency of IκB-β. IκB-α, IκB-β, and IκB-ε each complexed with RelA in uninfected murine lungs. Intratracheal instillation of LPS induced the degradation of IκB-α and IκB-β, as measured by the loss of immunoreactive proteins in non-nuclear fractions. Degradation was apparent by 2 h and sustained through 6 h. In contrast, net IκB-ε content increased over this period. The small amounts of IκB-α and IκB-β that were detected in nuclear fractions from the lungs also decreased over this time frame, whereas intranuclear NF-κB content (including both RelA and p50) increased. The hypophosphorylated form of IκB-β, which facilitates transcription induced by NF-κB, was not detected. Neutrophil recruitment and edema accumulation did not differ between wild type mice and gene-targeted mice deficient in IκB-β, suggesting that IκB-β is not specifically required for these responses. Altogether, these data suggest that RelA is liberated during LPS-induced pulmonary inflammation by the regulated degradation of both IκB-α and IκB-β. In the absence of IκB-β, IκB-α or other inhibitory proteins can regulate NF-κB functions essential to acute neutrophil emigration in the lungs.

Lower respiratory infections are the leading cause of lost disability-adjusted life years worldwide (1). Gram-negative bacteria are common causes of both community- and hospital-acquired pneumonias (2, 3). LPS from Gram-negative bacteria is recognized in the lungs by pattern recognition receptors (4) that initiate local innate immune responses, including neutrophil emigration and edema accumulation. Neutrophil emigration elicited by LPS or Gram-negative bacteria in the lungs requires the regulated expression of a variety of genes, including chemokines (5–7), adhesion molecules (8, 9), and early response cytokines (10). The transcription of many of these genes (including KC, macrophage inflammatory protein-2, intercellular adhesion molecule-1, and tumor necrosis factor-α) is mediated at least in part by the nuclear factor (NF)-κB family of transcription factors (reviewed in (11)).

The intrapulmonary deposition of bacteria or LPS induces the nuclear translocation of NF-κB complexes, including RelA and p50 subunits (12, 13). The RelA subunit is essential for effectively responding to bacterial stimuli in the lungs. The gene-targeted deletion of RelA renders mice susceptible to spontaneous pulmonary infections (14). Neutrophil emigration and the pulmonary expression of KC, macrophage inflammatory protein-2, and intercellular adhesion molecule-1 induced by intranasal insufflation of *Escherichia coli* LPS are inhibited by the absence of RelA (14). These data suggest that nuclear translocation of NF-κB RelA is critical to inducing the gene expression mediating neutrophil emigration elicited by LPS in the lungs.

NF-κB transcription factors are controlled by the IκB family of inhibitor proteins (15). IκB proteins bind to the Rel-homology domains of NF-κB proteins, masking the nuclear localization and DNA-binding sequences, and preventing NF-κB–mediated gene transcription. The stimulus-induced phosphorylation, ubiquitination, and proteasomal degradation of IκB proteins liberates the NF-κB complexes, which then translocate to the nucleus, bind specific DNA sequences, and induce the transcription of downstream genes.

Three IκB family members, IκB-α, -β, and -ε, are encoded by distinct genes and are capable of inhibiting NF-κB–mediated transcription. The extent to which these proteins have unique or overlapping functions remains unclear. They possess subtle differences in the quality of their biochemical interactions with individual NF-κB complexes (16–18), but no particular NF-κB complexes or NF-κB activities have been demonstrated to require distinct IκB proteins. Each of the IκB proteins is capable of inhibiting NF-κB translocation, DNA binding, and transcriptional activation when overexpressed (16–18). Degradation of each of the IκB proteins is associated with NF-κB translocation and expression of κB-associated genes. However, the kinetics of activation-induced degradation and resynthesis appears to differ for the three IκB proteins, with IκB-α being degraded and resynthesized most quickly and IκB-β most slowly (18–21). The IκB proteins are inducibly phosphorylated by multiple kinases (22–25); to our knowledge, no specific kinases have been selectively associated with distinct IκB proteins. The biochemical mechanisms responsible for the independent regulation of these IκB proteins remain to be elucidated.

The regulated expression of these three different proteins is likely critical to their independent functions. IκB-α deficiency results in neonatal lethality secondary to dysregulated NF-κB activity and spontaneous inflammatory disease (26), suggesting that IκB-α is essential to the basal inhibition of NF-κB. However, replacing the IκB-α gene with an IκB-β gene abrogates these effects, demonstrating that IκB-β can perform similar basal inhibitory functions as IκB-α, but

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Abbreviations: antibody, Ab; electrophoretic mobility shift assay, EMSA; nuclear factor, NF; red blood cell, RBC; wild type, WT.

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only when it is controlled by the *cis* regulatory elements of IκB-α (27). In contrast to IκB-α deficiency, gene-targeted mutation of IκB-β does not affect survival or result in spontaneous inflammatory disease (M.L. Scott and D. Baltimore, unpublished observations), suggesting that IκB-β is not essential to the basal inhibition of NF-κB.

