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In Vivo Inhibition of Nuclear Factor-κB Activation Prevents Inducible Nitric Oxide Synthase Expression and Systemic Hypotension in a Rat Model of Septic Shock[1]

Shu Fang Liu, Xiaobing Ye, and Asrar B. Malik[2]

We determined the in vivo function of LPS-induced nuclear factor-κB (NF-κB) activation in mediating inducible nitric oxide synthase (iNOS) mRNA and protein expression, and systemic arterial hypotension in a rat model of septic shock. LPS (8 mg/kg i.v.) challenge of rats activated NF-κB within 15 min in lung tissue, and the response persisted up to 4 h. NF-κB activation preceded the induction of iNOS mRNA. Pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB effective in cellular studies, prevented NF-κB activation in vivo and reduced iNOS mRNA expression and the increase in iNOS activity activated by LPS. At PDTC concentrations of 50, 100, and 200 mg/kg, the reductions in iNOS mRNA were 20, 46, and 48%, and the reductions in iNOS activity were 59, 66, and 75%, respectively. The PDTC concentration-dependent reductions in iNOS activity produced similar decreases in plasma nitrite/nitrate concentrations. PDTC also prevented the decrease in arterial blood pressure induced by LPS. These results demonstrate that activation of NF-κB is a critical in vivo regulatory mechanism mediating LPS-induced iNOS expression and the resultant systemic hypotension. ***The Journal of Immunology,* 1997, 159: 3976–3983.**

The nitric oxide synthase (NOS)[3] family of enzymes catalyze L-arginine to nitric oxide (NO) and L-citrulline. Three distinct isoforms of NOS have been characterized, and their genes have been cloned (1, 2). Neuronal NOS and endothelial NOS (ecNOS) are constitutively expressed, and NO produced by these enzymes activates several signal transduction pathways (1, 3). Inducible NOS (iNOS) is regulated by inflammatory mediators, and the excessive production of NO by iNOS has been implicated in the pathogenesis of inflammatory disease (1–4). Inducible NOS-mediated NO release plays a crucial role in the pathogenesis of septic shock (5–11). In response to LPS, host cells express the iNOS gene and protein (1, 2, 7, 8), resulting in the formation of large quantities of NO. NO contributes to the induction of systemic hypotension, vascular smooth muscle hyporeactivity to adrenergic mimics, and myocardial depression (5–12) that characterize septic shock (13, 14).

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor composed of a group of low m.w. nuclear proteins: c-*rel*, p50/p105 (NF-κB1), p65 (RelA), p52/p100 (NF-κB2), and RelB (15, 16). All NF-κB proteins exist as an inactive dimer in cytoplasm bound to I-κB, its inhibitory protein. Signaling stimulated by LPS and cytokines triggers the phosphorylation and degradation of I-κB, resulting in the dissociation of NF-κB from I-κB (15, 16). This allows NF-κB dimer to migrate to the nucleus, where it binds to promoters of NF-κB-regulated genes and initiates gene transcription (15, 16).

Promoter deletion and mutation studies in cultured cells have demonstrated that NF-κB plays a critical role in the transcriptional regulation of iNOS gene induced by LPS and cytokines (17–20). NF-κB is also required for the LPS-induced expression of several critical genes encoding other proteins involved in the pathogenesis of septic shock (15, 16): TNF-α, ILs (IL-1β, IL-2, IL-6, and IL-8), adhesion molecules (ICAM-1 and E-selectin), and cyclo-oxygenase-2 (15, 16). Lung homogenates from LPS-treated animals showed elevated NF-κB binding activity (21), indicating the in vivo activation of NF-κB. Despite evidence that NF-κB can activate "inflammatory genes" in vitro, its in vivo function as a transcription factor mediating LPS-induced iNOS expression and septic shock is not clear.

The antioxidant pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB activation in various cell types (22–24), prevented NF-κB activation induced by either PMA or TNF-α, but had no effect on the activation of transcription factors AP-1, octamer-binding protein-1, and cAMP response element-binding protein in response to the same stimuli (22, 24). Deletion or mutation of the NF-κB binding site on human ICAM-1 promoter abolished the inhibitory effect of PDTC on IL-1α-induced ICAM-1 promoter/CAT activity in human fibroblast cell lines (23). In the same cell lines, PDTC suppressed IL-1α-induced NF-κB activation as well as IL-1α-, IFN-γ-, and PMA-induced ICAM-1 expression in a concentration-dependent manner (23). PDTC also prevented the expression of several other NF-κB-dependent genes in vitro (23–27), including iNOS (17, 18, 20, 25, 28). In the present study we used PDTC to inhibit NF-κB activation in a rat model of septic shock and determined the in vivo function of NF-κB in mediating LPS-induced iNOS gene expression, NO production, and systemic hypotension.

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[3] Abbreviations used in this paper: NOS, nitric oxide synthase; NO, nitric oxide; ecNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; ICAM-1, intercellular adhesion molecule-1; PDTC, pyrrolidine dithiocarbamate; AP-1, activating protein-1; EMSA, electrophoretic mobility shift assay, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Materials and Methods

Animal protocols

Male Sprague Dawley rats were purchased from Charles River (Wilmington, MA) and housed in the institutional animal care facility. They were divided into experimental groups in a randomized manner and used for experiments when their body weights were in the 350 to 400 g range. All procedures were approved by the institutional animal care committee. Animals in the control and LPS groups were injected i.v. with either saline (1 ml/kg) or *Salmonella enteritidis* LPS (8 mg/kg in saline; Sigma Chemical Co., St. Louis, MO). Rats in the LPS plus PDTC groups were treated with different concentrations of PDTC before LPS challenge; PDTC (Sigma Chemical Co.) was injected i.p. at a concentration of 50, 100, or 200 mg/kg 1 h before the administration of LPS. Animals in the PDTC alone group were treated with PDTC (200 mg/kg i.p.) for an equivalent period of time. It has been shown that LPS at the dose of 5 mg/kg (i.v. or i.p.) causes no mortality in Sprague Dawley rats 24 or 48 h after LPS challenge (29, 30), and that i.p. or i.v. LPS challenge (10 mg/kg) produces a 24-h mortality of 25 or 50%, respectively (29, 30). The 6 h death rate with 10 mg/kg (i.p. or i.v.) is <20% (29, 30). We chose a sublethal LPS dosage (8 mg/kg) in our studies to maximize our ability to evaluate the inhibitory effects of PDTC on LPS-induced NF-κB activation and iNOS expression. The 5 h mortality rate with this LPS dose was 23%, which is comparable to the reported 6 h death rate of 20% with 10 mg/kg LPS (29, 30).

