The NF-κB Inhibitory Proteins IκBα and IκBβ Mediate Disparate Responses to Inflammation in Fetal Pulmonary Endothelial Cells

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Exposure to intrauterine inflammation impairs lung growth but paradoxically protects the neonatal pulmonary vasculature from hypoxic injury. The mechanisms mediating these contradictory effects are unknown. The objective is to identify the role of NF-κB in mediating cytoprotective and proinflammatory responses to inflammation in the fetal pulmonary endothelium. In newborn rats exposed to intra-amniotic LPS, we found increased expression of the NF-κB target gene manganese superoxide dismutase (MnSOD) in the pulmonary endothelium. Supporting these in vivo findings, LPS induced NF-κB activation and MnSOD expression in isolated fetal pulmonary arterial endothelial cells. In addition, LPS exposure caused apoptosis and suppressed cellular growth and induced P-selectin expression. LPS-induced NF-κB activation that proceeded through specific isoforms of the inhibitory protein IκB mediated these diverse responses; NF-κB signaling through IκBα degradation resulted in MnSOD upregulation and preserved cell growth, whereas NF-κB signaling through IκBβ degradation mediated apoptosis and P-selectin expression. These findings suggest that selective inhibition of NF-κB activation that results from IκBβ degradation preserves the enhanced antioxidant defense and protects the developing pulmonary vascular endothelium from ongoing inflammatory injury.  

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Chorioamnionitis is an inflammatory response to infection of the placental membranes, with prevalence inversely proportional to gestational age at delivery (1). Clinical chorioamnionitis significantly contributes to the development of bronchopulmonary dysplasia, a chronic lung disease of infancy (1, 2) and is associated with pulmonary hypertension and severe neonatal hypoxic respiratory failure in the newborn (3, 4). Those clinical findings link antenatal inflammation with endothelial dysfunction and injury in the developing lung, given that the endothelium critically regulates neonatal lung growth and the perinatal transition of pulmonary vasculature (5–9). The link between antenatal inflammation and pulmonary endothelial injury has been demonstrated in experimental chorioamnionitis, which causes fetal pulmonary vascular remodeling, pulmonary hypertension in the fetus and newborn, as well as impaired lung vascular and alveolar growth in the neonate (10–12). However, the underlying mechanisms that regulate the response of the developing pulmonary endothelium to antenatal inflammatory stress remain largely unknown.

Our recent study showed that exposure to either intra-amniotic LPS or postnatal hypoxia impairs lung vascular growth (13). Interestingly, intra-amniotic LPS protects the neonatal pulmonary vasculature against hypoxic injury. These findings indicate that intrauterine inflammatory stress induces both a cytoprotective response and disruptive effects on the growth of the developing pulmonary vasculature. However, the mechanisms underlying these paradoxical responses are unclear. Animal studies have reported that systemic or direct pulmonary exposure to LPS in neonatal or adult animals stimulates the expression of lung antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), thereby increasing the tolerance to hypoxic lung injury (14–17). Moreover, LPS exposure induces MnSOD in cultured pulmonary vascular endothelial cells (PAEC) isolated from the fetus as well as the adult (18, 19). However, whether intrauterine inflammatory stress induces MnSOD expression in pulmonary endothelium has not been studied. Moreover, the transcriptional mechanism through which LPS upregulates MnSOD expression in lung vascular endothelial cells is unknown.

