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Endotoxemia induces IκBβ/NFκB dependent ET-1 expression in hepatic macrophages

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Abstract

Elevated serum concentrations of the vasoactive protein ET-1 occur in the setting of systemic inflammatory response syndrome and contribute to distal organ hypoperfusion and pulmonary hypertension. Thus, understanding the cellular source and transcriptional regulation of systemic inflammatory stress-induced ET-1 expression may reveal therapeutic targets. Using a murine model of LPS-induced septic shock, we demonstrate that the hepatic macrophage is the primary source of elevated circulating ET-1, rather than the endothelium as previously proposed. Using pharmacologic inhibitors, ET-1 promoter luciferase assays, and by silencing and overexpressing NFκB inhibitory protein IκB expression, we demonstrate that LPS-induced ET-1 expression occurs via an NFκB dependent pathway. Finally, the specific role of the cRel/p65 inhibitory protein IκBβ was evaluated. Although cytoplasmic IκBβ inhibits activity of cRel containing NFκB dimers, nuclear IκBβ stabilizes NFκB/DNA binding and enhances gene expression. Using targeted pharmacologic therapies to specifically prevent IκBβ/NFκB signaling, as well as mice genetically modified to overexpress IκBβ, we show that nuclear IκBβ is both necessary and sufficient to drive LPS-induced ET-1 expression. Together, these results mechanistically link the innate immune response mediated by IκBβ/NFκB to ET-1 expression and potentially reveals therapeutic targets for patients with gram-negative septic shock.

Keywords

NF-kappa B; systemic inflammatory response syndrome; I-kappa B proteins; endothelin-1; macrophage; endothelium

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Introduction

Despite advances in our understanding of the pathophysiologic mechanisms underlying septic shock, morbidity and mortality related to this condition remain high.(1) The search continues for effective therapies to treat septic shock, as well as reliable biomarkers that guide effective intervention and predict outcomes. Multiple studies have demonstrated elevated circulating concentrations of the potent vasoconstrictor endothelin-1 (ET-1) in patients with pneumonia, acute respiratory distress syndrome, and septic shock.(2) Elevated ET-1 concentrations predict severity of illness and mortality in patients with pneumonia and septic shock.(2) Accordingly, it has been suggested that ET-1 could serve as a biomarker to help determine severity of illness, predict need for ICU admission, and mortality in these patients. Together, these findings have led to the intense study of the role that ET-1 plays in the pathogenesis of septic shock in hopes of developing new diagnostic tools and therapies.

Numerous studies in multiple species using endotoxemia to mimic septic shock have demonstrated a reliable and reproducible increase in circulating ET-1.(3–7) It remains unclear whether this response is beneficial or detrimental in the setting of sepsis. While increased ET-1 may help maintain blood pressure and organ perfusion,(8) it exacerbates renal and splanchnic ischemia, as well as pulmonary hypertension.(2) These dual effects may explain why animal studies have demonstrated that nonselective endothelin receptor blockade in the setting of systemic inflammatory stress has both therapeutic advantages and disadvantages. Treatment with nonselective endothelin receptor blockade can improve splanchnic and renal perfusion,(9) and attenuate pulmonary hypertension.(10, 11) However, this therapy has been shown to augment hypotension(12–15) and increase mortality.(12–14) Given these conflicting findings, no therapies targeting ET-1 activity in the setting of sepsis are used clinically.

Identifying the source of ET-1 production during endotoxemia, as well as the intracellular signaling pathways underlying its induction is thus desirable in order to identify more specific and optimized therapies to offset the detrimental effects of pathologic vasoconstriction, including pulmonary hypertension. While it is clear that endotoxemia increases circulating ET-1, the source is unclear. ET-1 was first isolated from endothelial cells,(16) and there is a general consensus that LPS-induced endothelial cell injury gives rise to elevated concentrations of circulating endothelin-1(2, 17) that acts in an autocrine and paracrine manner on smooth muscle cells to increase vasomotor tone.(2, 17, 18) Although some studies have shown that systemic LPS induces pulmonary ET-1 expression,(19, 20) this finding is inconsistent.(21–24) In fact, the most recent studies using more sensitive methods (e.g., RT-qPCR) to assess gene expression are split on whether systemic inflammatory stress induces pulmonary ET-1 expression.(22–24) Other experimental data challenge the paradigm of inflammatory stress-induced pulmonary endothelial ET-1 expression. Firstly, isolated endothelial cells demonstrate little to no LPS-induced ET-1 gene expression(5, 6, 25–27). Secondly, LPS-induced ET-1 expression from endothelial cells *in vitro* was small compared to what was observed *in vivo*.(6) Thirdly, the liver rather than the lung (20, 22, 23, 25, 28), and cultured macrophages rather than the endothelial cells (29, 30), most consistently demonstrate increased ET-1 expression in response to LPS. These findings support human data that indicate the lung is not a major site of ET-1 production in patients

with sepsis and suggests an non-endothelial, non-pulmonary source of increased circulating ET-1.(31)

Importantly, the rate-limiting step of ET-1 bioavailability is gene transcription.(16) Therefore, transcriptional activation of ET-1 may represent a potential therapeutic target. However, the transcriptional regulation of ET-1 in response to LPS has not been determined. The ET-1 promoter is known to have NFκB consensus sequence binding sites.(32) Importantly, whether LPS-stimulated ET-1 expression occurs via an NFκB-dependent mechanism remains unknown.

Here, we hypothesized that systemic LPS induces ET-1 expression via an NFκB dependent mechanism specifically in hepatic macrophages. Mice exposed to intraperitoneal (IP) LPS demonstrated increased hepatic ET-1 mRNA expression and circulating ET-1 concentrations. The hepatic macrophage was identified as the primary source of ET-1 expression using IHC and clodronate ablation of resident hepatic macrophages that abrogated LPS-induced ET-1 expression. *In vitro*, macrophages, but not endothelial cells, demonstrated LPS-induced ET-1 expression, and pharmacologic and genetic inhibition of NFκB activity attenuated LPS-induced ET-1 expression. Finally, either p65 or cRel overexpression was sufficient to induce ET-1 promoter luciferase activity, an effect abolished by deletion of known κB binding sites in the promoter region. Use of IκBβ expression plasmids and siRNA demonstrated a necessary and sufficient role for IκBβ in potentiating LPS-induced ET-1 expression. These findings provide significant novel mechanistic insights into ET-1 expression with endotoxemia, contrasting the previous paradigm of lung endothelial cell derived ET-1 by implicating an IκBβ/NFκB dependent mechanism in the hepatic macrophage. Our results thus provide novel potential therapeutic targets to specifically attenuate the detrimental effects of increased circulating ET-1 in critically ill patients.

Materials and Methods

Animal Model

Adult male ICR mice (6–10 week old, Taconic) and AKBI, or IκBβ knock-in, mice were exposed to either LPS (50 mg/kg; 0111:B4, Sigma Aldrich) or sterile saline by intraperitoneal injection. Mice were sacrificed using sodium pentobarbital, and blood was collected by cardiac puncture. Normal saline (5 ml) was perfused through the right ventricle, and liver and lung samples collected and processed as described below. Mouse blood was collected in sodium citrate (3.2%) coated syringes via cardiac puncture. Samples were left at room temperature for 20 minutes, then spun at 1000g for 15 min at 4C. Serum was aspirated and placed in −80C.

Clodronate ablation of hepatic macrophages

Liposomal clodronate (Clodrosome) or the same volume of control liposome suspension (Encapsome; Encapsula Nanosciences LLC, Nashville, TN), was administered IP to the animals as a full dose (40 mg/kg) the day before exposure to LPS (3mg/kg IP, 4 hrs). Successful ablation was demonstrated by IHC using the pan macrophage marker F4/80

which revealed >99% ablation and *Emr1* (encoding F4/80) expression was suppressed by >90% (data not shown).

Cell Culture and LPS exposure

RAW 264.7 macrophages (ATCC) and C57BL/6 mouse primary lung microvascular endothelial cells (LMEC, CellBiologics) were cultured according to manufacturer's instruction. Human Umbilical Vein Endothelial Cells (HUVEC, a generous gift from Dr. Vivek Balasubramaniam, University of Wisconsin, Madison, WI) were cultured as previously described.(33) Bone Marrow-Derived Macrophages (BMDM) were collected from 6–10 week old male ICR mice and were cultured as previously described.(34) Immortalized human microvascular endothelial cells (IHMVEC, a generous gift from Dr. Sean Colgan, University of Colorado, Aurora, CO) were grown to confluence with EGM2-MV media (Lonza). THP-1 monocytes were grown to confluence and differentiated with 5 ng/ml PMA for 48 hours prior to exposures. Cells were exposed to LPS (1 μg/ml, 055:B5, Sigma). RAW 264.7 cells were pretreated with the pharmacologic NFκB inhibitors Bay 11-7085 (1–20 μM, Sigma) or parthenolide (1–20 μM, Sigma) for 1 hour prior to LPS exposure and maintained in the medium throughout the exposure. In some experiments, conditioned medium was withdrawn from RAW 264.7 or HUVEC following LPS exposure and centrifuged at 5000g for 5 minutes to remove cellular debris and stored at −80C.

Isolation and measurement of mRNA levels

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) on 30 mg lung/liver tissues homogenized in RLT buffer in a Bullet Blender (1.0 mm zirconium oxide beads; Speed 7, 5 min; NextAdvance). For intrahepatic mononuclear cell isolation and mRNA isolation, livers were minced and incubated in Liberase^{R} (Hoffman La Roche, Germany) and DNAse (Sigma-Aldrich, St. Louis, MO) followed by centrifugation and separation over a Histodenz gradient (16%), and mRNA isolated using the RNeasy Mini Kit. cDNA was synthesized using a Verso cDNA Kit (Thermo Scientific). Gene expression was assessed using predesigned exon spanning primers using TaqMan gene expressions system (Applied Biosystems) on a StepOnePlus Real Time PCR System. Data was normalized to 18S rRNA using the cycle threshold (ΔΔCt) method.