Animals were killed by exsanguination, and tissues (lung, heart, and liver) were collected at 15, 30, or 60 min (for electrophoretic mobility shift assay (EMSA)) or 4 h post-LPS challenge (for Northern blot and iNOS activity assays). Animals in the PDTC alone group were killed at 1.25, 1.5, or 2 h (for EMSA) or at 5 h (for Northern blot and NOS activity assays), and tissues were collected. Blood samples (1 ml) were collected 4 h after LPS challenge or 5 h after PDTC administration for determination of plasma nitrite/nitrate concentrations. For the time-course studies, animals were killed and tissues were collected 5, 10, 15, 30, 60, 120, and 240 min after LPS injection. Tissues were snap-frozen in liquid nitrogen and kept at −70°C for later use.

Measurement of arterial blood pressure

Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and ventilated with rodent ventilator at a tidal volume of 1 ml/100 g body weight and a frequency of 60 breaths/min. A cannula containing heparin-saline was inserted into the left carotid artery for continuous monitoring of systemic blood pressure via a Statham P23 pressure transducer connected to a polygraph recorder (Gould, Inc., Cleveland, OH). After a 30-min equilibration period, LPS or PDTC was injected, and heart rate and arterial pressure were recorded for up to 5 h.

Nuclear protein extract and EMSA

Tissues were minced and incubated on ice for 30 min in 0.5 ml of ice-cold buffer A, composed of 10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% IGEPAL CA-630 (Sigma Chemical Co.), and 0.5 mM PMSF (Sigma Chemical Co.). The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifuging at 5000 × *g* at 4°C for 10 min. The crude nuclear pellet was suspended in 200 μl of buffer B (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 420 mM NaCl; 0.5 mM DTT; 0.2 mM EDTA; 0.5 mM PMSF; and 4 μM leupeptidin) and incubated on ice for 30 min. The suspension was centrifuged at 16,000 × *g* at 4°C for 30 min. The supernatant (nuclear proteins) was collected and kept at −70°C until use. The protein concentration was determined using the bicinchoninic acid assay kit with BSA as the standard (Pierce Chemical Co., Rockford, IL).

NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3) or iNOS NF-κB oligonucleotide probe (5′-CCAACTGGGGACTCTCCCTTTG-3) was end-labeled with [γ-^{32}P]ATP (Amersham Life Science, Arlington Heights, IL). Nuclear protein (10 μg) was incubated with 50,000 cpm of ^{32}P-labeled NF-κB or iNOS NF-κB consensus oligonucleotide for 30 min in a total volume of 15 μl in a binding buffer consisting of 10 mM Tris-Cl, pH 7.5; 1 mM $MgCl_2$; 50 mM NaCl; 0.5 mM DTT; 0.5 mM EDTA; 4% glycerol; and 1 μg of poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ). The specificity of the DNA/protein binding was determined by competition reactions in which a 50-fold molar excess of unlabeled iNOS NF-κB oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. In the supershift assay, Ab (1 μg) reactive to rat p50 or p65 protein or a combination of these two Abs (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture immediately after the addition of radiolabeled NF-κB probe. The reaction was stopped by adding 1 μl of gel loading buffer and was subjected to nondenaturing 4% PAGE in 0.25× Tris-borate/EDTA (TBE) buffer. The gel was vacuum-dried and exposed to x-ray film (Hyperfilm, Fuji, Tokyo, Japan).

Plasma nitrite/nitrate measurement

We used plasma nitrite/nitrate concentrations as an indicator of NO production. Plasma collected from each group of animal was ultrafiltrated using a 10^4 m.w. cut-off ultrafiltration unit (Fisher Scientific, Chicago, IL). The nitrate in the deproteinized plasma was reduced to nitrite using *Escherichia coli* nitrate reductase (Cayman Chemical Co., Ann Arbor, MI), and nitrite was measured with Greis reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4), using $NaNO_2$ as standard (31).

Northern blot analysis

Rat iNOS cDNA probe (741 bp) was amplified using a standard reverse transcription-PCR procedure. RNA (1 μg) from LPS-treated rat lungs was reverse transcribed into cDNA. The 741-bp iNOS cDNA fragment was amplified from RT-generated cDNA using designed primers corresponding to the published mouse iNOS cDNA sequences (32), purified by gel electrophoresis, and eluted from the gels using a Jetsorb DNA extraction kit (Genomed, Inc., Research Triangle Park, NC). The authenticity of the PCR product was confirmed by dideoxy chain termination sequencing.

The lungs and hearts were granulated and homogenized. Total RNA was extracted following the method described by Chomczynski and Sacchi (33). Poly(A)$^+$ mRNA was isolated using a poly(A) mRNA isolation kit (Promega Corp., Madison, WI). Approximately 3 μg of mRNA from each sample was separated on a 1.2% denaturing agarose gel and transferred onto Hybond-N nylon filter (Amersham Life Science). The filter was incubated at 42°C for at least 4 h in a prehybridization buffer that consisted of 50% formamide, 5× Denhardt's solution, 5× SSC, 200 μg/ml of sonicated denatured salmon sperm DNA, and 0.1% SDS. The filter was then hybridized with 1 × 10^6 cpm/ml ^{32}P-labeled rat iNOS probes at 42°C for 14 to 16 h. The blot was washed sequentially with decreasing concentrations of SSC/0.1% SDS and at increasing temperatures (final wash, 0.1× SSC/0.1% SDS at 60°C). The blots were exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY) in the presence of an intensifying screen for 2 days. Each filter was first hybridized to iNOS probe. After film exposure, the hybridized probe was stripped off, and the filter was subsequently rehybridized to the GAPDH probe (34).