The transcription factor NF-κB mediates cellular response to inflammatory stress. In quiescent cells, the IκB family of inhibitory proteins sequesters NF-κB dimers in the cytoplasm. The main cytoplasmic isoforms of IκB responsible for keeping NF-κB inactive are IκBα and IκBβ. Exposure to inflammatory stimuli results in the degradation of both IκBα and IκBβ, thereby releasing NF-κB dimers, allowing nuclear translocation and downstream gene expression (20). In addition to the well-studied NF-κB activity in immune cells, LPS-induced NF-κB activation has been reported in endothelial cells isolated from adult vasculature and human umbilical vein (21, 22). Moreover, NF-κB activation in endothelial cells mediates the expression of cytokines, chemokines, and adhesion molecules, which recruit circulating leukocytes into the inflamed tissue (23). In fact, endothelial NF-κB activity has been shown responsible for inflammatory lung injury and systemic vascular dysfunction in adult animals with endothoxemia (24, 25). Paradoxically, inflammatory stress-induced NF-κB activation also upregulates the transcription of cytoprotective...
genes, including antioxidant enzymes and anti-inflammatory mediators (26, 27). However, the role of inflammatory stress-induced endothelial NF-κB activation in directing proinflammatory and cytotoxic responses in the developing pulmonary vasculature is unknown.

To determine the mechanisms underlying the protective effect of antenatal inflammation against subsequent hyperoxic injury in the developing pulmonary vasculature, we hypothesized that intra-amniotic LPS enhances expression of MnSOD in fetal lung vascular endothelium. We further hypothesized that NF-κB activation regulates disparate responses of the fetal pulmonary vascular endothelium to inflammatory stress. We report that intra-amniotic LPS exposure increases MnSOD protein expression in the pulmonary vascular endothelium of newborn rats. In addition, in fetal PAEC, LPS exposure induces MnSOD expression but also causes apoptosis, suppresses cell growth and expression of vascular endothelial growth factor receptor 2 (VEGFR2) while inducing P-selectin expression. Moreover, we found that LPS-induced NF-κB activation mediates these diverse responses specifically through IκBα and IκBβ degradation; NF-κB signaling through IκBα degradation mediates MnSOD expression and is required to preserve cell growth and VEGFR2 expression, whereas NF-κB signaling through IκBβ degradation mediates apoptosis and P-selectin expression. To our knowledge, our findings demonstrate for the first time that selective inhibition of IκBβ-mediated NF-κB activation maintains the inflammatory stress-induced upregulation of antioxidant enzyme and preserves cellular growth, as shown in fetal pulmonary vascular endothelial cells.

Materials and Methods

Animals and intra-amniotic LPS administration

Fetal Sprague–Dawley rats were exposed to intra-amniotic injection of LPS (10 μg in 50 μl normal saline, L5418, Sigma-Aldrich) or saline (50 μl) at 20 gestration and delivered via cesarean section 2 d later as described previously (13).

Whole-lung homogenate and superoxide manganese dismutase expression and activity

Lung tissue harvested at birth was processed as previously described and assessed for superoxide manganese dismutase (SOD1), MnSOD, and SOD3 expression via Western blot analysis (13). SOD activity was determined in total lung homogenates using an SOD assay kit-WST (Dojindo Molecular Technologies) as described previously (28).

Immunohistochemical localization of pulmonary MnSOD

To assess MnSOD expression in pulmonary vascular endothelial cells, immunofluorescence double staining of MnSOD and von Willebrand factor (vWF) was performed on cryosections cut from OCT-embedded newborn rat lung blocks. The sections were blocked with 0.5% horse serum in 0.5% normal goat serum at room temperature and then were incubated with anti-MnSOD Ab (1:250, number 06-984; Millipore) and anti-vWF Ab (1:50, number MA1-82048; Thermo Pierce) at 4˚C overnight. On the following day, the sections were incubated with secondary Abs at 1:250 (Alexa Fluor 594 donkey-anti-rabbit for MnSOD and Alexa Fluor 488 donkey-anti-mouse for vWF; Molecular Probes) for 2 h at room temperature. The sections were then mounted with DAPI (Vector Laboratories) and imaged with an Olympus IX71 fluorescence microscope (Olympus America, Center Valley, PA). To further examine the architecture of pulmonary vasculature that was shown in immunofluorescence staining, adjacent cryosections were stained with H&E and were imaged under a light microscope.