Isolation of whole cell lysate and cytosolic and nuclear proteins

Liver and lung homogenate whole cell lysate—Lung/liver tissue was homogenized in T-PER (Thermo Scientific) lysis buffer with protease/phosphatase inhibitors (HALT, Thermo) using the Bullet Blender (1.0 mm zirconium oxide beads; Speed 7, 5 min).

Liver cytosolic and nuclear extracts—Lung/liver tissue was placed in cytosolic lysis buffer (NE-PER Kit, Pierce) with protease and phosphatase inhibitors and homogenized by Bullet Blender (1.0 mm zirconium Oxide beads; Speed 7, 5 min), followed by centrifugation (20000 g × 7 min). The cytosolic fraction was aspirated and the nuclear pellet washed in PBS and cytoplasmic extraction was repeated as above to ensure purity of the nuclear extract. The nuclear pellet was placed in nuclear lysis buffer with protease and phosphatase inhibitors and processed according to the manufacturer's instructions. Protein concentration was determined by Bradford Assay.

Cultured Whole Cell Lysate and Cytosolic and Nuclear Extracts—Whole cell lysates, cytosolic and nuclear extracts were collected from cultured cells, and protein concentration determined as previously described.(35)

IκBβ Overexpression and IκBα/IκBβ Silencing

RAW 264.7 cells were transfected with wild-type or dominant negative IκBα vectors (Clontech), or with a flag-tagged IκBβ vector (Genecopoeia, catalog number Ex-Mm04103-M12) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. To silence IκBα and IκBβ expression, RAW 264.7 cells were transfected with IκBα and IκBβ siRNA (Ambion) using Lipofectamine 2000 (Invitrogen).

Immunoblot Analysis

Whole cell, cytosolic and nuclear extracts were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen), transferred to an Immobilon membrane (Millipore) and blotted with antibodies. Densitometric analysis was performed using ImageLab (Bio-Rad).

Immunohistochemistry

OCT (Tissue-Tek, Sacura) embedded liver was cut (7 μm), fixed in acetone (−20 °C for 10 min), and incubated with antibodies. Sections were visualized on a Zeiss Axio Observer A1 microscope, photographed using a Zeiss AxioCam MRm3 5732 camera using the x40 objective lens with 1.6 optivar. Sequential images were processed and superimposed using Zeiss ZEN Pro 2011 software.

ET-1 ELISA

ELISA was performed using the ET-1 ELISA Kit (Enzo Life Sciences).

Construction and transfection of ET-1 promoter driven luciferase expression plasmids and luciferase assays

NFκB binding sites were identified at −1610, −1279, and −755 in the mouse ET-1 promoter using TFSearch software.(36, 37) ET-1 promoter fragments containing one, two, or all three NFκB binding sites were cloned to the pGL3-basic vector containing the luciferase reporter system. ET-1 promoter driven luciferase expression plasmids and either p65 or cRel expression plasmids (Genecopoeia) were transfected into WT MEF cells using Lipofectamine 2000 and assayed using the Dual Luciferase Reporter Assay System (Promega).

Statistical Analysis

For comparison between treatment groups, the null hypothesis that no difference existed between treatment means was tested by Student's *t*-test for two groups and two-way ANOVA for multiple groups with potentially interacting variables (genotype, LPS exposure), with statistical significance between and within groups determined by means of Bonferroni method of multiple comparisons (InStat, GraphPad Software, Inc). Statistical significance was defined as $p<0.05$.

Study Approval

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado (Aurora, CO).

Results

ET-1 expression is localized to hepatic macrophages in a LPS septic shock model

To determine the cellular source of elevated circulating ET-1 concentrations during endotoxemia, mice were exposed to IP LPS (50 mg/kg) over a 6 hour period. Consistent with previous reports, circulating peripheral serum ET-1 concentrations were significantly increased at 2, 4, and 6 hrs after LPS exposure compared to controls (Fig. 1A). Intriguingly, LPS treatment significantly decreased ET-1 mRNA expression in the lung, and significantly increased ET-1 mRNA expression in the liver when compared to untreated controls (Fig. 1B and 1C). We next determined the hepatic cellular source of ET-1. At 4 hours of LPS exposure, IHC revealed that hepatic ET-1 staining was increased and co-localized with hepatic macrophages (F4/80) and not with endothelial cells (CD31) (Fig. 1E, F). To further localize ET-1 expression to hepatic macrophages, we determined mRNA expression for ET-1 in purified intrahepatic mononuclear cells (ihMNCs) isolated from livers of LPS injected and control mice. Isolation of ihMNCs was chosen as this population of cells is inclusive of macrophage populations.(38) Compared to the ~20 fold increased ET-1 expression in whole liver from LPS exposed mice, expression of ET-1 in ihMNCs was increased ~60 fold compared to ihMNCs from untreated mice (Fig. 1F). In addition, clodronate-mediated ablation of hepatic macrophages almost completely abrogated LPS-induced hepatic ET-1 expression (Fig 1G). These results identify hepatic macrophages as a potential source of circulating ET-1 observed in response to endotoxemia.

Systemic administration of LPS induces hepatic NFκB activation

We next tested the hypothesis that LPS-induced ET-1 expression was mediated through NFκB signaling by evaluating levels of immunoreactive IκBα and IκBβ in liver of LPS exposed mice. Consistent with NFκB activation, levels of cytosolic IκBα and IκBβ were both significantly decreased by 2 hours of LPS exposure, and remained low through the 6 hour exposure (Fig. 2A–C). Furthermore, nuclear translocation of the NFκB subunits c-Rel, p65 and p50 correlated temporally with the degradation of the IκB family of NFκB inhibitory proteins (Fig. 2D, F–H). Purity of cytosolic and nuclear extracts was confirmed by assessing immunoreactive levels of the cytosolic protein GAPDH and the nuclear protein lamin B (Fig. 2E). Increased expression of a canonical NFκB target gene (IκBα) confirmed hepatic nuclear NFκB activity following LPS-exposure (Fig. 2I) that was present in isolated hepatic macrophages as well (Fig. 2J). These results demonstrate that LPS-induced hepatic NFκB signaling occurs in a time course that corresponds with increased transcription of ET-1. Furthermore, these data identify the hepatic macrophage as the primary source of circulating ET-1 during endotoxemia.

In vitro, LPS-induced NFκB activation occurs in both macrophage and endothelial cells

Considering that ET-1 expression in the liver of endotoxemic mice was restricted to macrophages and correlated with NFκB activation, we evaluated LPS-induced, NFκB mediated ET-1 expression in cultured endothelial cells and macrophages. All cell lines were confirmed to express TLR4 and MyD88, key components of the TLR4 signaling pathway, by RT-qPCR (data not shown). Following exposure to LPS, cultured macrophages (RAW 264.7, primary bone marrow-derived macrophages, BMDMs and THP-1) and endothelial cells (LMEC, HUVEC and IHMVEC) demonstrated degradation of the NFκB inhibitory proteins IκBα and IκBβ (Fig. 3A–D, Supplemental Figure 1A, B). Degradation of these inhibitory proteins corresponded with nuclear translocation of the NFκB subunit p65, indicative of LPS-induced NFκB activation (Fig. 3E–H). These findings confirm that LPS-induced degradation of IκB proteins and nuclear translocation of NFκB subunits occurred in all cultured macrophage and endothelial cell lines evaluated.

LPS-induced ET-1 expression occurs in macrophage but not endothelial cells

Following exposure to LPS, cultured primary (BMDM) and immortalized (RAW264.7, THP-1) macrophages and primary (LMEC and HUVEC) and immortalized (IHMVEC) endothelial cells all displayed a significant induction of the NFκB target gene IκBα (Fig. 4A, Supplemental Figure 1C, D). However, they differed markedly in LPS-induced ET-1 expression. In cultured macrophages, relative ET-1 mRNA expression was significantly increased up to >2000 fold (Fig. 4B, Supplemental Figure 1C). In contrast, no LPS-induced ET-1 mRNA expression was observed in endothelial cells (Fig. 4B, Supplemental Figure 1D). To confirm that increased gene expression was associated with increased protein synthesis, the concentration of ET-1 in conditioned medium was assessed by ELISA. Of note, ET-1 concentrations in medium collected from control HUVEC (45.3 +/− 7.2 pg/ml) were significantly higher than those measured in the medium collected from control RAW 264.7 macrophages (7.2 +/− 1.1 pg/ml). These findings suggest a higher production of ET-1 by endothelial cells under basal conditions. However, levels of ET-1 protein concentration increased >4 fold in medium from LPS-exposed RAW 264.7 macrophages, while no increase was observed in similarly exposed HUVEC (Fig. 4C). These findings demonstrate that LPS-induced NFκB activation results in increased expression of known NFκB target genes (eg, IκBα) in both macrophages and endothelial cells. However, only macrophages demonstrate LPS-induced ET-1 expression. These results raised the possibility that LPS-induced ET-1 expression in macrophages was either independent of NFκB, or occurred via an NFκB dependent mechanism specific to macrophages. Thus, the relationship between LPS-induced NFκB activity and ET-1 expression was further interrogated.

Inhibition of LPS-induced NFκB activation through IκBα overexpression attenuates ET-1 expression in macrophages

Because pharmacologic inhibition of NFκB may have unrecognized off-target effects, we used a genetic approach to inhibit LPS-induced NFκB activity in macrophages. Thus, RAW 264.7 cells were transfected with plasmids overexpressing wild-type (WT) and dominant negative (DN) IκBα. The dominant negative IκBα plasmid results in expression of IκBα in which serine 32/36 have been mutated to phenylalanine, preventing phosphorylation and

subsequent degradation. Using antibodies that distinguish native and WT IκBα from transfected IκBα demonstrated that both transfected WT and DN IκBα were resistant to LPS-induced degradation (Fig. 5A). Both WT and DN IκBα overexpression significantly attenuated LPS-induced ET-1 expression (Fig. 5B). These results implicate LPS-induced NFκB activation in the transcriptional regulation of ET-1.