Although all three of these IκB proteins concentrate NF-κB proteins in the cytoplasm, both IκB-α and IκB-β appear in the nucleus as well. The regulation and function of these IκB proteins within this organelle may differ. IκB-α does not completely mask the nuclear localization signals of RelA, and hence, IκB-α is regularly imported into nuclei along with NF-κB proteins (28–30). However, a nuclear export signal on IκB-α efficiently returns the complexes to the cytoplasm via the Crm1 export receptor (28–30). Thus, IκB-α regularly shuttles through the nucleus, and export of NF-κB complexes displaced from the DNA by IκB-α may be critical to turning off gene expression induced by NF-κB. IκB-β can also be found in the nucleus, but the mechanisms of its import and export are different from IκB-α and remain to elucidated (28, 29, 31–33). IκB-β in particular may increase in the nucleus subsequent to cell stimulation (31, 33). The intranuclear form of IκB-β appears to be hypophosphorylated, and incapable of inhibiting NF-κB–mediated gene expression (31). Since it prevents binding of NF-κB by other IκB proteins (31), hypophosphorylated IκB-β may enhance rather than inhibit gene expression mediated by NF-κB.

We hypothesized that the different IκB proteins perform distinct functions in regulating inflammatory responses to LPS in the lungs. The present study characterizes IκB-α, -β, and -ε in the lungs during pulmonary inflammation induced by *E. coli* LPS. The functional requirements for IκB-β in regulating pulmonary inflammation were specifically examined using mice with targeted deletion of the IκB-β gene.

Materials and Methods

Mice

IκB-β–deficient mice were generated by standard procedures using homologous recombination in embryonic stem cells. The targeting of mouse *Nfkbib* deleted the genomic DNA encoding nucleotides 253 to 987 of the IκB-β cDNA (GenBank Accession U19799, National Center for Biotechnology Information, Bethesda, MD). This targeted deletion eliminated $>$ 60% of the coding region, including all ankyrin repeats. IκB-β–deficient mice and wild type (WT) mice of similar random hybrid genetic background (C57BL/6 $\times$ 129/Sv) were maintained under specific pathogen-free conditions in a full barrier facility. C57BL/6 mice were purchased from Taconic (Germantown, NY). Mice were 6 to 10 wk of age at the time of experiments. All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals.

NF-κB and IκB Proteins in the Lungs

NF-κB translocation in murine lungs was measured as previously described (10, 13). Mice were anesthetized by intramuscular injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg). The trachea was surgically exposed, and an angiocatheter was inserted via the trachea into the left bronchus. Sterile saline containing 100 μg *E. coli* LPS serotype O55:B5 (Sigma, St. Louis, MO) and 5% colloidal carbon, to mark the site of instillation, was instilled in a volume of 50 μl per mouse. Mice were killed by inhalation of a halothane overdose at the indicated times. Colloidal carbon-containing lung lobes from mice instilled with LPS, as well as left lung lobes from mice that did not receive LPS instillation, were excised, snap-frozen in liquid nitrogen, and stored at −80°C until protein extraction. Nuclear and non-nuclear proteins were collected from the frozen lung samples (10, 13), and total protein concentrations were measured using a bicinchonic acid assay with bovine serum albumin as the standard.

For electrophoretic mobility shift assay (EMSA), nuclear proteins (0.5 mg/ml) incubated with 3.5 nM γ[^{32}P]ATP-labeled NF-κB consensus oligonucleotide (Promega, Madison, WI). In supershift assays, nuclear proteins (0.3 mg/ml) were incubated with 3.5 nM γ[^{32}P]ATP-labeled NF-κB consensus oligonucleotide and an antibody (Ab) against one subunit of NF-κB (0.2 mg/ml) for 30 min prior to electrophoresis. All polyclonal Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), including the following: sc-7151 against RelA, sc-1192 against p50, sc-71 against c-Rel, sc-226 against RelB, and sc-298 against p52. Protein-oligonucleotide complexes were separated from protein-free oligonucleotides by polyacrylamide gel electrophoresis and detected by autoradiography. Independent experiments with proteins collected from the lungs of different mice yielded consistent results.

For most Western analyses, nuclear and non-nuclear proteins (30 μg/well) were separated on 4–12% gradient gels by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P PVDF membranes. Membranes were probed with the following polyclonal Ab (Santa Cruz Biotechnology): sc-371 against IκB-α, sc-945 against IκB-β, and sc-7156 against IκB-ε. After washing, primary Ab associated with the membranes were detected on autoradiographic film by horseradish peroxidase-conjugated secondary Ab and the ECL+Plus chemiluminescent system (Amersham Pharmacia Biotech; Piscataway, NJ). Densitometric data were collected and analyzed using Scion ImagePC software (Scion; Frederick, MD).

For Western analyses designed to differentiate the electrophoretic mobilities of basally phosphorylated and hypophosphorylated IκB-β, gel electrophoresis conditions which separated these isoforms were empirically determined. Nuclear and non-nuclear proteins (30 μg/well) were separated by SDS-PAGE over 20 cm in 10% polyacrylamide at a constant current of 24 mA. Proteins were transferred to Immobilon-P PVDF membranes, and Western analyses were performed as above. To render IκB-β hypophosphorylated, protein samples were incubated with calf intestinal phosphatase (Promega) at 0.5 U/μl for 30 min at 37°C prior to electrophoresis.