Isolation and culture of fetal ovine PAEC

Proximal fetal ovine PAEC were isolated as previously described (29), and endothelial cell phenotype was confirmed by positive immunostaining for vWF, endothelial NO synthase, vascular endothelial cadherin, and VEGFR2, positive uptake of acetylated low-density lipoprotein, and negative staining for desmin. Cells from passages 4 to 8 were used for all experiments, and cells from individual animals were kept separate throughout all experiments.

Cell culture and LPS exposure

Fetal ovine PAEC were grown in DMEM supplemented with 10% FBS, 1% antibiotics/antimycotics (Invitrogen), and 1% nonessential amino acid solution (Sigma-Aldrich) and maintained at 37˚C in 5% CO2 and 95% room air. In all experiments, cells were seeded at 15,000 cells/cm2 in plastic culture dishes and allowed to adhere overnight prior to exposure. For LPS exposures, the medium was aspirated from the cells (~80% confluent), and fresh medium containing LPS (L6529; Sigma-Aldrich) was added.

Treatment of PAEC with the NF-κB inhibitors parthenolide and BAY-7085

Fetal ovine PAEC were treated with inhibitors of the NF-κB pathway, BAY-7085 and parthenolide (Sigma-Aldrich). We chose to separately test two different inhibitors of NF-κB activation to help ensure that any effect observed was due to NF-κB inhibition rather than off-target effects. BAY-7085 was diluted in ethanol. Parthenolide was diluted in DMSO. PAEC were pretreated with either BAY-7085 or parthenolide for 1 h prior to LPS exposure and maintained in the medium throughout the LPS exposure.

Preparation of cell lysate, cytotoxic and nuclear extractions

Cell lysate, cytotoxic or nuclear extracts were prepared as described previously (30).

Cell fractionation and immunoblot analysis

Cell lysate or cytotoxic or nuclear extracts were electrophoresed (4–12% polyacrylamide gel; Invitrogen). Proteins were transferred to an Immobilon membrane (Millipore) and blotted with anti-IκBα (sc-371), anti-IκBβ (sc-9130), anti-P-selectin (sc-6943), anti-SOD3 (sc-67809), or anti-lamin B (sc-6216) Abs (Santa Cruz Biotechnology); anti-c-Rel (4774), anti-VEGFR2 (2479), and anti-caspase-3 (9665) Abs (Cell Signaling Technology); anti-p50 (ab7971) and anti-MnSOD (60-984) or anti-tubulin (05-829) Ab (Millipore). Densitometric analysis was performed using ImageJ.

Evaluation of nuclear NF-κB binding by EMSA

A 32P-labeled oligonucleotide with the consensus sequence for NF-κB (5'-AGTTGAGGACAGCTTCCAGGC-3') (Promega, Madison, WI) was used as a probe to evaluate NF-κB binding ability as described previously (31). To identify nonspecific binding of nuclear proteins, competition reactions were performed by addition of either 50-fold excess of the nonradiolabeled NF-κB consensus sequence or 50-fold excess of nonradiolabeled mutated NF-κB consensus sequence (5'-AGTTGAGGC-ACTTCCAGGC-3') (Santa Cruz Biotechnology, Santa Cruz, CA) to the reaction mixtures prior to electrophoresis. In separate experiments, to identify the NF-κB subunit proteins in the binding complex, 2.5 μl p50 or p65 Abs (Calbiochem) was incubated with nuclear proteins for 1 h at 37˚C prior to addition of the radiolabeled probe.

Evaluation of caspase-3 activity

Caspase-3 activity was assessed as a measure of apoptosis as described previously (30).

Growth assay

Fetal PAEC were plated at 1 × 105 cells/well into six-well plates and allowed to adhere overnight. The following day (day 0) the medium was changed to DMEM with 2.5% FBS plus LPS (0.1–100 ng/ml), with and without BAY 11-7085 (1 μmol/l) or parthenolide (0.1 μmol/l) pretreatment. These doses were chosen after dose–response experiments showed no effect of BAY 11-7085 or parthenolide on baseline growth after prolonged (3 d) exposure when compared with control (data not shown). Medium was changed daily, and cell counts were performed on days 0 and 3 after removal of trypsin digestion. All conditions were run in triplicate. Growth studies with treatment were performed in DMEM with 2.5% FBS, based on previous studies that determined that this was the lowest serum concentration that supported fetal PAEC survival with some proliferation (32).