NOS activity

Tissues were homogenized in homogenizing buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-ME, 2 μM leupeptin, and 1 mM PMSF). Crude supernatant was obtained by centrifuging the homogenate at 10,000 × *g* for 10 min. The protein concentration in the supernatant was determined using a bicinchoninic acid assay kit with BSA as the standard (Pierce). NOS activity was measured by the ability of the supernatant to convert L-[^{3}H]arginine (Amersham Life Science) to L-[^{3}H]citrulline. In brief, 20 μl of crude supernatant was incubated in the presence of L-arginine/L-[^{3}H]arginine (10 μM, 5 kBq), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (3 μM), and L-valine (60 mM, to inhibit arginase activity) at 37°C for 20 min. The reaction was stopped by adding 1 ml of ice-cold stopping buffer (20 mM HEPES, 2 mM EGTA, and 2 mM EDTA). The reaction mixture was applied to a Dowex 50W (Na^+ form) column (Bio-Rad Laboratories, Hercules, CA), and the eluted L-[^{3}H]citrulline was measured in Beckman scintillation counter (Beckman Instruments, Fullerton, CA). Each sample was assayed for NOS activity under three conditions: in the presence of 2 mM calcium (Ca^{2+}-dependent NOS activity), in the absence of calcium and in the presence of 1 mM EGTA (Ca^{2+}-independent NOS activity), and in the presence of 100 μM N^G-nitro-L-arginine methyl ester (a selective NOS inhibitor). The N^G-nitro-L-arginine methyl ester-inhibitable component was taken as the specific NOS activity.

Statistical analysis

Bands for iNOS and GAPDH on Northern blot autoradiograph were quantitated using a laser densitometry (Howtek, Hudson, NH) linked to a computer analysis system (PDI, Huntington Station, NY). The relative iNOS mRNA levels are expressed as a percentage of their corresponding GAPDH bands. The data are presented as the mean ± SEM. Statistical analysis of results was performed using the Kruskal-Wallis rank test followed by the Mann-Whitney *U* test for stepward comparison.

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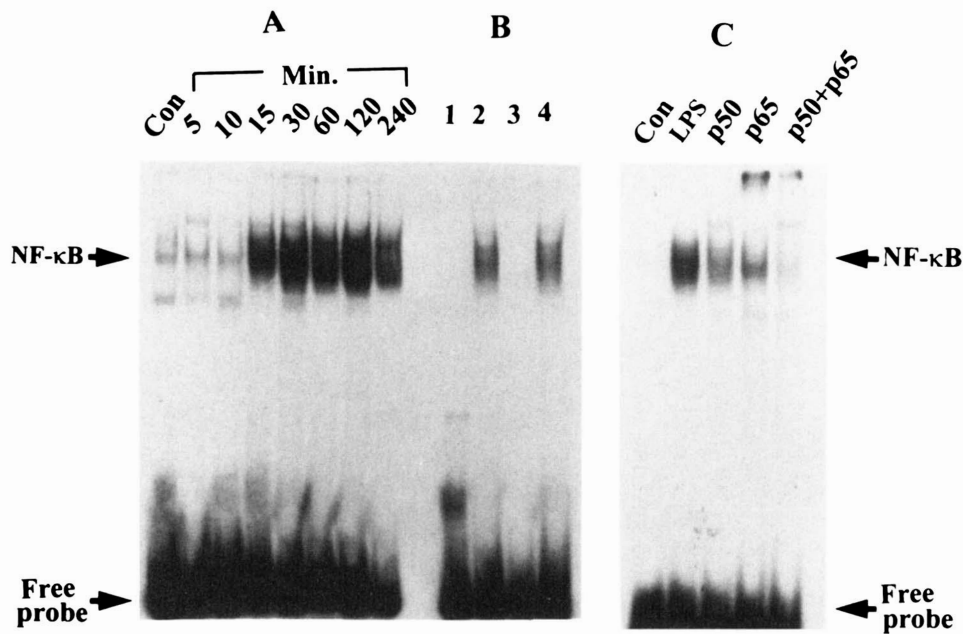

FIGURE 1. Autoradiograph of EMSA showing LPS-induced NF-κB activation in lung tissue. *A*, Time course of LPS-induced NF-κB activation. Rats were challenged with LPS (8 mg/kg i.v.) for the indicated time (in minutes). Nuclear protein was extracted from lungs of control (Con) and LPS-treated rats, and subjected to EMSA and autoradiography as described in *Materials and Methods*. The positions of NF-κB/DNA complex and free probe are marked. *B*, Competition study. *Lane 1*, NF-κB probe without nuclear extract; *lane 2*, nuclear extract from the lung of a rat treated with LPS for 15 min; *lanes 3* and *4*, the same sample as that in *lane 2* but including a 50-fold molar excess of unlabeled NF-κB probe (*lane 3*) or AP-2 probe (*lane 4*). *C*, Supershift assay of nuclear extract from a control lung (Con) and a lung from an LPS-treated rat (15 min). The NF-κB/DNA binding reaction was performed in the absence (LPS) and the presence of Abs to p50 (p50), p65 (p65), or a combination of these two Abs (p50+p65). A shift was observed with p50 or p65 Ab. Note that the combination of p50 and p65 Abs fully shifted this complex.