Silencing NFκB inhibitory protein induces ET-1 expression in macrophages

To assess the specific effect that loss of IκB expression has ET-1 expression, RAW 264.7 macrophages were subjected to siRNA transfection of the NFκB cytoplasmic inhibitors IκBα and IκBβ. We used transfection of both IκBα and IκBβ siRNA as both of these proteins act as cytoplasmic inhibitors, and the expression of both must be silenced to allow nuclear translocation of NFκB subunits. Transfection of IκBα and IκBβ siRNA resulted in a 20% and 60% decrease of immunoreactive IκBα and IκBβ, respectively (Fig. 5C). Analysis of mRNA expression demonstrated similar levels of knock-down (Fig. 5B). With this level of inhibitory protein knock-down, ET-1 expression significantly increased >30 fold, even in the absence of external stimulation with LPS (Fig. 5D). These results show that specific activation of NFκB via silencing of IκB inhibitory proteins was sufficient to increase ET-1 gene expression, further implicating NFκB in ET-1 gene expression in macrophages.

Involvement of NFκB in ET-1 gene promoter activity

Analysis of the 2000 bp upstream of the murine ET-1 transcription start site revealed three cRel/NFκB consensus sequences (−1610, TGGGCATTCC; −1279, GGAATGCCCT; −755, GGGGATCGTCCCCT).(37, 39) To confirm the role of NFκB in inducing ET-1 expression, wild-type MEF cells were transfected with ET-1 promoter luciferase reporter plasmids. Three different reporter plasmids were used, containing all three (full, −1701), two (NFκB#2/NFκB#3, −1483) or only the most proximal (NFκB #3, −755) binding sites. Co-transfection with either p65 or cRel induced luciferase activity when the full ET-1 promoter containing all 3 NFκB sites (full, −1701) was present (Fig. 6A). Co-transfection with either p65 or cRel significantly induced luciferase activity when two NFκB consensus sequence binding sites were present (NFκB#2/NFκB#3, −1483), however luciferase activity was attenuated compared to the full length promoter reporter plasmid (Fig. 6B). Finally, the activity at the ET-1 promoter luciferase reporter plasmid containing only the most proximal NFκB consensus sequence binding site (NFκB #3, −755) was not induced by p65 or cRel co-transfection (Fig. 6C). These results show that NFκB induces ET-1 promoter activity by interaction with κB sites within the ET-1 promoter.

Pharmacologic inhibition of LPS-induced NFκB activation attenuates ET-1 expression in macrophages

Having demonstrated that LPS-induced NFκB activity occurred in RAW 264.7 macrophages and that both cRel and p65 induce ET-1 promoter activity, we sought to further define the role of IκBβ in LPS-induced ET-1 expression. The NFκB inhibitory protein IκBβ is the preferred binding partner of cRel containing NF-κB dimers.(40) We have previously demonstrated that low-dose parthenolide prevents LPS-induced IκBβ degradation, while preserving NFκB signaling that occurs through IκBα.(41) First, the effect of parthenolide and BAY 11-7085, pharmacologic inhibitors of NFκB activity, on LPS-induced ET-1

expression was assessed. RAW 264.7 macrophages were pretreated with either BAY 11-7085 or parthenolide for 1 hour prior to LPS (1 mcg/ml, 1 hour) exposure. Both BAY 11-7085 and parthenolide inhibited LPS-induced IκBα and IκBβ degradation in a dose-dependent manner (Fig. 7A). Consistent with this finding, LPS-induced expression of the NFκB target gene IκBα was significantly attenuated in a dose-dependent manner (Fig. 7B). Furthermore, both BAY 11-7085 and parthenolide attenuated LPS-induced ET-1 mRNA expression and protein synthesis in a dose-dependent manner (Fig. 8C and D). Consistent with an IκBβ/NFκB dependent mechanism, the ability of parthenolide to significantly inhibit LPS-induced ET-1 expression occurred at doses (5 μM, Fig 7A) where only IκBβ degradation was inhibited. These results demonstrate that pharmacologic inhibition of LPS-induced IκBβ was sufficient to attenuate ET-1 expression in cultured macrophages.

We next sought to determine the mechanisms underlying the profound effect on LPS-induced ET-1 expression observed by specifically inhibiting IκBβ degradation. Parthenolide pre-treatment attenuated cRel, p65 and IκBβ nuclear translocation, with a return to levels similar to baseline at 4 hours of exposure (Fig. 8A). Furthermore, IκBβ overexpression alone resulted in a significant increase in ET-1 expression, similar to those observed with LPS exposure (Fig. 8B). Cells transfected with IκBβ expression plasmid demonstrated both cRel and IκBβ nuclear translocation (Fig. 8C). These results demonstrate that IκBβ is sufficient to drive ET-1 expression. Furthermore, cells demonstrating a 50% reduction in IκBβ mRNA and protein expression (Fig. 8D, E) after their transfection with IκBβ siRNA, also demonstrated a significant inhibition of LPS-induced ET-1 expression. These data, together with data from our ET-1 promoter ET-1 promoter luciferase activity assay support a mechanistic role of NFκB in mediating LPS-induced ET-1 expression *in vitro*.

In vivo suppression of IκBβ mediated NFκB activation attenuates LPS-induced ET-1 expression

To determine the effect of attenuated LPS-induced IκBβ/NFκB activation *in vivo*, we utilized IκBβ knock-in, or AKBI mice. The AKBI mice have IκBβ cDNA inserted behind the IκBα promoter, and thus over-express IκBβ and do not express IκBα (Fig. 9A). They are phenotypically indistinct from wild-type littermate controls.(42) Consistent with IκBβ overexpression and rapid cytoplasmic IκBβ re-accumulation via IκBα promoter-driven expression following LPS-induced degradation, AKBI mice demonstrated an abbreviated duration of cytoplasmic IκBβ degradation, with a return to baseline levels by four hours of exposure (Fig. 9B, C). This is in contrast to what was observed in LPS-exposed WT mice, where hepatic IκBβ levels remained low throughout the 6-hour exposure (Fig. 2A, C). Accordingly, LPS-induced elevations in serum ET-1 concentration and hepatic mRNA was attenuated in AKBI mice (Fig. 9D, E). Importantly, mortality was not statistically different between endotoxemic WT and AKBI; however, AKBI mice demonstrated 20% survival at 40 hours of exposure while WT mice demonstrated 100% mortality (Fig 9F). Furthermore, we have previously reported that AKBI mice are resistant to oxidative stress and hyperoxic lung injury.(35, 43, 44) Given the potential interaction between oxidative stress and NFκB signaling, we assessed the expression of the two major anti-oxidant NFκB target genes, manganese superoxide dismutase (MnSOD) and ferritin heavy chain, and two major pro-oxidant NFκB target genes, xanthine oxidase/dehydrogenase (XOR) and cyclooxygenase-2

(COX-2).(45) Importantly, expression of these NFκB targets was not different between endotoxemic WT and AKBI mice (Supplemental Figure 2). This suggests that a difference in end organ oxidative stress related to NFκB target gene expression an unlikely explanation of differential ET-1 expression. Finally, BMDMs isolated from AKBI mice show a significant attenuation of LPS-induced ET-1 mRNA expression when compared to similarly exposed WT BMDM (Fig. 9G). Finally, we investigated the relationship between LPS-induced inducible nitric oxide synthase (iNOS) expression and ET-1 expression in WT and AKBi mice. It is well recognized that nitric oxide (NO) induces ET-1 expression, and that iNOS is an NFκB target gene.(45, 46) Importantly, LPS-induced hepatic iNOS expression was not different between WT and AKBI mice (Supplemental Figure 3A), nor was LPS-induced iNOS expression different between WT and AKBI BMDM (Supplemental Figure 3B). These results argue against differential expression of iNOS contributing to observed differences in ET-1 expression in WT and AKBI mice.

Discussion

Here we have identified the hepatic macrophage as the primary cellular source of ET-1, while ruling out the lung as a significant source of ET-1, in a murine model of lethal endotoxemia. Furthermore, we have demonstrate that in macrophages, LPS-induced ET-1 expression occurs via an IκBβ/NFκB dependent mechanism *in vivo* and *in vitro*. Our findings thus challenge the current paradigm of lung endothelial cells as the primary source of ET-1 in systemic inflammatory stress. These findings have significant implications for potential therapeutic interventions aimed at attenuating pulmonary hypertension associated with septic shock and ARDS, especially those associated with gram negative organisms.

Our data furthermore provide novel mechanistic insight into the source and transcriptional regulation of ET-1 in the setting of endotoxemia. The long-standing observation that levels of circulating ET-1 concentrations increase in response to endotoxemia is consistent and reliable across multiple species – including humans, sheep, pigs, dogs, rats, mice.(2–7) The association between endotoxemia, increased ET-1 and pulmonary hypertension has generated interest in attenuating the pulmonary vasoconstriction through the use of endothelin receptor blockade. While the results of these studies have consistently shown that endothelin receptor blockade attenuates increased PVR seen in endotoxic shock,(10, 11, 47) this therapy is not without risk. These risks include worsening systemic acidosis,(10) hypotension,(13, 15) and mortality.(13, 14) Thus, while it is clear that increased circulating ET-1 concentrations in the setting of endotoxemia contributes to pulmonary hypertension and cardiac dysfunction, how to safely address these physiologic changes remains unclear.

Clearly identifying the cellular source and signaling mechanisms dictating increased ET-1 expression is prerequisite to develop more targeted therapeutic approaches for pulmonary hypertension and cardiac dysfunction complicating septic shock. Previous studies have provided compelling evidence *that under normal homeostatic conditions*, the lung demonstrates the highest level of ET-1 expression when compared to other organs,(48) fostering the hypothesis that the pulmonary endothelium is the source of elevated ET-1 concentrations. However, other experimental data have challenged this hypothesis, chiefly because the ability to demonstrate pulmonary ET-1 expression in response to systemic LPS

was inconsistent.(19–24) Here, we identified that pulmonary ET-1 expression, as evaluated by RT-qPCR, decreases in response to endotoxemia at early time points, while hepatic ET-1 expression increases. Our results are consistent with previous studies evaluating ET-1 expression in the lungs of LPS-exposed rats by RT-qPCR,(22) and challenge the hypothesis that the lung is an important source of increased circulating ET-1 in the setting of endotoxemia.