Statistical analysis

For comparison between treatment groups, the null hypothesis that no difference existed between treatment means was tested by ANOVA for multiple groups or t test for two groups (InStat). Statistical significance

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(p < 0.05) between and within groups was determined by means of Bonferroni method of multiple comparisons.

**Results**

**Antenatal exposure to LPS increases SOD activity and MnSOD expression**

Our recent study demonstrated that exposure to intra-amniotic LPS protects against hyperoxia-induced neonatal pulmonary vascular injury (13). Because higher SOD activity attenuates hyperoxic injury (14–17), the expression of pulmonary SOD in newborn mice following exposure to intra-amniotic LPS was evaluated.

Intra-amniotic LPS resulted in a significant increase in total pulmonary SOD activity when compared with saline-injected controls on DOL 1 (Fig. 1A). On Western blot analysis, intra-amniotic LPS increased the protein expression of MnSOD (SOD2), whereas it decreased SOD1 expression, and SOD3 did not change (Fig. 1B, 1C). These data show that specific upregulation of MnSOD expression is responsible for the increased pulmonary SOD activity in newborn rats after exposure to antenatal LPS.

The source of increased MnSOD expression in the newborn lung examined by immunofluorescence staining. In saline-exposed newborn rats, MnSOD was expressed almost exclusively in the

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Exposure to intraamniotic LPS induces MnSOD expression and activity in newborn rat lung. (A) Total pulmonary SOD activity measured in whole-lung homogenate from newborn rats exposed to intra-amniotic LPS. Saline, intra-amniotic saline injection; LPS, intra-amniotic LPS injection. Values are means ± SE, n = 4 animals/group. *p < 0.05 versus saline control. (B) Representative Western blot showing SOD1, MnSOD, and SOD3 in whole-lung homogenate from newborn rats following exposure to intra-amniotic saline versus LPS. β-Actin is shown as a loading control. (C) Densitometric evaluation of SOD1, MnSOD, and SOD3 protein expression in whole-lung homogenate from newborn rats exposed to intra-amniotic saline versus LPS. *p < 0.05 versus saline control. Values are means ± SE, n = 4 animals/group. (D) Representative immunofluorescence and H&E staining of newborn rat lungs following intra-amniotic injection of saline (Da)–(Dd) or LPS (De)–(Dh), all at original magnification ×200. MnSOD was stained in red, vWF in green, and DAPI in blue.
bronchial epithelium (Fig. 1A–D). However, LPS-exposed newborn rats demonstrated increased intensity of MnSOD signal (Fig. 1Dg and 1Dc versus 1Dg), part of which was colocalized with vWF, a marker of endothelial cells (Fig. 1Dh and 1Dd versus 1Dh). H&E staining on the adjacent lung sections confirmed the architecture of pulmonary vasculature (Fig. 1Da and 1De), which was double positive for MnSOD and vWF as shown in immunofluorescence staining of LPS-exposed rat lungs. These findings indicate that increased MnSOD expression in the pulmonary endothelium contributes to elevated MnSOD in the fetal lung in response to intra-amniotic LPS.

**Increased MnSOD protein expression in fetal PAEC exposed to LPS**

To further evaluate the in vivo observation of increased MnSOD expression in the developing pulmonary endothelium following intraamniotic LPS, we exposed fetal PAEC to LPS. Following exposure to LPS for 2–4 h, protein levels of MnSOD increased significantly (Fig. 2A, 2B). These findings support the in vivo data showing that exposure to antenatal LPS results in increased MnSOD expression in the fetal pulmonary endothelium.