Results

Temporal relationship between NF-κB activation and iNOS expression in vivo

We injected rats i.v. with LPS for different durations to establish the temporal relationship between NF-κB activation and iNOS mRNA expression. We analyzed NF-κB/DNA binding activities and iNOS expression by EMSA and Northern blotting. Figures 1*A* and 2 are autoradiographs showing the time course of LPS-induced NF-κB/DNA binding activity and iNOS mRNA expression. A low level of NF-κB/DNA binding activity was detected basally in nuclear proteins from control lungs (Fig. 1*A*). This DNA binding activity slightly increased in nuclear extracts from lungs 5 and 10 min after LPS challenge (Fig. 1*A*), but increased significantly in nuclear extracts obtained from lungs treated with LPS for 15, 30, 60, 120, and 240 min, respectively (Fig. 1*A*).

The specificity of the NF-κB/DNA binding complex was evident by the complete displacement of the NF-κB/DNA binding complex in the presence of a 50-fold molar excess of unlabeled NF-κB probe in the competition reaction (Fig. 1*B*, *lane 3*). In contrast, a 50-fold molar excess of unlabeled AP-2 oligo probe had no effect on this DNA binding activity (Fig. 1*B*, *lane 4*). A slower and a faster migrating complex was seen in some assays, but this was not induced by LPS.

The composition of the NF-κB complex activated by LPS was determined using Abs reactive with the rat p50 and p65 subunits of NF-κB proteins. Addition of either anti-p50 or anti-p65 Ab to the binding reaction resulted in a marked reduction of the NF-κB band intensity and led to the appearance of a slow migrating band (Fig. 1*C*, p50 and p65). Simultaneous addition of both Abs to the binding reaction prevented the formation of the DNA/NF-κB complex and resulted in two slower migrating bands (Fig. 1*C*, p50 and p65), suggesting that this complex contained predominately p50 and p65 subunits of the NF-κB protein family.

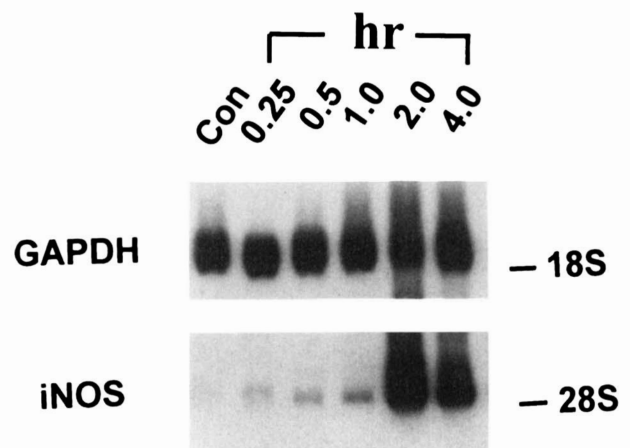

FIGURE 2. Northern blot autoradiogram showing the time course of LPS-induced iNOS mRNA expression in the lung. GAPDH mRNA served as the internal control. Con, control; 0.25, 0.5, 1.0, 2.0, and 4.0 represent 15 min, 30 min, 1 h, 2 h, and 4 h after LPS injection. This is a representative blot of three experiments performed using three animals at each time point.

Using the rat iNOS cDNA probe, we detected a 4.4-kb iNOS transcript in RNAs in all LPS-treated lungs and a low level of iNOS transcript in control lungs (Fig. 2). The iNOS transcript increased in lungs of rats challenged with LPS for 30 min or longer (Fig. 2). The iNOS mRNA abundance increased further 1 h after LPS administration and peaked at 2 h (Fig. 2).

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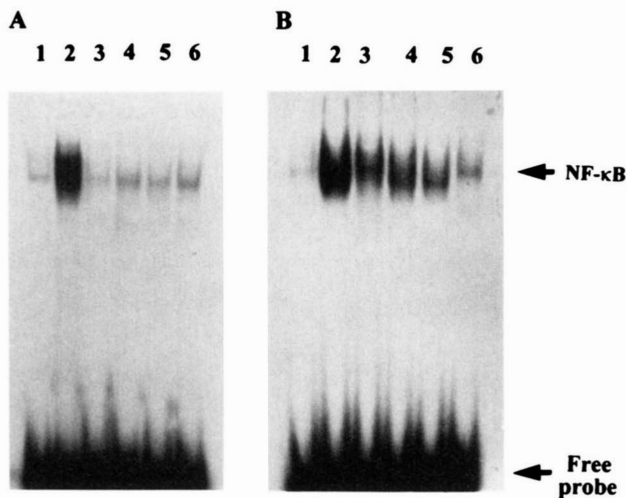

FIGURE 3. Autoradiograph of EMSA showing inhibition by PDTC of LPS-induced NF-κB activation in the lungs of rats challenged with LPS for 15 (*A*) or 60 (*B*) min. Lungs were isolated 15 or 60 min after LPS administration. PDTC was injected 1 h before LPS. Nuclear extracts were subjected to EMSA and autoradiography as described in *Materials and Methods. Lane 1,* Control; *lane 2,* LPS alone; *lane 3,* LPS plus 50 mg/kg PDTC; *lane 4,* LPS plus 100 mg/kg PDTC; *lane 5,* LPS plus 200 mg/kg PDTC; *lane 6,* PDTC alone (200 mg/kg). The positions of NF-κB/DNA complex and free probe are marked. This autoradiographs are representative of three separate experiments performed using three animals in each group.

PDTC inhibits LPS-induced NF-κB activation in vivo

We used PDTC (which was shown to be a selective inhibitor of NF-κB activation in cells (22–24)) to study the in vivo function of NF-κB activation in the induction of iNOS in this rat model. We compared the appearance of the NF-κB/DNA complex in nuclear extracts from lungs of control rats; rats challenged with LPS for 15, 30, or 60 min; and rats treated with 50, 100, or 200 mg/kg PDTC for 1 h followed by LPS challenge for 15, 30, or 60 min. As shown in Figure 3, LPS caused a significant increase in the level of NF-κB/DNA complex 15 or 60 min after LPS challenge (this also occurred at 30 min after LPS challenge; data not shown). PDTC at each concentration abolished the response in rats challenged with LPS for 15 min (Fig. 3*A*), but the inhibitory effect of PTDC was dose dependent and was less in rats challenged with LPS for 60 min (Fig. 3*B*). The results with 30 min of LPS challenge were the same as those with 60 min (data not shown). PDTC (at 200 mg/kg) alone slightly increased the level of NF-κB/DNA complex compared with that in control experiments, consistent with the observation in cultured cells that higher PDTC concentrations can independently induce a low level of NF-κB activation (22).