Instead, we have identified the liver as the primary organ and the hepatic macrophage as the primary cellular source of increased circulating *ET-1* in response to endotoxemia. Endothelin-1 was first isolated from endothelial cells (16) and was subsequently confirmed to be a primary source of this vasoactive protein under normal physiologic conditions,(2, 18, 32, 48) however its source in perturbed states has not been clearly defined. Importantly, the stimulus and signaling pathways leading to endothelin-1 transcription and secretion remained largely unknown. Our results demonstrate that endotoxemia induces ET-1 gene and protein expression exclusively in hepatic macrophages and not as previously postulated in endothelial cells. Moreover, the observation that hepatic ET-1 expression was largely absent after clodronate-mediated ablation of macrophages, further suggests that in the absence of macrophages other hepatic cell types cannot compensate for ET-1 expression and conclusively identifies the hepatic macrophage as the primary generator of LPS induced ET-1 expression. Of note, our *in vitro* studies confirm that endothelial cells demonstrate little to no LPS-induced ET-1 gene expression while macrophages (both primary and cell lines) mount robust ET-1 gene and protein expression in response to LPS. Of note, all cell lines used in this report express TLR4 (as determined by RT-qPCR, data not shown) and are thus responsive to LPS, further highlighted by the observation that they display increased expression of the NFκB target genes IκBα. These *in vitro* findings indicate that endothelial cells are refractory to ET-1 expression in response to LPS-mediated NFκB signaling. In contrast, and consistent with previous reports, we demonstrate that ET-1 expression robustly occurs in murine macrophages in response to LPS. The apparent increase in ET-1 expression in macrophages in culture is robust and consistent across species.(29, 30) The apparent discrepancy in terms of ET-1 transcription in macrophages and endothelial cells in response to LPS-NFκB raised the possibility that LPS-induced ET-1 expression in macrophages was either independent of NFκB, or occurred via an NFκB dependent mechanism specific to macrophages. We thus sought to define the mechanisms underlying increased ET-1 transcription in macrophages in greater detail. Understanding these mechanisms is critical, as gene transcription is the rate-limiting step in ET-1 secretion,(16, 32)

Interestingly, NFκB has been identified as critical for the expression of ET-1 in endothelial and smooth muscle cells in response to advanced glycation end products, leptin, oleic acid, interferon-γ and TNF-α.(49–53) While it is interesting that these stimuli can activate NFκB and drive ET-1 expression, this does not specifically implicate this transcription factor in LPS-induced gene expression. Importantly, NFκB regulated gene expression is *stimulus and cell type specific*, thus findings in *cytokine-exposed* endothelial and smooth muscle cells do not prove that *LPS-induced* NFκB signaling drives ET-1 gene expression in macrophages. (54, 55) Importantly, this report adds that LPS-induced NFκB activity – and thus the innate immune response – regulates ET-1 production and secretion. Interestingly, we observed that

NFκB-dependent, LPS-induced ET-1 expression occurs only in macrophages and not endothelial cells. Thus, the observation that LPS-induced NFκB activity upregulates ET-1 gene expression in macrophages, but not endothelial cells, may allow targeted therapeutic approaches to attenuate the pathologic effects of elevated ET-1 associated with systemic inflammatory stress.

In the current study, we have demonstrated a central role played by IκBβ in mediating LPS-induced ET-1 expression. In quiescent cells, the IκB family of NFκB inhibitory proteins maintain inactivated NFκB dimers in the cytoplasm.(56) Both IκBα and IκBβ are ubiquitously expressed throughout the body.(57) Following exposure to inflammatory stimuli (eg, LPS), IκBα and IκBβ degradation occurs, allowing NFκB nuclear translocation.(56) Importantly, the inhibitory protein IκBβ affects dimer composition of nuclear NFκB translocation and target gene expression. Because IκBβ preferentially binds cRel containing NF-κB dimers, and these dimer combinations bind to unique DNA sequences, specific downstream genes are targeted.(40, 54) Additionally, following their initial degradation, both newly synthesized IκBα and IκBβ enter the nucleus. The IκBα isoform contains a nuclear export sequence (NES), allowing it export DNA-bound NFκB complexes from the nucleus.(58) In contrast, IκBβ lacks a NES,(59) and remains in the nucleus to stabilize NFκB-DNA binding and enhance target gene expression.(60) Previous reports have linked IκBβ expression to LPS induced ET-1 expression.(40) The NFκB subunit cRel appears to be critical for ET-1 expression following exposure to systemic inflammatory stress. Neither $cRel^{-/-}$ mice or cRel/p50 double knock-out mice demonstrate LPS-induced ET-1 expression.(61, 62)

These mechanistic insights may allow more targeted interventions that attenuate – rather than completely abrogate – LPS-induced NFκB activity. To this point, preclinical studies using endotoxemia to mimic septic shock have demonstrated both beneficial and detrimental effects of global inhibition of NFκB signaling. These findings have motivated calls for interventions that modulate or dampen NFκB activity following exposure to inflammatory stress.(63) Targeting IκBβ mediated NFκB activity represents one such strategy. Previous studies have shown that the induction of pro-inflammatory target gene expression (TNFα, IL1β, IL6) in adult wild-type mice exposed to intraperitoneal LPS is attenuated in $IκBβ^{-/-}$ mice.(64, 65) The attenuated pro-inflammatory target gene expression results in improved survival from endotoxemia. Additionally, previous reports of the IκBβ overexpressing (AKBI) mice used in this study demonstrate attenuated angiotensin II and IL1β induced pro-inflammatory cytokine expression and cardiovascular inflammation.(66) These findings suggest that similar effects on NFκB target genes can be observed with either preventing signaling through overexpression or complete absence of this key inhibitory protein. These consistencies argue that specifically targeting IκBβ-mediated NFκB activation, and thereby attenuating - but not completely blocking - NFκB activity, may represent a novel therapy for septic shock. The detailed mechanisms underlying the differential regulation of ET-1 in response to LPS demonstrated between endothelial and macrophage cells remains to be determined and are subject of ongoing studies in our laboratory, with the hypothesis that subtle modifications in the NFκB transcriptome is dictated by IκB expression, duration of NFκB activation, and nuclear translocation of specific NFκB dimer pairs that target unique consensus sequence binding sites.(54)

The current study has several limitations. First, we did not assess physiologic variables beyond mortality in the WT and AKBI mice following exposure to lethal endotoxemia. Importantly, statistical analysis revealed that mortality was not different in response to endotoxemia between the two strains. However, AKBI mice demonstrated 20% survival at 40 hours of exposure while WT mice demonstrated 100% mortality. It is possible that significant differences would be revealed by performing dose-response experiments. Furthermore, in other rodent models, cardiovascular pertubations are observed within 2 hours of LPS administration at doses lower than what was used in the current study.(67) This time course would be consistent with early activation of hepatic NFκB as observed in our study and subsequent ET-1 expression. We did not perform robust physiologic assessments of WT and AKBI mice exposed to lethal endotoxemia because the AKBI globally over-express IκBβ and thus demonstrate perturbation in the expression of multiple NFκB target genes. It remains to be determined how targeting hepatic macrophage ET-1 expression would affect hemodynamics in the setting of systemic inflammatory stress and these studies are ongoing using appropriate *in vivo* models. However, identifying the source and transcriptional regulation of LPS-induced ET-1 expression allows us to target specific cells and signaling pathways in future studies. Systemic inflammatory stress did not induce pulmonary ET-1 expression through 6 hours of exposure. It remains possible that pulmonary macrophages respond similarly to hepatic macrophages at later time points and/or in response to cytokines released by hepatic macrophages; similarly, the pulmonary endothelium may display ET-1 expression at later time points or as a result of paracrine cytokine signals derived from hepatic macrophages. Additionally, it remains to be determined if pulmonary ET-1 increases with prolonged exposure to inflammatory stress, and whether the resulting pulmonary hypertension contributes to mortality. Finally, although we did assess ET-1 expression in other tissue beds, we only evaluated the cellular source of this expression in the liver. For example, renal ET-1 expression is increased endotoxemic mice(5–6 fold, data not shown), but not to the degree that is observed in the liver (15–20 fold). Further studies are needed to determine if this LPS-induced ET-1 expression in other tissue beds is derived from macrophages, or whether this observation is dependent upon the resident tissue. These are important questions unanswered by our study.