For immunoprecipitations, left lung lobes were homogenized in the lysis buffer containing antiproteases designed by Shenkar and colleagues for immunoprecipitation of mouse lung proteins (34), and cleared by centrifugation. Aliquots from each extract were saved for Western analyses. Protein concentrations were determined using the bicinchonic acid assay (Sigma), and aliquots containing 8 mg of lung protein were rotated end-over-end with primary Ab at a final concentration of 5 μg/ml, overnight at 4°C. Ab-binding complexes were precipitated with Protein A-sepharose (Sigma); protein samples which did not precipitate with protein A-sepharose (supernatant fractions) were collected for comparison. Precipitates were washed three times with lysis buffer containing antiproteases, and suspended in loading dye. Equal volumes of protein samples were separated by SDS-PAGE, and the presence of specific proteins in a given preparation (extract, supernatant, or precipitate) was assessed by Western analyses as described above. Immunoprecipitations using proteins collected from the lungs of different mice yielded reproducible, consistent results.

Neutrophil Emigration and Edema Accumulation

Mice received intratracheal instillations of LPS as described above. After 6 h, mice were killed by inhalation of a halothane overdose. Control WT mice received no intratracheal instillations. The hearts were tied off to maintain pulmonary blood contents, and peripheral blood samples were collected from the inferior vena cava. Excised lungs were fixed by intratracheal instillation of 6% glutaraldehyde at a pressure of 23 cm H_2O. Emigrated and sequestered neutrophils were quantified by morphometric analyses of histologic lung sections (10, 35). Investigators were blinded to the genotype of the mice during counting procedures.

Circulating neutrophils were quantified in peripheral blood samples. After red blood cell (RBC) lysis, leukocytes were counted using a hemacytometer, and differential distributions were assessed in blood smears stained with LeukoStat (Fisher Scientific, Pittsburgh, PA).

Pulmonary edema, as measured by the extravascular accumulation of ^{125}I-albumin, was quantified as previously described (10, 35). Specific activities of ^{125}I-albumin and ^{51}Cr-RBC were measured in blood and plasma samples and in excised, fixed lungs from each mouse. Hematocrits were calculated from ^{125}I-albumin activities in the blood and plasma samples. Pulmonary blood volume was derived from the ^{51}Cr-RBC activity in the lung and blood samples. Volumes of total plasma equivalents in the lungs were calculated from the ^{125}I-albumin activity in the lung and the plasma samples, and volumes of intravascular plasma were calculated using the hematocrits and the pulmonary blood volumes. Volumes of extravascular plasma equivalents in the lungs were derived from the difference between the volumes of total plasma equivalents and the volumes of intravascular plasma. Edema fluid accumulation was expressed as μl per lung of extravascular plasma equivalents.

Statistical Analysis

For comparing NF-κB and IκB levels at multiple time points, all groups were compared by ANOVA, and intergroup comparisons were performed using *post hoc* Scheffé's tests. For emigration and edema studies, WT and IκB-β–deficient mice were compared using Student's *t* test. All pooled data were presented as mean and SE values. Differences were considered significant when $P < 0.05$.

Results

NF-κB Translocation Elicited by LPS in the Lungs

E. coli LPS induced the nuclear translocation of NF-κB complexes in murine lungs. By 2 h after the intratracheal instillation of *E. coli* LPS, a significant increase in NF-κB binding activity was detectable in the nuclear fractions (Figure 1A). The NF-κB activity in the nuclear fractions was further increased after 6 h (Figure 1A). The electrophoretic mobilities of the NF-κB complexes differed in the nuclear fractions from lungs collected 2 h compared with that collected 6 h after the instillation of LPS (Figure 1A). In particular, faster moving complexes became apparent after 6 h, while the slowest moving complexes became less prominent.

The identities of NF-κB proteins translocating to the nucleus in response to *E. coli* LPS were investigated using supershift analyses. Ab against c-Rel, RelB, and p52 did not supershift bands at either time point (Figure 1B). Bands were supershifted by Ab against RelA or p50 at both time points (Figure 1B), indicating that these proteins translocated to the nucleus in response to the pulmonary deposition of LPS. The band labeled Complex II in Figure 1B was shifted only by Ab against p50, suggesting that this band contains p50/p50 homodimers. Ab against RelA decreased the intensity of a distinct band at either time point (Complex I, present after 2 h, and Complex III, present after 6 h; Figure 1B), but the electrophoretic mobility of the supershifted band differed at the two time points. The intensities of Complexes I and III in Figure 1B were also decreased by Ab against p50. These data suggest that Complexes I and III both contain RelA/p50 heterodimers. The greater electrophoretic mobility of Complex III compared with either Complexes I or II suggests that RelA may be partially degraded in nuclear fractions from lungs collected 6 h after LPS instillation, perhaps as a result of neutrophil proteases, as has been previously described (12, 36, and unpublished observations).