**LPS induces NF-κB activity in fetal PAEC**

Because LPS is known to induce NF-κB activity, and cytokines induce MnSOD expression via NF-κB activation (33, 34), NF-κB activity was assessed in fetal PAEC. Cytoplasmic IκBα and IκBβ degradation occurred within 30 min of exposure to LPS (10 ng/ml; Fig. 3A). Densitometric evaluation of IκB levels following LPS confirmed significant decreases of IκB levels starting at 30 min of exposure and continuing through 8 h for IκBα and 4 h for IκBβ (Fig. 3A).

To confirm that degradation of IκB was associated with nuclear translocation of NF-κB subunits, nuclear extracts were subjected to Western blot analysis and probed for the NF-κB subunit p65. Corresponding with the timing of IκB degradation, nuclear p65 increased significantly after 30 min of exposure to LPS and remained elevated through 4 h of exposure (Fig. 3B). Furthermore, EMSA revealed increased NF-κB DNA binding at 1 h of exposure to LPS, which was persistent through 18 h of exposure (Fig. 3C, lanes 2 and 4, respectively). Supershift analysis confirmed presence of the NF-κB subunits p65 (Fig. 3C, lane 7) and p50 (Fig. 3C, lane 8). These results confirm that LPS induces IκB degradation, subsequent NF-κB nuclear translocation, and DNA binding in fetal PAEC.

**NF-κB inhibitors BAY 11-7082 and parthenolide have differential effects on IκB-mediated NF-κB activation in fetal PAEC**

Having demonstrated that LPS-induced NF-κB activity occurs in fetal PAEC, the effect of NF-κB inhibitors was assessed. Parthenolide and BAY 11-7085, both IκB kinase (IKK) inhibitors, were used. Fetal PAEC were pretreated with either BAY 11-7085 or parthenolide for 1 h prior to LPS (10 ng/ml, 1 h) exposure. BAY 11-7085 completely inhibited IκBα and IκBβ degradation at a dose of 4 μmol/l (Fig. 4A). In contrast, parthenolide did not inhibit IκBα degradation at any dose, whereas IκBβ degradation was inhibited in a dose-dependent fashion starting at 0.5 μmol/l (Fig. 4B). Doses of parthenolide > 2 μmol/l were not assessed by Western blot analysis because visible cytotoxicity was noted prohibiting adequate protein collection. On the basis of these experiments, doses of BAY 11-7085 (4 μmol/l) and parthenolide (2 μmol/l) were used for all further experiments.

Having identified these doses, the time course of LPS-induced NF-κB activation following BAY 11-7085 and parthenolide pretreatment was determined. In fetal PAEC pretreated with BAY 11-7085, degradation of both IκBα and IκBβ was prevented through 4 h of exposure to LPS (Fig. 5A and 5D, 5E). Prevention of IκB degradation by BAY 11-7085 was associated with complete inhibition of p65, p50, and c-Rel nuclear translocation in LPS-exposed PAEC (Fig. 5A and 5C and 5F–H). These data suggest that pretreating with BAY 11-7085 completely prevents NF-κB activation. In contrast to BAY 11-7085, parthenolide did not affect IκBα degradation but selectively attenuated LPS-induced IκBβ degradation (Fig. 5B and 5D, 5E). This effect of parthenolide resulted in attenuated p65 and p50 nuclear translocation (Fig. 5B and 5F, 5G) and complete inhibition of c-Rel nuclear translocation in LPS-exposed PAEC (Fig. 5C and 5H). Of note, cells pretreated with parthenolide prior to LPS demonstrated significantly less p65 and p50 nuclear translocation when compared with LPS treatment alone (Fig. 5B and 5F, 5G). Furthermore, p65 nuclear translocation was significantly higher in cells pretreated with parthenolide when compared with BAY 11-7085 pretreatment prior to exposure to LPS at all time points (Fig. 5F). On the basis of previous reports that c-Rel containing NF-κB dimers bind preferentially to IκBβ (35–41), our data suggest that parthenolide specifically inhibits LPS-induced c-Rel nuclear translocation through preventing IκBβ degradation. Accordingly, the presence of LPS-induced p65 and p50 nuclear translocation likely represents intact NF-κB signaling through IκBα degradation.