We conclude that in the setting of endotoxemia, subsequent IκBβ/NFκB activation induced ET-1 expression in the hepatic macrophage. These findings challenge our current understanding of the mechanisms linking ET-1 and the pathogenesis of septic shock. This work has clearly defined the source and transcriptional regulation of ET-1 in response to endotoxemia, and could potentially help guide therapies to improve the outcomes of patients with septic shock. By targeting specific cells and signaling pathways, clinicians may be able to target therapies to offset the detrimental effects of splanchnic, renal and pulmonary vasoconstriction while maintaining blood pressure and improving the outcomes of patients with sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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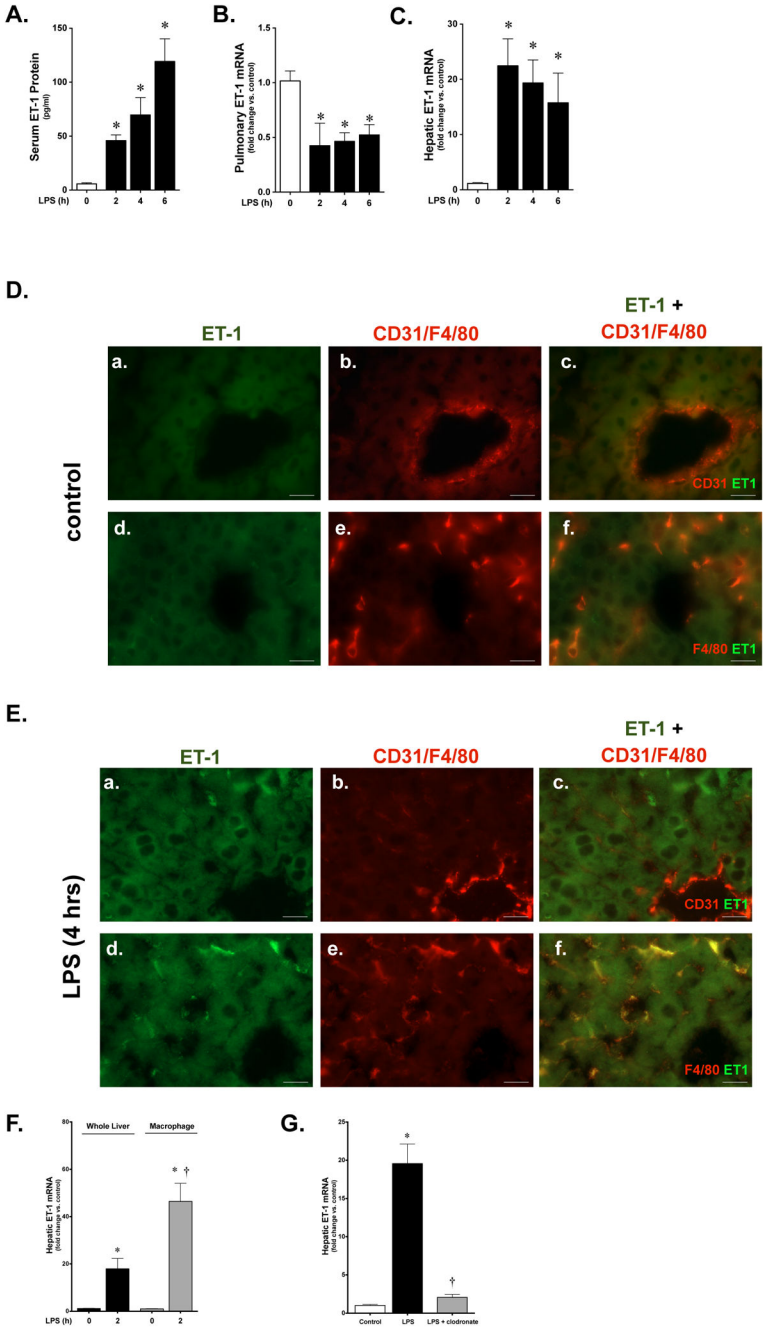

Figure 1. Endotoxic shock induces hepatic macrophage ET-1 expression

(A) Serum ET-1 levels (B) Pulmonary ET-1 mRNA expression and (C) Hepatic ET-1 mRNA expression in mice exposed to LPS (50 mg/kg, IP). Values are means ± SE (n=6/time point); h, hours. * = $p<0.05$ vs. unexposed controls. (D–E) Representative immunofluorescence staining of (D) control and (E) LPS-exposed mouse liver. ET-1 was stained in green (a, d) and endothelial (CD31; b, c) and macrophage (F4-80; e, f) markers stained in red. Internal scale bar 20uM. (F) Fold change in LPS-induced ET-1 mRNA expression in whole liver and isolated intrahepatic mononuclear cells. Values are means ±

SE (n=4/time point); h, hours. *=p<0.05 vs. unexposed controls; †=p<0.05 vs. whole liver LPS exposed. (G) Fold change in LPS-induced ET-1 expression after clodronate ablation of hepatic macrophages. Values are means ± SE (n=6/time point); h, hours. * = p<0.05 vs. unexposed controls; †=p<0.05 vs. LPS exposed.

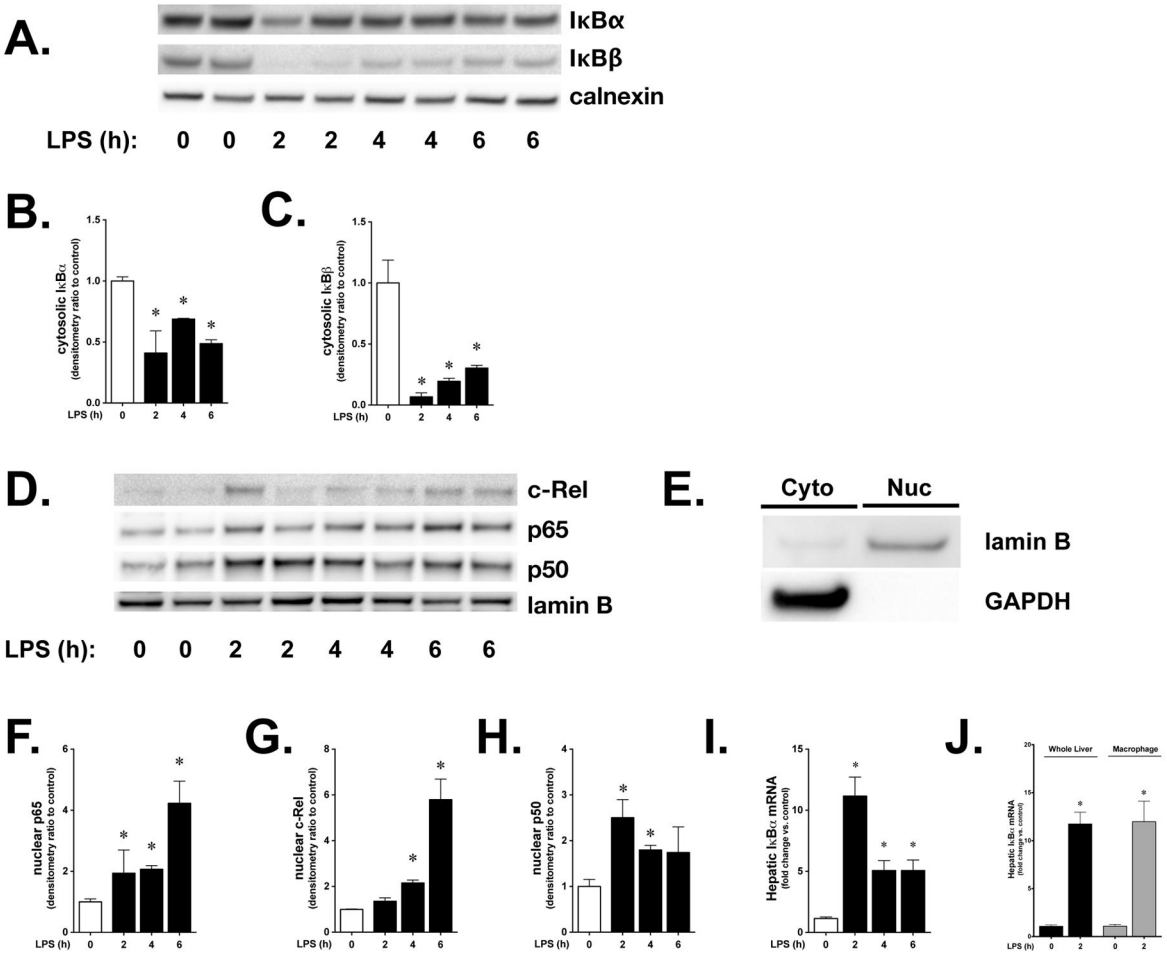

Figure 2. Endotoxic shock induces hepatic NFκB activation

(A) Representative Western blot showing IκBα and IκBβ in hepatic cytosolic extracts following exposure to LPS (IP, 50 mg/kg). Calnexin is shown as loading control. (B–C) Densitometric evaluation of cytosolic (B) IκBα and (C) IκBβ normalized to calnexin and expressed as a ratio to unexposed control. Data expressed as means ± SEM (n=3/time point); h, hours; *, p <0.05 vs. unexposed control. (D) Representative Western blot showing c-Rel, p65, and p50 in liver nuclear extracts from mice exposed to IP LPS (IP, 50 mg/kg). Lamin B is shown as loading control. (E) Representative Western blot demonstrating purity of cytosolic and nuclear extracts from liver. GAPDH was used as a cytosolic marker, while Lamin B was used as a nuclear marker. (F–H) Densitometric evaluation of nuclear (E) c-Rel (F) p65 (G) p50 normalized to lamin B and expressed as a ratio to unexposed control. Data expressed as means ± SEM (n=3/time point); h, hours; *, p <0.05 vs. unexposed control. (I) Fold change in hepatic IκBα mRNA expression in mice exposed to LPS (IP, 50 mg/kg). Data expressed as means ± SEM (n=3/time point); h, hours; *, p <0.05 vs. unexposed control. (J) Fold change in LPS-induced IκBα mRNA expression in whole liver and isolated intrahepatic mononuclear cells. Values are means ± SE (n=4/time point); h, hours. *=p<0.05 vs. unexposed controls; †=p<0.05 vs. whole liver LPS exposed.