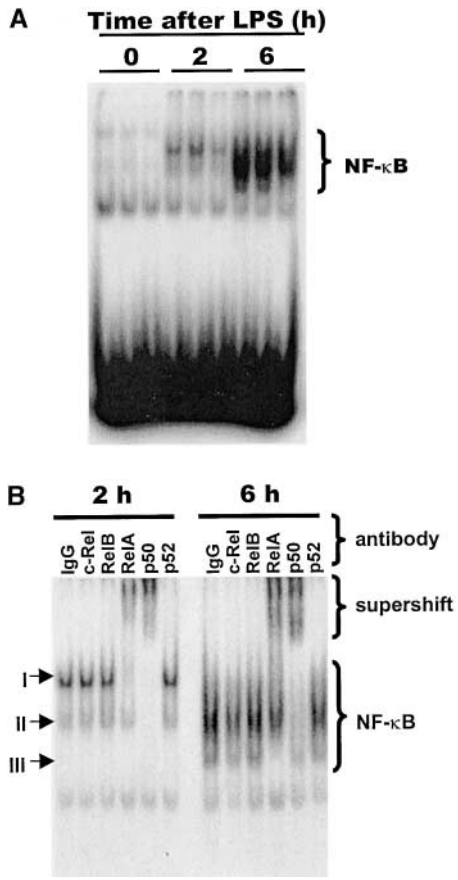


Figure 1. Nuclear translocation of NF-κB proteins induced by LPS in the lungs. (*A*) Increased levels of NF-κB proteins in nuclear fractions from the lungs of C57BL/6 mice after the intratracheal instillation of *E. coli* LPS. NF-κB proteins were identified by their ability to bind a consensus oligonucleotide probe using EMSA. (*B*) Complexes induced to translocate in the lungs include both RelA and p50 at each time point examined after LPS instillation. Components of NF-κB Complexes I, II, and III were identified by the ability of polyclonal Ab to supershift complexes (please see Results). All EMSA and supershift experiments using protein from the lungs of different mice were repeated with consistent results.

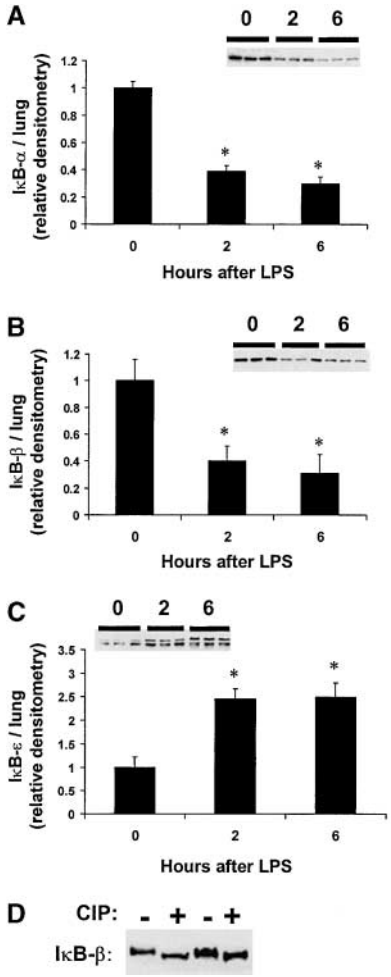


Figure 2. Altered IκB levels in response to LPS in the lungs. IκB levels in the non-nuclear fractions from the lungs of C57BL/6 mice were detected by Western analyses 0, 2, and 6 h after LPS instillation, and quantitated using densitometry. Graphs depict the mean and SE from six mice, with *insets* demonstrating representative immunoblots with three mice at each time point. *Asterisks* (*) indicate significant differences ($P < 0.05$) compared with hour 0. (*A*) IκB-α levels decreased within 2 h and remained low at 6 h after LPS instillation. (*B*) IκB-β levels decreased within 2 h and remained low at 6 h after LPS instillation. (*C*) IκB-ε levels did not decrease but increased 2 and 6 h after LPS instillation. (*D*) Phosphorylated IκB-β in murine lungs. IκB-β was detected by Western analyses after incubation of non-nuclear fractions from lungs of two different 0 h C57BL/6 mice with calf intestinal phosphatase, as indicated (+). Adjacent (−) *lanes* show the same proteins not treated with phosphatase. Increased electrophoretic mobility induced by phosphatase was interpreted as evidence of IκB-β phosphorylation.

Selective Degradation of IκB Proteins Elicited by LPS in the Lungs

The content of IκB proteins was assessed in the non-nuclear fractions from the same lungs as that of the NF-κB analyses. IκB-α was readily detected in the lungs prior to instillation of LPS. By 2 h after instillation, LPS in the air spaces induced a significant loss of IκB-α, to 39 ± 4% of the basal content (Figure 2A). IκB-α content remained low, with only 30 ± 5% of the basal content detected 6 h after LPS instillation (Figure 2A). Thus, LPS in the lungs induced a rapid, substantial, and sustained loss of IκB-α.

TABLE 1
Loss of nuclear IκB-α and IκB-β after LPS instillation to the lungs

	0 h	2 h	6 h
IκB-α*	1.00 ± 0.05	0.28 ± 0.04†	0.13 ± 0.01†
IκB-β*	1.00 ± 0.10	0.49 ± 0.08†	0.21 ± 0.02†

*IκB levels in nuclear fractions, collected from the lungs of C57BL/6 mice 0, 2, and 6 h after LPS instillation, detected by Western analyses. Data are mean ± SE relative densitometric values from four to five mice per time point.
† $P < 0.05$ compared to 0 h.

LPS similarly affected IκB-β content in the lungs. Like IκB-α, IκB-β levels were decreased to 40 ± 11% and 31 ± 14% of basal content, 2 and 6 h after LPS instillation, respectively (Figure 2B).