**NF-κB inhibitors BAY 11-7082 and parthenolide have differential effects on NF-κB-mediated MnSOD expression in fetal PAEC**

Given the in vitro data demonstrating that LPS exposure induced MnSOD activity and protein expression in the fetal lung, the effect of NF-κB blockade on MnSOD expression in fetal PAEC was assessed. A significant increase in MnSOD expression was seen in
fetal PAEC exposed to LPS (Fig. 6A, 6B). Pretreatment with BAY 11-7085 completely abrogated this upregulation (Fig. 6A, 6B). In contrast, pretreatment with parthenolide did not affect LPS-induced MnSOD expression (Fig. 6A, 6B). These results suggest that LPS-induced NF-κB activity that proceeds through IkBb is responsible for MnSOD expression.

NF-κB inhibitors BAY 11-7085 and parthenolide prevent NF-κB–mediated target proinflammatory gene expression and apoptosis in fetal PAEC

Having demonstrated a differential effect of BAY 11-7085 and parthenolide on NF-κB signaling, the expression of proinflammatory NF-κB–regulated genes and apoptosis was assessed. Both parthenolide and BAY 11-7085 pretreatment prior to LPS exposure prevented expression of P-selectin (Fig. 6C, 6D). In addition, both BAY 11-7085 and parthenolide pretreatment prevented LPS-induced apoptosis in fetal PAEC as assessed by caspase-3 cleavage by Western blot analysis (Fig. 6C and 6E). The results on Western blot analysis were corroborated by caspase-3 activity assay (Fig. 6F). These results suggest that preventing NF-κB activity through IkBb attenuates inflammation and apoptosis following exposure to LPS. Thus, the proinflammatory component of LPS-induced NF-κB activation is attenuated by preventing IkBb degradation, whereas the protective acute-phase response of MnSOD expression is preserved by allowing NF-κB signaling to proceed through IkBa.
Parthenolide, but not BAY 11-7085, attenuates LPS inhibition of PAEC growth and impaired VEGFR2 expression

Having noted that LPS-induced apoptosis was inhibited by both BAY 11-7085 and parthenolide, the effect on cell growth was assessed. Fetal PAEC demonstrated a dose-dependent decrease in cell number through 3 d of LPS exposure (Fig. 7A). The effect of NF-κB inhibition on LPS-induced decrease in cell growth was assessed after BAY 11-7085 and parthenolide pretreatment. Parthenolide pretreatment abrogated the LPS-induced decrease in cell number (Fig. 7B) that was associated with preserved VEGFR2 expression (Fig. 7C, 7D). In contrast, although BAY 11-7085 was able to inhibit apoptosis, pretreatment was not able to prevent the LPS-induced decrease in cell growth (Fig. 7B) and was associated with decreased VEGFR2 expression (Figs. 7C, 7D). A summary
of the NF-κB signaling events resulting in these findings is depicted in the schematic in Fig. 8.

**Discussion**

This study highlights several novel findings. First, we found that intra-amniotic LPS exposure during late gestation increases MnSOD expression in pulmonary vascular endothelium of newborn rats. Furthermore, we showed that upregulation of MnSOD in fetal pulmonary vascular endothelial cells in response to LPS is dependent on NF-κB activation. Third, we demonstrated the differential roles of IκBa and IκBβ in regulating downstream effects of LPS-induced NF-κB activity on fetal PAEC: NF-κB activation
through IκBa degradation mediates LPS-upregulated MnSOD, whereas NF-κB signaling through IκBβ degradation mediates LPS-induced apoptosis and P-selectin expression. Moreover, we found that IκBa degradation is required for protecting growth of fetal PAEC against LPS, likely through preservation of VEGFR2 expression and induction of MnSOD expression. These findings collectively support our hypothesis that endothelial NF-κB activation regulates disparate responses of fetal pulmonary artery endothelium toward inflammatory stress.