PDTC reduces LPS-induced iNOS expression

We treated animals with 50, 100, or 200 mg/kg PDTC 1 h before they were challenged with LPS for 4 h and compared the iNOS mRNA abundance in heart homogenates of rats treated with saline (control) or LPS alone. As the time course and characteristics of LPS-induced iNOS mRNA expression were identical in hearts, lungs, and other organs examined (8, 35), we used cardiac tissue to study the dose-dependent effects of PDTC on LPS-induced iNOS mRNA expression. The iNOS mRNA abundance increased markedly after LPS challenge (Fig. 4*A*). PDTC significantly reduced the LPS-induced iNOS expression, whereas PDTC alone did not affect its expression (Fig. 4*A*). We quantified the iNOS and GAPDH band intensities using densitometry and normalized the iNOS bands to their corresponding GAPDH bands. Neither LPS nor PDTC had an effect on GAPDH mRNA transcription. However, LPS increased the iNOS/GAPDH ratio by ninefold, whereas PDTC inhibited this response by 20, 46, and 48% at concentrations of 50, 100, and 200 mg/kg, respectively (Fig. 4*B*).

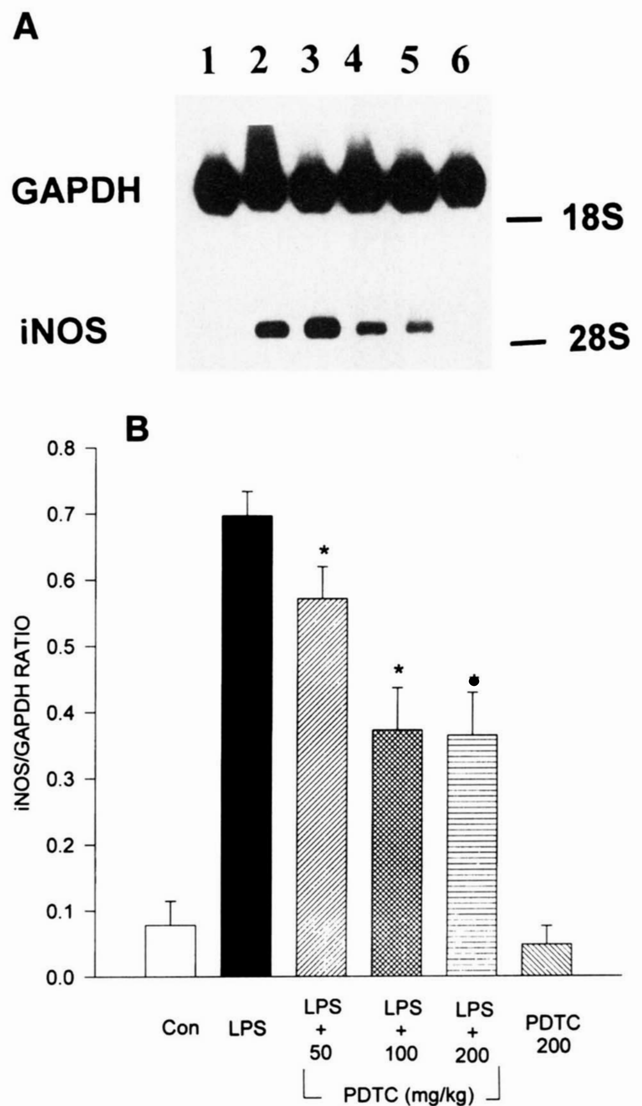

FIGURE 4. PDTC inhibits iNOS mRNA expression. *A*, Northern blot autoradiograms showing the inhibition by PDTC of iNOS mRNA expression induced by LPS in the heart. GAPDH mRNA served as the internal control. *Lane 1,* Control; *lane 2,* RNA from animals treated with LPS plus 50 mg/kg PDTC; *lane 3,* RNA from animals treated with LPS alone; *lane 4,* RNA from animals treated with LPS plus 100 mg/kg PDTC; *lane 5,* RNA from animals treated with LPS plus 200 mg/kg PDTC; *lane 6,* RNA from animals treated with 200 mg/kg PDTC alone. *B*, Relative iNOS mRNA levels in hearts of rats treated with saline (Con), LPS, LPS plus different dosages of PDTC, or PDTC alone, as quantified by densitometry and expressed as the iNOS/GAPDH ratio (OD ratio of iNOS to GAPDH). Pretreatment with 50, 100, and 200 mg/kg of PDTC significantly reduced LPS-induced iNOS mRNA levels. PDTC alone had no effect. *, Indicates $p < 0.05$ compared with LPS alone. Values are the mean ± SEM of four to six animals in each group.

PDTC reduces LPS-induced Ca^{2+}-independent NOS activity

We assessed the iNOS activity in homogenates of lung, heart, and liver from control, LPS, LPS plus PDTC, and PDTC alone groups.