In contrast, LPS instillation did not decrease IκB-ε levels in the non-nuclear fractions recovered from whole lungs. Ab against IκB-ε detected multiple bands in the expected 45 kD size range in protein fractions from mouse lungs with or without LPS instillation (Figure 2C). Multiple isoforms of IκB-ε have been detected in mouse cell lines, more prominently than in human cell lines, and the slower migrating bands have been demonstrated to be hyperphosphorylated forms of IκB-ε (18). The biological significance of this IκB-ε phosphorylation is unknown. In the present study, all isoforms, but perhaps especially the slower migrating hyperphosphorylated isoforms, of IκB-ε proteins detected by Western blotting increased in the lungs 2 and 6 h after the instillation of *E. coli* LPS (Figure 2C). Thus, in contrast to the other IκB proteins, *E. coli* LPS resulted in a net gain, rather than loss, of IκB-ε in the lungs.

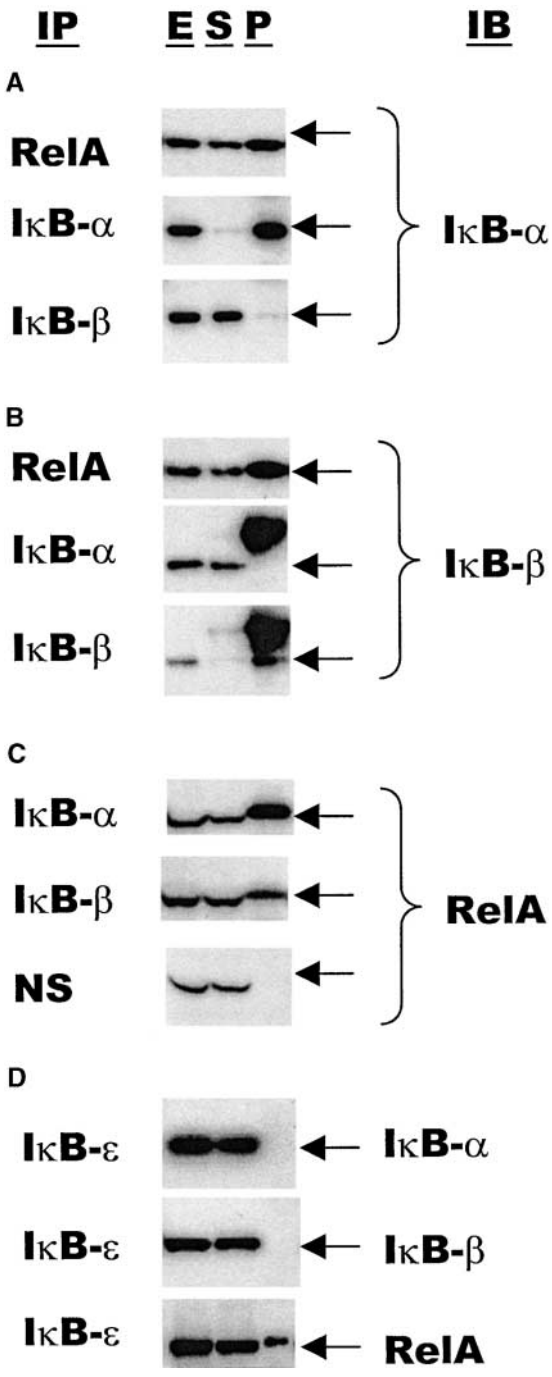
Nuclear IκB Proteins

Although both are concentrated in the cytoplasm, IκB-α and IκB-β each appear in the nuclear compartment as well (28, 29, 31–33). Furthermore, IκB-β content in the nucleus increases subsequent to stimulation in some settings (31, 33). The levels of IκB proteins in the nuclear fractions collected from mouse lungs before and after LPS instillation were examined by Western analyses. Although the majority of IκB-α and IκB-β in the lungs was cytoplasmic, both proteins were detected in the nuclear fractions prior to LPS instillation. In contrast, IκB-ε in the nuclear compartments was present, if at all, at levels too low to reliably visualize or quantitate. These results are consistent with the transit of IκB-α and IκB-β through the nuclear compartment in resting lungs. Contents of both IκB-α and IκB-β decreased in the nuclear fraction after LPS instillation (Table 1). IκB-ε remained at levels too low to reliably quantify. Thus, none of the IκB proteins, including IκB-β, accumulated in the nuclei after LPS instillation to the lungs.

Basal Phosphorylation of IκB-β in the Lungs

A hypophosphorylated form of IκB-β may emerge subsequent to stimulation of NF-κB translocation (31, 32). Elec-

trophoretic mobility was used to differentiate hypophosphorylated forms of IκB-β from basally phosphorylated forms. Incubation with phosphatase prior to electrophoresis increased the electrophoretic mobility of IκB-β collected from the non-nuclear fractions of mouse lungs (Figure 2D). These data demonstrate that hypophosphorylated IκB-β could be differentiated from basally phosphorylated IκB-β using this assay. Furthermore, the results indicate that the majority of IκB-β in resting lungs was basally phosphorylated. No differences in electrophoretic mobility were detected between IκB-β proteins in nuclear compartments and those in non-nuclear compartments, before or 6 h after LPS instillation (data not shown). Thus, these studies did not demonstrate an accumulation of hypophosphorylated IκB-β induced by LPS in the lungs.