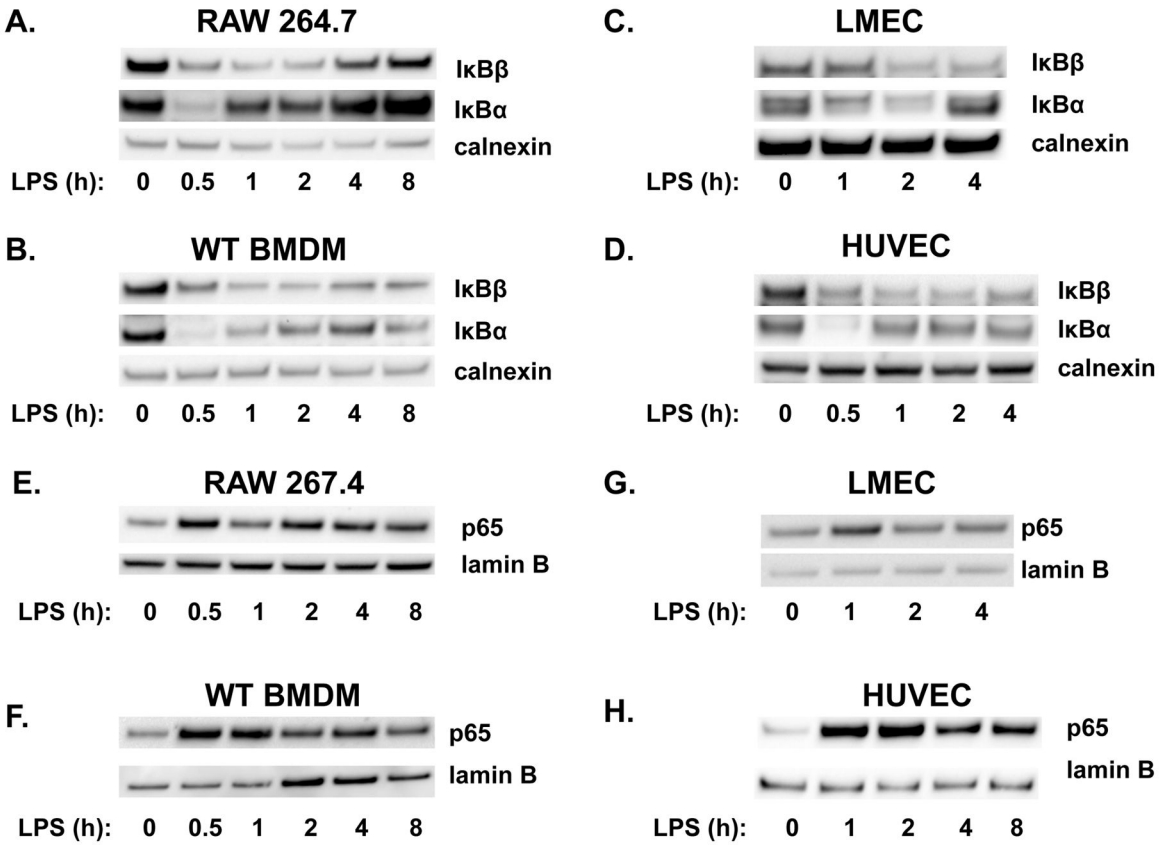

Figure 3. LPS induces IκB degradation and NFκB nuclear translocation in both macrophage and endothelial cell lines

(A–D) Representative Western blot showing IκBα and IκBβ in cytosolic extracts from (A) RAW 264.7 cells, (B) murine BMDM, (C) LMEC and (D) HUVEC exposed to LPS (1 μg/mL), with calnexin as loading control. (E–H) Representative Western blot showing p65 in nuclear extracts from (E) RAW 264.7 cells, (F) murine BMDM, (G) LMEC and (H) HUVEC exposed to LPS (1 μg/mL), with lamin B as loading control.

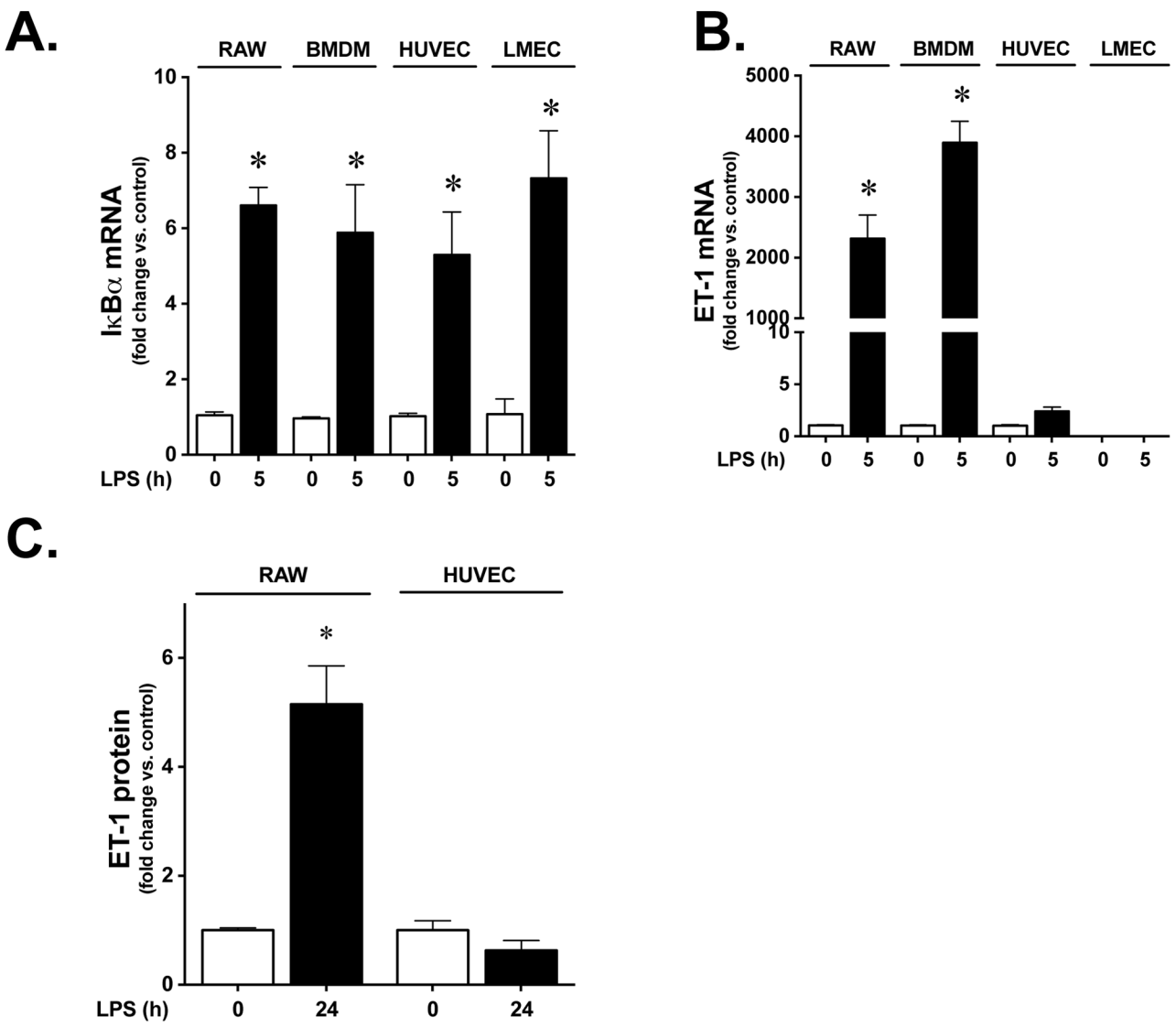

Figure 4. LPS induces expression of NFκB target genes both macrophage and endothelial cell lines, but ET-1 expression occurs in only macrophages

(A–B) Fold change in LPS-induced (A) IκBα and (B) ET-1 gene expression in macrophage and endothelial cells. Values are means ± SEM (n=4/time point); h, hours; *, $p < 0.05$ vs. unexposed control. (C) Fold change in ET-1 protein secretion from RAW 264.7 cells and HUVEC following exposure to LPS measured in cell medium by ELISA. Values are means ± SEM (n=4/time point); h, hours; *, $p < 0.05$ vs. unexposed control.

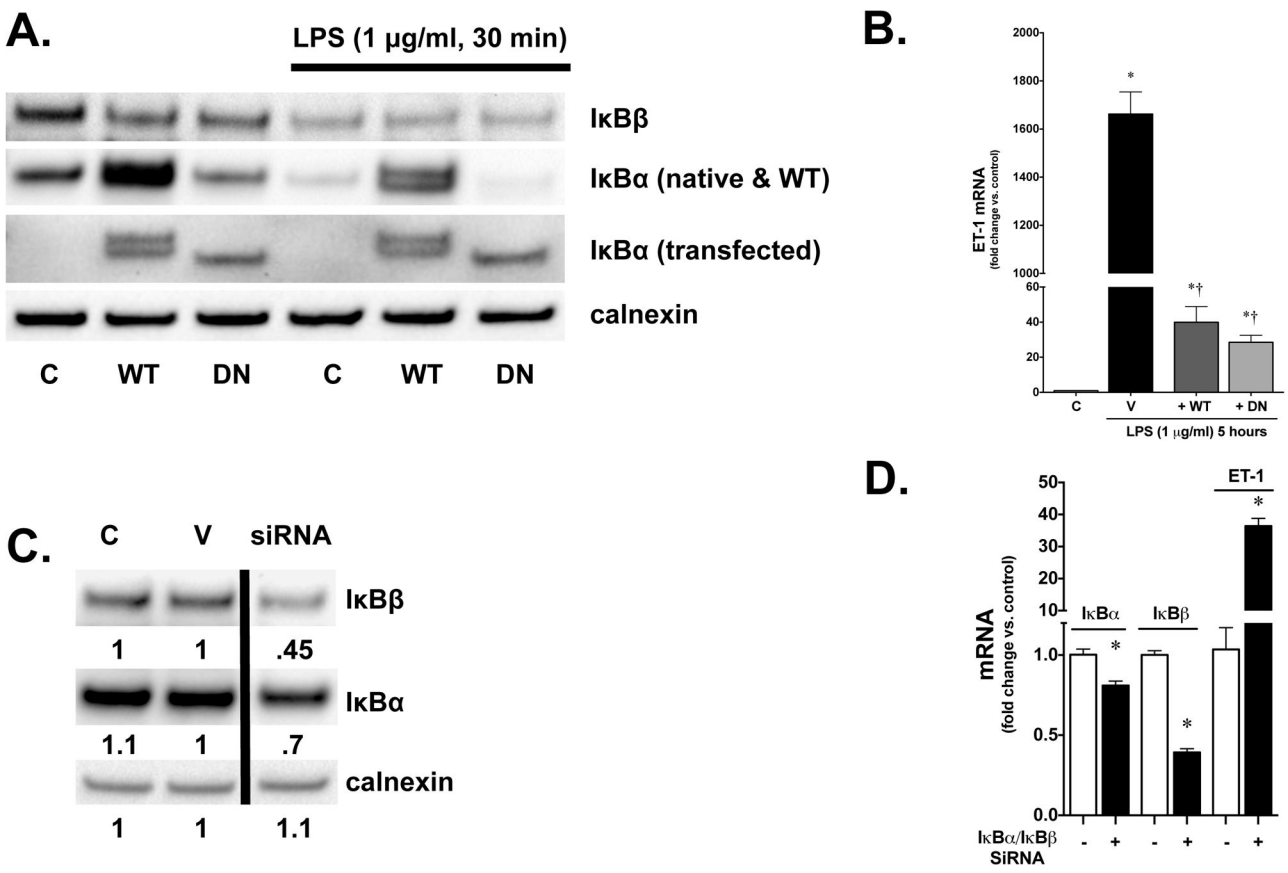

Figure 5. Overexpression of the NFκB inhibitory protein IκBα attenuates, while knockdown of the NFκB inhibitory proteins IκBα and IκBβ potentiates LPS-induced ET-1 gene expression in RAW 264.7 marcophages