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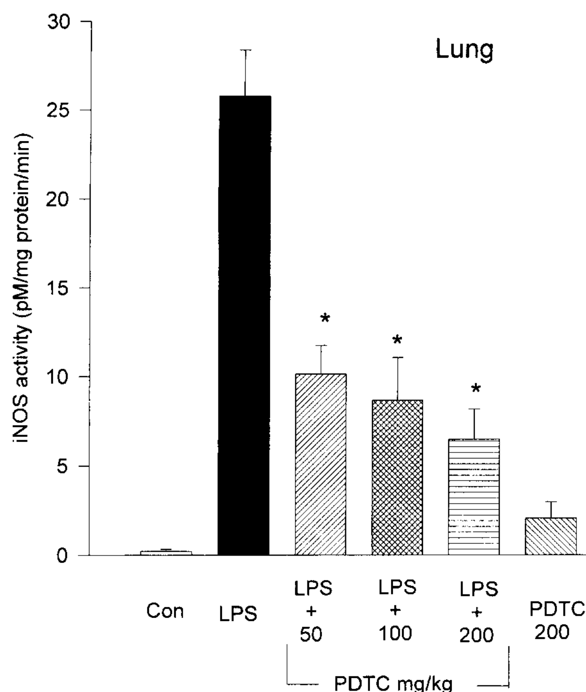

FIGURE 5. Calcium-independent (iNOS) NOS activity in crude supernatants of lung homogenates from rats treated with saline (Con), LPS, LPS plus different dosages of PDTC, or PDTC alone. The calcium-independent NOS activity was determined by the production of L-citrulline from L-arginine (picomoles per milligram per minute) in the absence of calcium and in the presence of 1 mM EGTA and excess concentrations of cofactors (see *Materials and Methods*). Pretreatment with 50, 100, and 200 mg/kg of PDTC significantly reduced LPS-induced iNOS activity in a dose-dependent manner. *, Indicates $p < 0.01$ compared with LPS alone. Values are the mean ± SEM of eight determinations using lungs from four animals in each group.

As shown in Figures 5 and 6, iNOS activity was low in control tissues, but increased markedly in tissues of LPS-challenged rats. PDTC produced dose-dependent inhibition of LPS-induced iNOS activity in lungs (Fig. 5). The results showed that PDTC at doses of 50, 100, and 200 mg/kg caused 59, 66, and 75% reductions of iNOS activity, respectively. The inhibition by PDTC of LPS-induced iNOS mRNA and iNOS activity was studied in the same groups of animals. We tested only one dose of PDTC in the hearts and livers; PDTC (100 mg/kg) reduced LPS-induced iNOS activity by approximately 40% in both heart and liver (Fig. 6). PDTC alone had no significant effect on iNOS activity in all three tissues tested (Figs. 5 and 6).

To determine whether PDTC-induced inhibition of iNOS activity was the result of a direct effect of PDTC on iNOS catalytic activity, we incubated crude supernatant from LPS-challenged heart and lung homogenates with two concentrations of PDTC and assayed for iNOS activity. The iNOS activity in a control reaction and those in reactions containing 50 and 100 μM PDTC were 2.5 ± 0.3, 2.3 ± 0.2, and 2.4 ± 0.3 pmol/mg protein/min in heart homogenates and 23.1 ± 4.5, 18.5 ± 1.8, and 19.8 ± 2.3 pmol/mg protein/min in lung homogenates, respectively. This result indicates that PDTC itself did not interfere with iNOS catalytic activity.

PDTC inhibits NO production in LPS-challenged rats

We determined the effects of PDTC on alterations in the plasma nitrite/nitrate concentration (a final oxidation product of NO), which was used as indicator of NO production. Control animals showed low concentrations of plasma nitrite/nitrate. The plasma nitrite/nitrate concentration increased by 17-fold in LPS-challenged animals (Fig. 7). Pretreatment with PDTC inhibited the LPS-induced increase in plasma nitrite/nitrate concentration in a dose-dependent manner (reductions of 45, 66, and 75% at PDTC concentrations of 50, 100, and 200 mg/kg, respectively). There was a direct relationship between the inhibition of iNOS activity and the reduction in NO production (Figs. 5 and 7). PDTC alone had no effect on the basal plasma nitrite/nitrate concentration (Fig. 7).

PDTC prevents LPS-induced systemic arterial hypotension

We determined the effects of inhibition of iNOS mRNA and activity by PDTC on LPS-induced arterial hypotension. We measured mean arterial blood pressure in rats of control, LPS, LPS plus 100 mg/kg PDTC, and PDTC alone groups. Mean arterial pressures were not significantly different in the four groups before treatments (Fig. 8, *left bars*); rats developed marked systemic hypotension 4 h after LPS challenge. Pretreatment with PDTC prevented the decrease in arterial blood pressure in response to LPS challenge (Fig. 8, *right bars*).

Discussion

We determined the in vivo function of NF-κB activation in mediating LPS-induced iNOS expression and septic shock in a rat model. Several in vitro studies have defined LPS- and cytokine-responsive elements on the iNOS gene promoter and have identified NF-κB as the primary transcription factor regulating iNOS expression after LPS or cytokine challenge (17–20). In the present study we demonstrated that LPS activates NF-κB in vivo and that its activation, in turn, induces transcription of the iNOS gene and expression of the iNOS protein. LPS challenge activated NF-κB within 15 min, and the response peaked at 30 min and remained elevated up to 4 h post-LPS challenge, indicating the sustained nature of NF-κB activation in this model. The iNOS steady state mRNA activity increased at 1 h, peaked at 2 h, and remained elevated 4 h after LPS. These data established that activation of NF-κB precedes iNOS gene expression in lungs of rats challenged with LPS and that the response can be maintained at an elevated level for several hours.

We used PDTC, an inhibitor of NF-κB activation (22–24, 27), to evaluate the contribution of NF-κB in mediating in vivo iNOS gene expression. Pretreatment of rats with PDTC ranging from 50 to 200 mg/kg body weight disrupted the LPS-induced NF-κB/DNA binding activity and significantly reduced iNOS mRNA expression, iNOS activity, and the elevation in plasma nitrite/nitrate concentrations. PDTC prevented the LPS-induced systemic hypotension. These results indicated that in vivo activation of NF-κB induced by LPS is a critical step in the development of septic shock.