Complexes Between IκB Proteins and RelA in the Lungs

LPS induced the degradation of both IκB-α and IκB-β in the lungs (Figure 2) and the nuclear translocation of p50/p50 and p50/RelA (Figure 1). RelA is essential for LPS-induced neutrophil emigration in the lungs (14). To determine whether either or both of these IκB proteins physically interacted with RelA in the lungs, reciprocal co-immunoprecipitations were performed. Immunoprecipitation of either IκB-α or IκB-β coprecipitated RelA, but did not coprecipitate the other IκB protein (Figures 3A and 3B). Immunoprecipitation of RelA coprecipitated both IκB proteins (Figure 3C). Thus, in resting lungs, RelA forms complexes with IκB-α and with IκB-β. These data suggest that the LPS-induced degradation of either IκB-α or IκB-β liberates RelA to translocate to the nucleus in the lungs.

Although IκB-ε levels were not decreased during this inflammatory response, we immunoprecipitated IκB-ε to determine whether IκB-ε formed complexes with RelA in mouse lungs. The immunoprecipitation of IκB-ε coprecipitated RelA, but not IκB-α or -β (Figure 3D). Thus, conditions in which IκB-ε is degraded in the lungs may also result in the nuclear translocation of RelA.

Responses to Intrapulmonary LPS in the Absence of IκB-β

The above data implicate the regulated degradation of IκB-α and/or IκB-β as potential regulatory steps determining NF-κB function and acute inflammatory responses elicited by bacterial LPS in the lungs. To determine whether IκB-β has unique essential roles in regulating pulmonary responses to LPS, biochemical and functional responses to

Figure 3. Biochemical interactions of RelA with IκB proteins. Whole lung lobes were homogenized, and protein complexes containing RelA, IκB-α, IκB-β, or IκB-ε were immunoprecipitated using Ab specific for the proteins indicated under *IP*. An additional lung homogenate was precipitated with nonspecific (*NS*) IgG. Proteins present in the whole lung extract (*E*), proteins which were not precipitated but remained in the supernatant (*S*), and proteins which were precipitated (*P*) were separated by SDS-PAGE. Separated proteins were transferred to membranes, and the presence of IκB-α, IκB-β, or RelA in the protein samples was assessed by immunoblot (as indicated under *IB*). All immunoprecipitations were repeated with proteins from the lungs of multiple mice with consistent results. (*A*) IκB-α was precipitated by IP of RelA or IκB-α, but not IκB-β. (*B*) IκB-β was precipitated by IP of RelA or IκB-β, but not IκB-α. Because Ab used to precipitate IκB-α and -β (but not RelA) were from same species as IκB-β–specific Ab used in IB, the Ig used for IP heavy chains were revealed by secondary Ab staining, apparent in the *precipitate lanes* running slightly higher than the *IκB-β band*. (*C*) RelA was precipitated by IP of IκB-α or IκB-β, but not by non-specific IgG. (*D*) RelA, but not IκB-α or IκB-β, was precipitated by IP of IκB-ε.

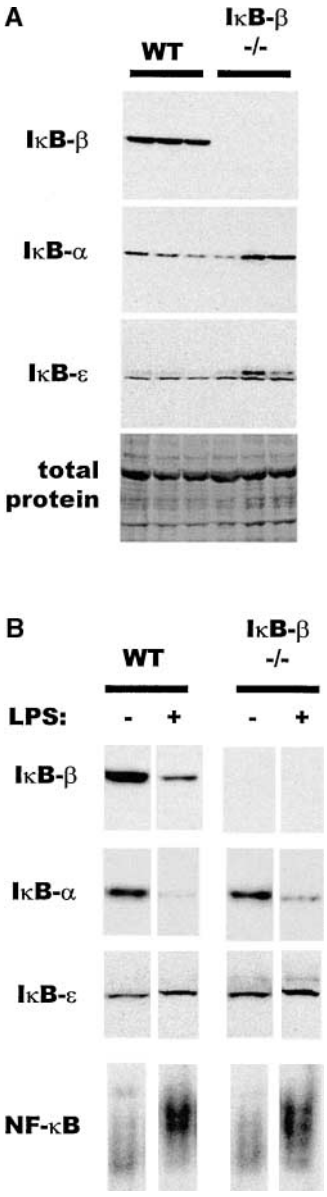


Figure 4. IκB and NF-κB in IκB-β–deficient mice. (*A*) IκB expression detected by Western analyses of non-nuclear fractions from the lungs of mice prior to LPS instillation. Membranes were immunoblotted with polyclonal Ab against the indicated IκB protein, or were stained for total protein using BLOT-Fast-Stain (Genotech; St. Louis, MO). (*B*) Effect of LPS instillation on IκB protein levels and NF-κB nuclear translocation in the lungs of WT and IκB-β–deficient mice. IκB protein levels were detected in the non-nuclear fractions by Western analyses using polyclonal Ab against the indicated IκB proteins. NF-κB levels in the nuclear fractions were detected by EMSA.