(A) Representative Western blot showing IκBβ and IκBα in whole cell lysates from cells transfected with either IκBα expression plasmid (WT) or dominant-negative IκBα expression plasmid (DN) and exposed to LPS (1 μg/mL, 1h). One IκBα antibody (sc-371, Santa Cruz) detects only native and WT IκBα, while the other (#4814, Cell Signaling) detects only transfected IκBα. Calnexin is shown as loading control. (B) ET-1 gene expression in cells transfected with either IκBα expression plasmid (+WT) or dominant-negative IκBα expression plasmid (+DN) and exposed to LPS (1 μg/mL, 5h). Values are means ± SEM (n=4/time point); C, control; V, vehicle; *, $p < 0.05$ vs. unexposed control; †, $p < 0.05$ vs. LPS exposed. (C) Representative Western blot showing IκBβ and IκBα in whole cell lysates from RAW 264.7 following transfection with IκBα and IκBβ siRNA. (D) IκBα, IκBβ and ET-1 gene expression in RAW 264.7 following transfection with IκBα and IκBβ siRNA. C, control; V, vehicle; siRNA, siRNA transfected. Values are means ± SEM (n=4/time point); *, $p < 0.05$ vs. non-transfected control.

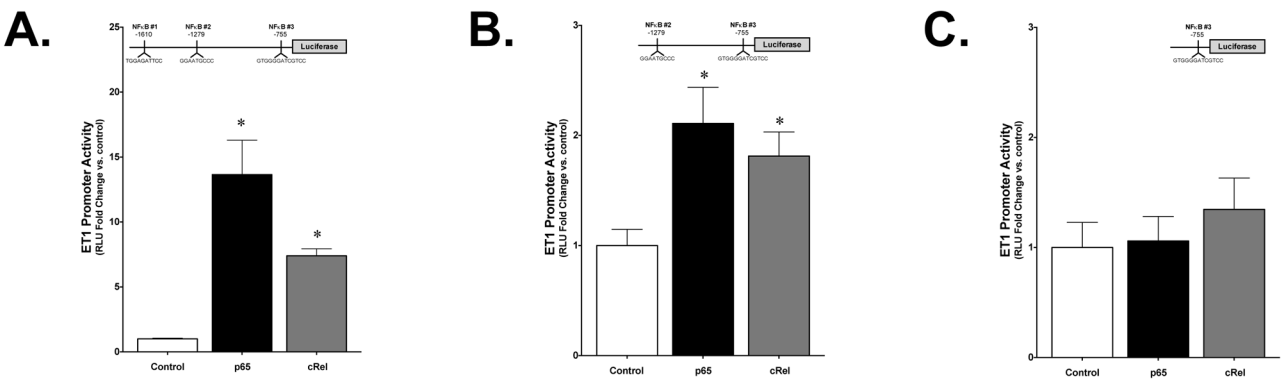

Figure 6.

The NFκB subunits p65 and cRel are sufficient to drive ET-1 promoter activity. WT MEF cells were transfected with the (A) Full length ET-1 promoter (−1701) containing 3 NFκB consensus sequence binding sites (−1610, −1269, −755), (B) Truncated (−1483) ET-1 promoter containing 2 NFκB binding sites (−1269, −755), or (C) Truncated (−861) ET-1 promoter containing one NFκB binding site (−755). Schematic representations of the 5′-promoter regions of the ET-1 promoter constructs, with NFκB binding sites are provided. Graphs represent relative luciferase units (RLU) normalized by Renilla luciferase and standardized to control. Values are means ± SEM (n=3/time point); control, ET-1 promoter transfection; p65, ET-1 promoter + p65 transfection; cRel, ET-1 promoter + cRel transfection; *, $p < 0.05$ vs. untransfected control.

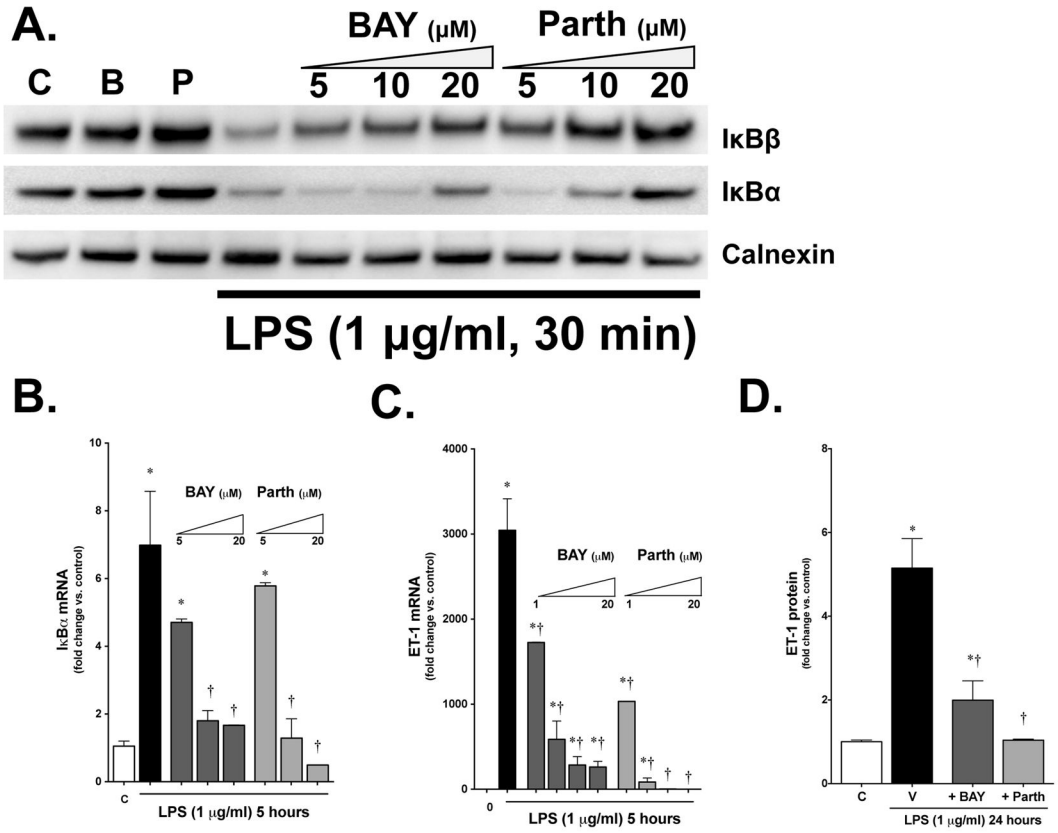

Figure 7.

The NFκB inhibitors BAY-7085 and parthenolide inhibit LPS-induced NFκB activation and the expression of downstream target genes including ET-1 in RAW 264.7 marcophages (A) Representative Western blot showing IκBβ and IκBα in whole cell lysates from cells pretreated with BAY-7085 (5–20 μmol/L, 1h) or pathenolide (5–20 μmol/L, 1h) prior to LPS exposure (1 μg/mL, 1h). Calnexin is shown as a loading control. (B) IκBα and (C) ET-1 gene expression in RAW 264.7 pretreated with BAY-7085 (5–20 μmol/L, 1h) or pathenolide (5–20 μmol/L, 1h) prior to LPS exposure (1 μg/mL, 1h). Values are means ± SEM (n=4/time point); h, hours; *, $p < 0.05$ vs. unexposed control; †, $p < 0.05$ vs. LPS exposed. (D) Fold change in ET-1 protein secretion from RAW 264.7 cells pretreated with BAY-7085 (10 μmol/L, 1h) or pathenolide (10 μmol/L, 1h) prior to LPS exposure (1 μg/mL, 24h) measured in cell medium by ELISA. Values are means ± SEM (n=4/time point); h, hours; *, $p < 0.05$ vs. unexposed control; †, $p < 0.05$ vs. LPS exposed.