Schreck et al. (22) demonstrated that PDTC inhibited LPS-, TNF-α-, and PMA-induced NF-κB activation, but had no effect on activation of transcription factors, AP-1, octamer-binding proteins, cAMP response element-binding proteins, and promoter-selective transcription factor (Sp-1). This inhibitory effect of PDTC was dose dependent and reversible, and was not cell selective since it occurred in several cell lines (22). The selectivity of PDTC was supported by the finding that PDTC suppressed NF-κB activation, ICAM-1 mRNA and protein expression, and ICAM-1 promoter/CAT activity in human fibroblast cells stimulated with IL-1α (23). Deletion or mutation of the NF-κB binding site on the ICAM-1

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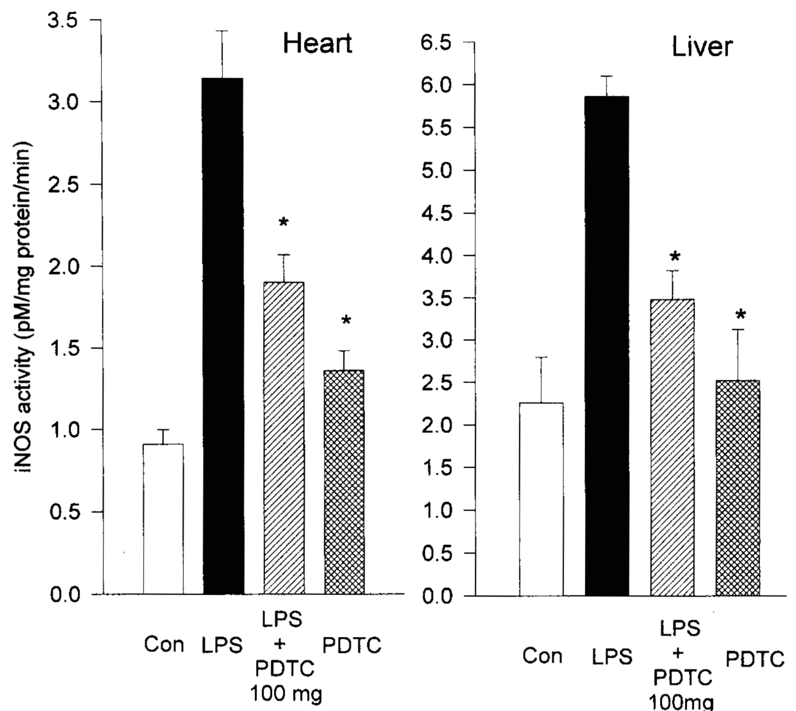

FIGURE 6. The calcium-independent (iNOS) NOS activity in crude supernatants of heart and liver homogenates from animals treated with saline (Con), LPS, LPS plus PDTC (100 mg/kg), or PDTC alone. The calcium-independent NOS activity was determined by the production of L-citrulline from L-arginine (picomoles per milligrams per minute) in the absence of calcium and in the presence of 1 mM EGTA and excess concentrations of cofactors. PDTC significantly reduced LPS-induced iNOS activity in both organs. *, Denotes $p < 0.01$ compared LPS alone. Values are the mean ± SEM of eight determinations using hearts or livers from four animals in each group.

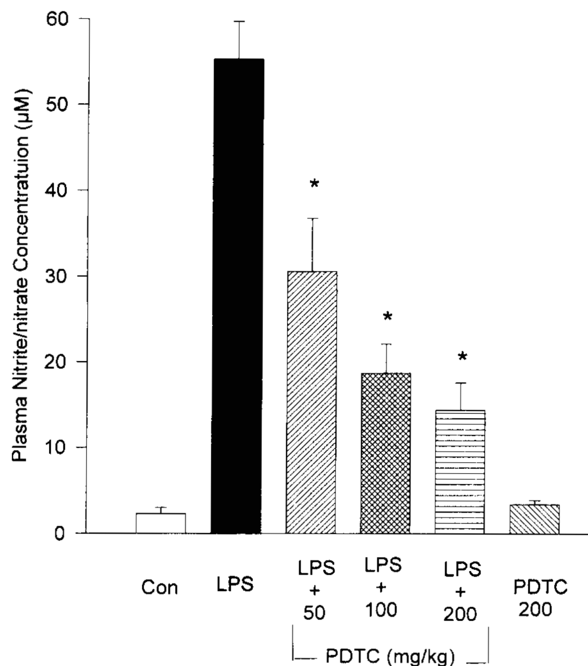

FIGURE 7. Plasma nitrite/nitrate concentrations in control animals (Con), in animals 4 h after LPS (8 mg/kg i.v.) challenge, in animals treated with LPS plus different dosages of PDTC (50, 100, and 200 mg/kg i.p. 1 h before the administration of LPS), and in animals treated with PDTC alone (200 mg/kg). *, Denotes $p < 0.01$ compared with animals challenged with LPS alone. Values are the mean ± SEM of four to six animals in each group.

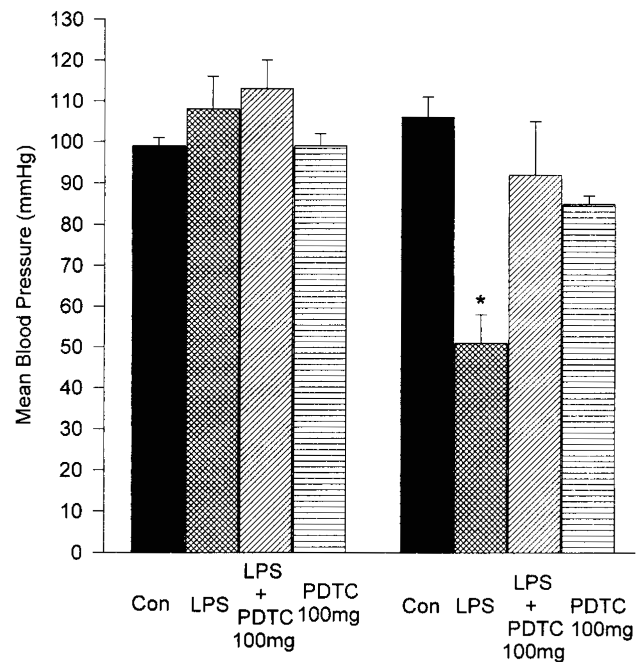

FIGURE 8. Effects of 100 mg/kg PDTC on LPS-induced hypotension. LPS was given by i.v. infusion (8 mg/kg), and PDTC was injected i.p. 1 h before the administration of LPS. *Left bars* are the basal mean arterial pressures before LPS or PDTC injections. *Right bars* are the mean arterial pressures 4 h after LPS challenge. *, Denotes $p < 0.05$ compared with the control, LPS plus PDTC, and PDTC alone groups. Values are the mean ± SEM of five to seven animals.