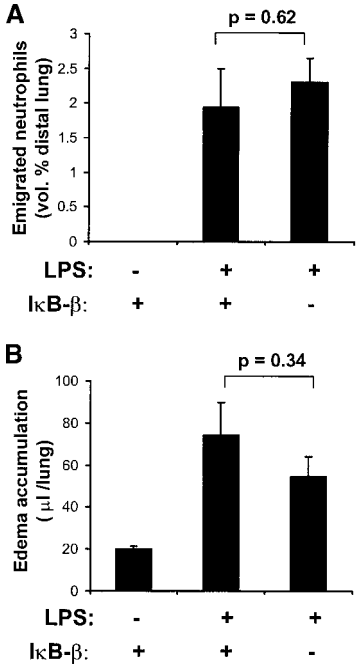


Figure 5. Pulmonary inflammation induced by LPS in the lungs of WT and IκB-β–deficient mice. LPS was instilled intratracheally, and the lungs were collected after 6 h. Baseline values were collected from WT mice. Data depict mean ± SE from four to five mice per group. (*A*) Neutrophil emigration was not affected by IκB-β deficiency. Emigrated neutrophils were quantified as neutrophils within alveolar air spaces, expressed as a percent of the total volume of distal lung, using morphometric analyses of histologic sections. (*B*) Edema accumulation was not affected by IκB-β deficiency. Edema accumulation was quantified as the volume of extravascular plasma equivalents, expressed as μl/lung, using radiotracer analyses.

intratracheally instilled *E. coli* LPS were compared in WT mice and mice deficient in IκB-β due to gene targeting.

Gene targeting resulted in the complete loss of immunoreactive IκB-β (Figure 4A). IκB-α and IκB-ε were detected in non-nuclear fractions from the lungs of IκB-β–deficient mice (Figure 4A), at levels similar to or greater than that in WT mice. IκB-β remained undetectable after LPS instillation to IκB-β–deficient mice (Figure 4B). As in WT mice, LPS instillation to IκB-β–deficient mice diminished levels of IκB-α but not IκB-ε (Figure 4B). The intratracheal instillation of LPS resulted in NF-κB translocation in IκB-β–deficient mice, which persisted at least 6 h, as was the case in WT mice (Figure 4B). Thus, over this time frame, IκB-β does not appear essential to the nuclear accumulation of NF-κB proteins induced by LPS in the lungs.

Potential roles of IκB-β in regulating pulmonary inflammation were examined by comparing neutrophil emigration and edema accumulation induced by *E. coli* LPS in the lungs of WT and IκB-β–deficient mice. Neutrophil emigration was induced by LPS instillation (Figure 5A), as measured by morphometric quantitation of neutrophils in the alveolar air spaces. The quantities of emigrated neutrophils did not differ between WT and IκB-β–deficient mice (Figure 5A), suggesting that IκB-β was not essential for this process. Circulating neutrophil numbers also did not differ between WT and IκB-β–deficient mice with LPS-induced pulmonary inflammation ($1.5 \pm 0.3 \times 10^6$ and $1.7 \pm 0.3 \times 10^6$ per ml blood in WT and mutant mice, respectively). Similarly, pulmonary edema, as measured by the accumulation of extravascular albumin in the lungs, was induced by the intratracheal instillation of LPS (Figure 5B). Again, the amount of extravasated albumin did not differ between WT and IκB-β–deficient mice (Figure 5B), suggesting that IκB-β was not essential for this process. Therefore, IκB-β did not perform unique and required roles in regulating these acute inflammatory responses to LPS in the lungs when studied using gene-targeted mutant mice.

Discussion

The present study demonstrates that LPS in the lungs resulted in decreased levels of IκB-α and IκB-β, consistent with the hyperphosphorylation, ubiquitination, and proteasomal degradation of each of these proteins. The LPS-induced nuclear translocation of NF-κB could therefore be regulated by either or both of these proteins. Coimmunoprecipitations demonstrated that NF-κB RelA, which is essential for neutrophil emigration and antibacterial host defenses (14), complexed with both IκB-α and IκB-β proteins. In contrast

to RelA, neither IκB-α nor IκB-β accumulated in the nuclear fractions after LPS instillation. These data suggest that the LPS-induced nuclear translocation of RelA in the lungs results from the degradation of both IκB-α and IκB-β.

In contrast to IκB-α and -β, the levels of IκB-ε did not decrease in response to LPS in the lungs. These data suggest that IκB-ε is regulated differently than IκB-α and -β in this setting, and that net IκB-ε degradation is not responsible for NF-κB translocation in the lungs up to 6 h after LPS instillation. Interestingly, IκB-ε and RelA did form complexes in mouse lungs, suggesting that the degradation of pulmonary IκB-ε in settings other than LPS-induced inflammation may contribute to the nuclear translocation of RelA. IκB-ε degradation can be induced *in vitro* (18), although the degradation of IκB-ε in the lungs has not been demonstrated to our knowledge.