To our knowledge, this is the first study reporting that LPS activates NF-κB in fetal vascular endothelial cells. The pulmonary vascular endothelium is not only a prime target of inflammatory insults (42) but also plays a pivotal role in orchestrating vascular and alveolar growth in the developing lung (5–8). The present study reports that LPS-induced NF-κB activation mediates multiple downstream cellular responses. First, we demonstrate that LPS-activated NF-κB expression upregulates MnSOD, which builds on previous work showing that NF-κB is involved in the induction of MnSOD by proinflammatory stimuli (33, 34). Second, NF-κB signaling provides a transcriptional mechanism to explain the previously reported apoptotic response to LPS in fetal PAEC (18). Third, the current study reports that LPS-activated NF-κB in fetal PAEC mediates the induction of P-selectin, which has been found significantly contributing to inflammatory lung injury in endotoxemia (43). The ability of LPS-induced NF-κB activation to regulate the expression of both cytoprotective and proinflammatory genes in fetal PAEC, as demonstrated in the current study, uncovers the transcriptional regulation that links the oth-

FIGURE 7. Parthenolide attenuates LPS-induced inhibition of fetal PAEC proliferation. (A) Fetal PAEC cell number expressed as percentage of control following exposure to LPS (range, 0.1–100 ng/ml) for 3 d. Values are means ± SE of three independent experiments for each group. *p < 0.05 versus unexposed control. (B) Fetal PAEC cell number expressed as percentage of control following exposure to either BAY 11-7085 (1 nmol/l), parthenolide (0.1 μmol/l), LPS (1 ng/ml), or combination for 3 d. Values are means ± SE of three independent experiments for each group. *p < 0.05 versus control, †p < 0.05 versus LPS and LPS + BAY exposed. (C) Representative Western blot showing VEGFR2 protein in whole-cell lysates from fetal PAEC pretreated with BAY-7085 (4 μmol/l, 1 h) or parthenolide (2 μmol/l, 1 h) prior to LPS exposure (10 ng/ml, 6 h). Calnexin is shown as a loading control. (D) Densitometric evaluation of VEGFR2 in fetal PAEC pretreated with NF-κB inhibitors (1 h) and exposed to LPS (10 ng/ml, 6 h). Values are means ± SE of three independent experiments for each group. *p < 0.05 versus control, †p < 0.05 versus LPS-7085 pretreatment, ‡p < 0.05 versus time-matched LPS and BAY + LPS exposed.
Mechanisms underlying the differential roles of IκBα and IκBβ in regulating fetal lung endothelial response to inflammatory stress may reflect the known differences between IκBα and IκBβ. The penultimate step of NF-κB nuclear translocation and DNA binding converges on the inhibitory proteins IκBα and IκBβ (20). Although both IκBα and IκBβ inhibit NF-κB activation, signaling that proceeds through these proteins has unique implications on target gene expression. Because IκBα and IκBβ preferentially bind unique NF-κB dimer combinations, and each dimer combination has different affinity for DNA sequences, specific genes are targeted (35, 36). In addition, following degradation, newly synthesized IκBα enters the nucleus and removes DNA-bound NF-κB complexes through its nuclear export sequence, thereby terminating the activity of NF-κB dimers on transcribing the target genes (20). In contrast, IκBβ has no nuclear export sequence and, once in the nucleus, facilitates and stabilizes DNA binding of NF-κB dimers, resulting in expression of specific proinflammatory target genes (38–40, 44, 45). This has important implications on the expression of NF-κB target genes, because the duration of NF-κB nuclear localization plays a role in determining which target genes are expressed, including proinflammatory chemokines (41, 46). Thus, the effect of specifically inhibiting LPS-induced IκBβ degradation in fetal pulmonary endothelial cells may be due to inhibiting the nuclear translocation of specific NF-κB dimers or simply from truncating the duration and blunting NF-κB activity. By using knockout or transgenic mice with controlled expression of IκBα and IκBβ, respectively, future animal studies will determine whether selective inhibition of endothelial IκBβ degradation during inflammatory stress may attenuate fetal lung injury from antenatal inflammation while preserving normal lung development.