promoter abolished the inhibitory effect of PDTC on IL-1α-induced ICAM-1 promoter/CAT activity (23). Other studies showed that PDTC prevented the expression of NF-κB-dependent genes in several cell types (23–27). PDTC also inhibited the expression of iNOS induced by LPS or cytokines in vitro (17, 18, 20, 25, 28). In the present study we showed that PDTC suppressed NF-κB activation induced by LPS in vivo, and thus, we used it as a reagent for the analysis of NF-κB activation and NF-κB-regulated iNOS expression in this rat model.

We observed that PDTC had a greater inhibitory effect on NF-κB activation induced by 15 min of LPS exposure than on iNOS expression; that is, 200 mg/kg PDTC caused 85% inhibition

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of NF-κB activation, but only 48 and 66% inhibition of iNOS mRNA expression and iNOS activity, respectively. This result suggested that in addition to NF-κB, other transcription factors are also involved in mediating LPS-induced iNOS expression. This finding differs from those of in vitro studies in which NF-κB activation alone was fully responsible for induction of iNOS gene by LPS or cytokine in murine macrophages (18) and rat smooth muscle cell lines (17). The present results are consistent with promoter deletion studies in primary cultured rat aortic and pulmonary smooth muscle cells (19, 20) in which mutations or deletions of NF-κB sites in the iNOS promoter incompletely inhibited the promoter activity in response to IL-1β (19, 20). Our results indicated that NF-κB is an important transcriptional factor, but it is not the sole in vivo regulator for LPS-induced iNOS gene expression.

The PDTC-inhibitable NF-κB complex induced by LPS in lungs was partly supershifted by either anti-p50 or anti-p65 Ab, and was fully shifted by a combination of these two Abs, suggesting that the NF-κB complex responsible for iNOS induction by LPS consisted of both p50 and p65 subunits. This finding also differs from results in cultured mouse macrophage and rat vascular smooth muscle cell lines (17, 18). The LPS- and cytokine-induced NF-κB complex is composed of p50, p65, and c-*rel* in macrophages and p65 and a 50-kDa protein (which is not recognized by Ab against p50) in vascular smooth muscle cells (17, 18).

Although PDTC significantly reduced iNOS expression, it did not decrease ecNOS expression. We showed that PDTC prevented the characteristic down-regulation of ecNOS mRNA induced by LPS (S. F. Liu et al., unpublished observation). Therefore, the protective effect of PDTC in reducing LPS-induced NO production cannot be ascribed to a decrease in ecNOS expression.

The discovery that NO is a critical mediator of septic hypotension (4–12) has prompted the use of NO synthase inhibitors for the treatment of septic shock (5, 6, 10). Although these inhibitors restored the blood pressure (5, 6, 10) and corrected the depressed vasoactive response to norepinephrine and other vasoconstrictors (9, 11), they are nonselective. NOS inhibitors may promote tissue injury because they inhibit both high output iNOS and low output neuronal NOS and ecNOS. NOS inhibitors also induced the expression of endothelial adhesion molecules (36, 37) and thereby may promote neutrophil adhesion and migration across the endothelium (36–39), both of which are steps involved in the mechanism of LPS-induced tissue injury. NOS inhibitors enhanced splenocyte TNF-α and IFN-γ production in response to staphylococcal endotoxin B (40) and facilitated LPS-induced gastrointestinal damage (41). In contrast to these effects of NOS inhibitors, the targeting of NF-κB with agents such as PDTC may be a more effective strategy in the treatment of septic shock, because inhibition of NF-κB activation selectively prevented the increase in iNOS activity and iNOS-mediated NO production.

Systemic administration of LPS leads to the release of multiple proinflammatory cytokines, including TNF-α, IFN-γ, and IL-1β (13), all of which are potent NF-κB activators (15, 16). These cytokines were also effective inducers of iNOS expression and were synergistic with LPS in mediating iNOS expression (1, 2, 15–20). These cytokines may play an important role in sustaining NF-κB activation and iNOS expression in the present study. However, these cytokines are unlikely to mediate the early phase of NF-κB activation and iNOS expression. As we demonstrated, LPS challenge activated NF-κB within 15 min, whereas plasma levels of these cytokines were not elevated until 60 min post-LPS treatment (29, 42, 43). LPS, by binding to LPS binding protein, can directly activate CD14 receptor and can thereby induce NF-κB activation (15). Moreover, NF-κB is a crucial mediator of LPS-induced TNF-α, IFN-γ, and IL-1β expression (15, 16, 24). The NF-κB activation induced by 15-min LPS exposure was fully prevented by all concentrations of PDTC, whereas the NF-κB activation induced with 60-min LPS exposure was inhibited to a lesser extent and in a dose-dependent manner by PDTC. The finding that a similar level of inhibition was not seen 1 h after LPS administration suggests that the release of secondary mediators by LPS produce a delayed onset activation of NF-κB such that PDTC becomes a less effective inhibitor with time. This finding may also help to explain why PDTC did not fully prevent the expression of iNOS mRNA and iNOS activity in response to LPS.

In summary, we have shown that the activation of NF-κB as early as 15 min after LPS challenge activates in vivo transcription of the iNOS gene and leads to expression of iNOS and generation of NO. The results indicate that these sequential events are critical in the induction of septic shock in the rat model and suggest the LPS challenge in vivo can also activate factors that can amplify the response (perhaps due to release of secondary mediators) such that the inhibitor of NF-κB activation, PDTC, becomes less effective with time after LPS challenge. Nevertheless, a therapeutic strategy involving the inhibition of NF-κB activation may prove effective in septic shock.

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