The nuclear accumulation of NF-κB over several hours may specifically require signaling to IκB-β proteins (19, 20, 31). This postulate is based in part on evidence that, after stimulation by LPS or cytokines, IκB-α re-attains prestimulation values rapidly (typically within 1–2 h), whereas IκB-β remains at decreased levels for longer times (typically $\geqslant$ 4 h). Such distinctive kinetics have been observed after LPS stimulation both *in vitro*, with the 70Z/3 cell line and with cultured murine peritoneal macrophages (19, 21), and *in vivo*, in the liver and in the lungs after intraperitoneal injection (21, 37). However, in the present study, the intratracheal instillation of LPS decreased the pulmonary levels of both IκB-α and IκB-β for at least 6 h. After LPS instillation to the lung, IκB degradation was likely initiated by receptors recognizing LPS, but it may have been prolonged by sequential activation of additional receptors (such as adhesion molecules and cytokine receptors) during neutrophil emigration from the pulmonary capillaries into the alveolar air spaces.

Pulmonary inflammation induced by intra-alveolar IgG immune complexes in rats is associated with a different pattern of IκB regulation than that observed in the present study with pulmonary inflammation induced by intratracheal LPS. Intrapulmonary formation of IgG immune complexes induces a slower degradation of IκB-α, with IκB-α content in the lungs decreasing progressively until it eventually reaches a nadir 4 h after the intratracheal instillation of IgG (38), and no decrease in IκB-β protein (39). In contrast, the present study demonstrates that LPS induced a rapid drop in the levels of IκB-α, apparent less than 1 h after LPS instillation (data not shown) and reaching a nadir by 2 h. Furthermore, IκB-β content decreased in parallel with IκB-α content after LPS instillation. Thus, IκB proteins display distinct patterns of regulation and may serve distinct functions in pulmonary inflammations induced by IgG immune complexes and *E. coli* LPS.

In addition to stimulating NF-κB translocation subsequent to its degradation, a hypophosphorylated form of IκB-β can actively promote NF-κB translocation and NF-κB–mediated gene expression. Hypophosphorylated IκB-β proteins competitively inhibit the interaction of NF-κB proteins with other IκB proteins, but they do not prevent the nuclear translocation or transcriptional activity of NF-κB (31). The hypophosphorylated form of IκB-β may contribute to the excessive inflammation in the airways of patients with cystic fibrosis, as mutation in the CFTR gene in transformed bronchial epithelial cell lines confers an increase in hypophosphorylated IκB-β and in IL-8 expression induced by tumor necrosis factor-α *in vitro* (40). In the present study, phosphatase treatment increased the electrophoretic mobility of IκB-β from the lungs, demonstrating that the protein was phosphorylated. The IκB-β detected after LPS instillation possessed the same electrophoretic mobility as IκB-β proteins from uninflamed lungs. These data do not suggest the emergence of a prominent hypophosphorylated (and faster migrating) form of IκB-β induced by LPS in the lungs. Further, the genetic deficiency of IκB-β did not prevent the nuclear accumulation of NF-κB proteins through 6 h after LPS instillation, and neither neutrophil emigration nor edema accumulation were compromised by IκB-β deficiency over this period. Thus, hypophosphorylated forms of IκB-β are not essential to promoting NF-κB functions critical to the acute inflammatory responses elicited by LPS in the lungs.

As with any work based on targeted mutation as a means to study the function of a gene, other genes may potentially be regulated in ways that are not typical of animals without the mutation, appropriating functions normally mediated by the targeted gene. The genetic deficiency of IκB-α results in increased expression of IκB-ε, as detected by Western blots of the non-nuclear fractions from embryonic fibroblasts (18), although the overexpressed IκB-ε is incapable of performing IκB-α functions that are essential to homeostasis *ex utero* (26). In the present study, two of three IκB-β–deficient mice demonstrated increased levels of both IκB-α and IκB-ε compared with WT mice, as detected by Western blots of the non-nuclear fractions from uninstilled lungs. Thus, IκB-α and/or IκB-ε may have been altered in the lungs of the IκB-β–deficient mice prior to LPS instillation. It is possible that alterations in IκB-α, IκB-ε, or other genes, resulting indirectly from targeted mutation of the IκB-β gene, may have prevented the identification of IκB-β functions that are critical to regulation of acute inflammatory responses in the lungs of normal mice. The present results indicate that IκB-β does not possess unique attributes, unavailable to other gene products, that are essential to mediating neutrophil emigration and edema accumulation elicited by intrapulmonary LPS.

Altogether, the present results and previous studies suggest that IκB proteins play diverse roles in different settings of pulmonary inflammation. The intratracheal instillation of LPS elicits signaling events (and downstream cellular and physiological changes) that are directly relevant to infection of the distal lung with Gram-negative bacteria. The present data are, to our knowledge, the first to report the effects of intrapulmonary LPS on IκB proteins. These findings indicate that the nuclear translocation of RelA and p50 induced by LPS in the lungs is associated with the degradation of both IκB-α and IκB-β, but not IκB-ε. Because both IκB-α and IκB-β complex with RelA, the translocation of this NF-κB subunit induced by LPS in the lungs requires the degradation of both of these IκB proteins. Studies with IκB-β–deficient mice demonstrate that IκB-β proteins do not possess unique properties which are essen-

tial to NF-κB translocation or to acute inflammation induced by *E. coli* LPS in the lungs. Thus, IκB-α or other proteins, in the absence of IκB-β, can regulate the NF-κB functions induced by intrapulmonary LPS that are required for acute neutrophil emigration.

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