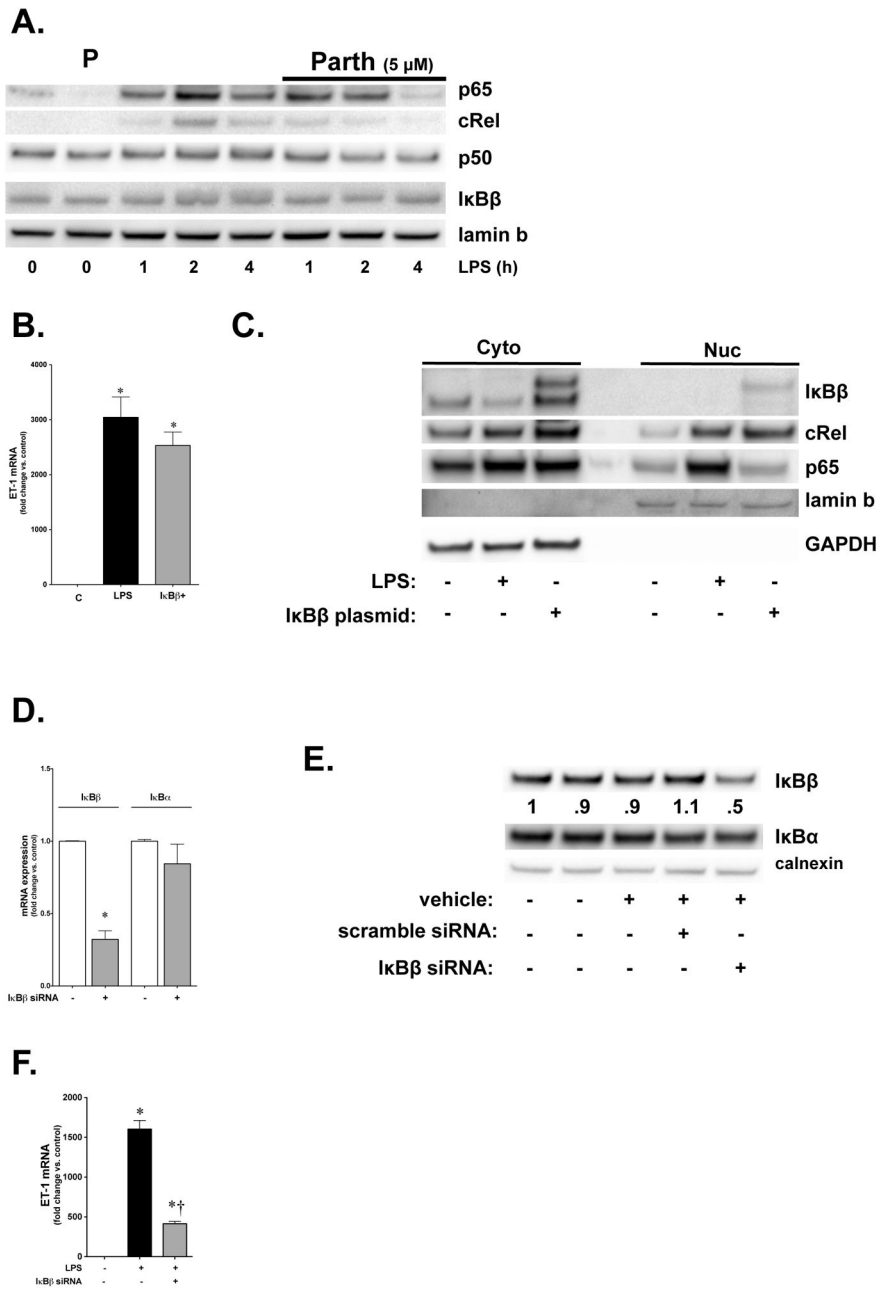

Figure 8. The NFκB inhibitory protein IκBβ is necessary and sufficient to drive ET-1 expression

(A) Representative Western blot showing c-Rel, p65, p50 and IκBβ in nuclear extracts from RAQ 264.7 cells exposed to LPS (1 μg/mL, 1–4 h), or LPS after pretreatment with parthenolide (5 μM, 1 hr) with lamin B as loading control. C, unexposed control; P, parthenolide alone; h, hours. (B) Relative ET-1 mRNA expression in RAW 264.7 macrophages following transfection with IκBβ expression plasmid. Values are means ± SEM. n=9. *, $p<0.05$ vs. untransfected control. (C) Representative Western blot showing IκBβ and cRel in cytosolic and nuclear extracts from RAW 264.7 macrophages either exposed to LPS (1 μg/mL, 4 h) or transfected with IκBβ expression plasmid, with GAPDH

as cytosolic loading control and lamin B as nuclear loading control. (D) Relative IκBα and IκBβ mRNA expression in RAW 264.7 macrophages following transfection with IκBβ siRNA. Values are means ± SEM. n=9. *, $p<0.05$ vs. untransfected control. (E) Representative Western blot showing IκBα and IκBβ in cytosolic extracts from RAW 264.7 cells following transfection with IκBβ siRNA, with calnexin as loading control. Densitometric evaluation is provided. (F) Relative LPS-induced (1μg/mL, 5 hrs) ET-1 mRNA expression in RAW 264.7 macrophages following transfection with IκBβ siRNA. Values are means ± SEM. n=6. *, $p<0.05$ vs. unexposed control; †, $p<0.05$ vs. LPS exposed.

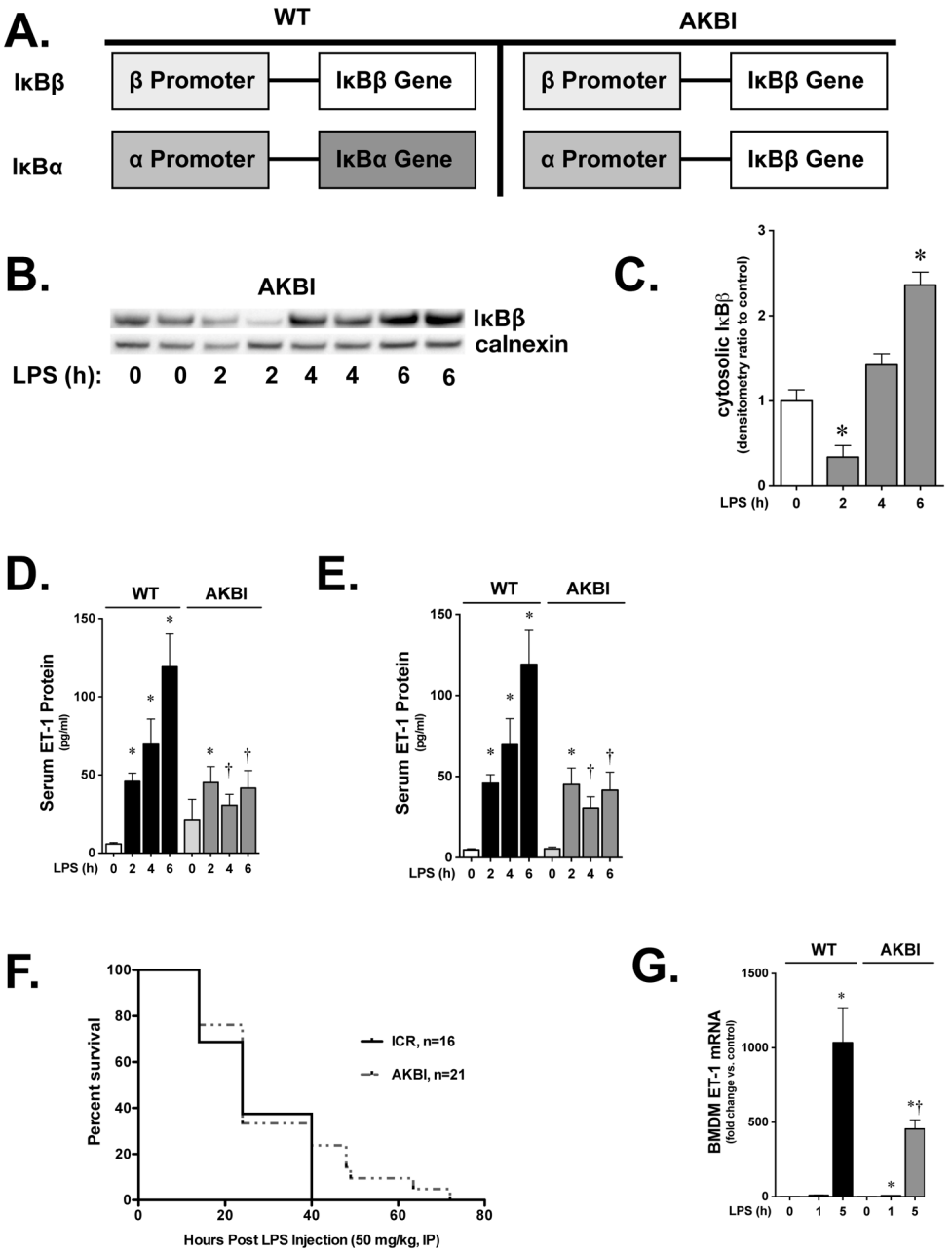

Figure 9. Inhibition of IκBβ/NFκB signaling attenuates LPS-induced ET-1 expression *in vivo*

(A) Schematic of IκB expression patterns in AKBI mice. The IκBα gene has been replaced by IκBβ cDNA. The IκBα promoter controls the expression of the IκBβ transgenic loci. Thus, AKBI overexpress IκBβ, without expressing IκBα (B) Representative Western blot showing IκBβ in hepatic cytosolic extracts from AKBI mice following exposure to LPS (IP, 50 mg/kg). Calnexin is shown as loading control. (C) Densitometric evaluation of cytosolic IκBβ normalized to calnexin and expressed as a ratio to unexposed control. Data expressed as means ± SEM (n=3/time point); h, hours; *, p <0.05 vs. unexposed control. (D) Serum ET-1 levels in WT and AKBI mice exposed to LPS (50 mg/kg, IP). * = p<0.05 vs. unexposed controls; †, p<0.05 vs. paired WT exposure. Data expressed as means ± SE (n=8/

control, 4/additional time point); h, hours. (E) Hepatic ET-1 mRNA expression in WT and AKBI mice exposed to LPS (50 mg/kg, IP). * = $p<0.05$ vs. unexposed controls; †, $p<0.05$ vs. paired WT exposure. Data expressed as means ± SE (n=4/time point); h, hours. (F) Kaplan-Meier survival analysis of WT and AKBI mice exposed to LPS (50 mg/kg, IP). Values are expressed as the percentage of surviving animals. (G) LPS-induced ET-1 gene expression in WT and AKBI bone marrow derived macrophage following exposure to LPS (1 μg/ml). Values are means ± SEM (n=4/time point); h, hours; *, $p<0.05$ vs. unexposed control; †, $p<0.05$ vs. paired WT exposure.