The anti-inflammatory properties of the sesquiterpene lactone parthenolide have been attributed to its effects on NF-κB. Reported mechanisms of action include inhibition of IKK activity or p65 DNA binding (47). Previous studies have demonstrated that parthenolide can inhibit LPS-induced NF-κB activation in vitro and in vivo (48–54). However, in these studies, the effect of parthenolide on LPS-induced IκBα degradation was not assessed. Conflicting data exist on whether parthenolide inhibits LPS-induced IκBα degradation, whereas the effect on IκBβ degradation has never been reported. Parthenolide inhibits LPS-induced IκBα degradation and NF-κB activation in RAW 264.7, peripheral blood mononuclear, and vascular smooth muscle cells (55–58). In contrast to these findings, others have found parthenolide can prevent LPS-induced NF-κB activation despite only transient or absent inhibition of IκBα degradation (59, 60). Our data show conclusively that parthenolide inhibits IκBβ degradation, NF-κB nuclear translocation, and NF-κB target gene expression in fetal pulmonary endothelial cells. Thus, the differences between our findings and those previously reported are most likely stimulus dependent, dose dependent, cell type specific, and developmentally regulated in cells and tissues being studied. The discovery of differential effects of BAY-7085 and parthenolide on the IκB isoforms was unexpected, but we believe the data are in concert with previous findings regarding the roles of IκBα and IκBβ in mediating LPS-induced NF-κB activation. In addition, it must be acknowledged that the use of pharmacologic inhibitors in this study raises the question of “off-target” effects on various signaling pathways and the cell-type specificity of these effects. For example, parthenolide can affect MAPK signaling stimulated by LPS (61, 62) as well as ERK and AP-1 activity stimulated by TNF-α and IL-1β (63). Thus, the interpretation of our data must be tempered by the reality that there may be yet undiscovered effects of parthenolide and BAY-7085 on intracellular signaling.
and their effects on specific types of cells not completely understood. However, these findings suggest that there may be therapeutic advantages to selectively targeting the proinflammatory consequences of NF-κB activation that occur with IκB ε degradation.

Our studies are limited in that we used only pharmacologic agents to inhibit inflammatory stress-induced NF-κB activity in the fetal PAECs. We chose to use pharmacologic inhibition, rather than transient overexpression or silencing of the IκB isoforms for multiple reasons. First, overexpression of either IκBα or IκB ε would lead to nearly complete inhibition of LPS-induced NF-κB activity because of the cellular abundance of inhibitory protein. Moreover, silencing expression of either IκBα or IκB ε alters both constitutive and induced NF-κB activity (64).

In conclusion, the current study demonstrates that LPS-induced NF-κB activation mediates both cytoprotective and destructive responses in fetal pulmonary vascular endothelial cells. LPS-induced NF-κB activity achieves this differential transcriptional regulation through specific isoforms of IκB proteins; NF-κB signaling via IκBε degradation upregulates MnSOD and preserves cell growth, whereas NF-κB activation through IκBα degradation induces apoptosis and P-selectin expression. The disparate responses mediated by NF-κB signaling in fetal PAEC parallel the antioxidant and injurious effects of experimental chorioamnionitis on the neonatal pulmonary vasculature. We speculate that selective inhibition of endothelial NF-κB activation that results from IκBε degradation may prevent endothelial dysfunction and preserve the enhanced antioxidant defense in the developing pulmonary vasculature exposed to intrauterine inflammatory stress.

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Disclosures

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