

Hypertonicity Inhibits Lipopolysaccharide-Induced Nitric Oxide Synthase Expression in Smooth Muscle Cells by Inhibiting Nuclear Factor κ B

SANDEEP C. PINGLE, JOSEPH F. SANCHEZ, DANIEL M. HALLAM, ANDREA L. WILLIAMSON, SANJAY B. MAGGIRWAR, and VICKRAM RAMKUMAR

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois (S.C.P., D.M.H., V.R.); and Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York (J.F.S., A.L.W., S.B.M.)

Received September 16, 2002; accepted February 24, 2003

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

The expression of inducible nitric-oxide synthase (iNOS) in vascular smooth muscle cells leads to prolonged vasorelaxation in vivo and contributes to the profound vasodilation induced by bacterial lipopolysaccharide (LPS) in septic shock. This induction of iNOS depends, in large part, on activation of nuclear factor (NF)- κ B. Hypertonicity regulates the activity of NF- κ B in different cell lines; as such, we propose that it should also regulate the expression of iNOS. Thus, the goal of this study was to determine whether hypertonicity regulates iNOS expression and function in smooth muscle cells and to elucidate the mechanism(s) underlying this process. Treatment of hamster ductus deferens (DDT₁MF-2) cells and porcine aortic smooth muscle cells with either mannitol (50 mM) or NaCl (50 mM) reduced LPS-stimulated iNOS expression and nitric oxide release. Both of these agents also reduced the activation of

NF- κ B induced by LPS, tumor necrosis factor- α and interleukin-1 β in smooth muscle cells. This inhibitory action was caused by suppression of I κ B- α phosphorylation, a prerequisite for ubiquitination and degradation of this protein, and showed additivity with *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG-132), an inhibitor of proteasomal degradation of I κ B- α . Furthermore, exposure to mannitol inhibited the activity of I κ B kinase, an enzyme involved in phosphorylation of I κ B- α . Mannitol was unable to affect the induction of iNOS produced by overexpression of RelA in DDT₁MF-2 cells, suggesting that this agent does not have additional downstream inhibitory actions on this activated NF- κ B subunit. Taken together, these data suggest that these hypertonic solutions may prove useful as anti-inflammatory agents, especially against conditions associated with increased NF- κ B activity.

Hypertonic stress activates a number of cell signaling processes that ensure cell viability in the presence of prolonged stress. In cells of the renal inner medulla, hypertonic solutions increase the activity of extracellular signal-regulated kinase/mitogen activated protein kinase pathway, leading to the activation of genes involved in the synthesis or transport of osmolytes (Burg et al., 1997). Hypertonic stress induced by urea regulates the activity of phosphatidylinositol-3 kinase (Zhang et al., 2000), phospholipase C- γ (Cohen et al., 1996), and the transcription and translation of the immediate early gene transcription factor, Egr-1, in renal inner medullary cells (Cohen et al., 1994). Induction of hyperosmotic stress in NMuMg mammary epithelial cells increases the expression

of the serum- and glucocorticoid-inducible protein kinase via a p38 mitogen-activated protein kinase signaling pathway (Bell et al., 2000). Other proteins that are also induced by hypertonic stress include the 70-kDa heat shock protein (hsp70) family (Cohen et al., 1991) and aquaporin (Jenq et al., 1999). In the event that osmotic stress is sustained and/or compensatory measures (as described above) prove ineffective, activation of cell-signaling pathways, such as the c-Jun NH₂-terminal kinase pathway (Malek et al., 1998), can initiate the apoptotic cascade.

Less is known concerning the involvement of nuclear factor (NF)- κ B in mediating cellular responses to hyperosmotic stress. Exposure of normal skin fibroblasts to hyperosmotic concentrations of sucrose or NaCl inhibited NF- κ B activity induced by TNF- α (Andrieu et al., 1995). Similarly, hypertonicity produced by mannitol reduced the activation of NF- κ B and IL-8 production elicited by *Helicobacter pylori* (Kim et

This study was funded by National Institutes of Health grants R01-HL56316-01 (to V.R.), R01-NS39039, and P01-MH64570 (to S.B.M.) and by funds from the Central Research Committee (Southern Illinois University School of Medicine).

ABBREVIATIONS: NF- κ B, nuclear factor κ B; IKK, I κ B kinase; iNOS, inducible nitric-oxide synthase; NO, nitric oxide; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor- α ; IL, interleukin; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DAF-2 DA, 4,5-diaminofluorescein diacetate; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RelA_{FL}, full-length RelA; MG-132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal.

al., 1999). In contrast, exposure to hyperosmotic glucose solutions elicited NF- κ B activation in vascular smooth muscle cells (Hattori et al., 2000). In rat hepatoma cells, whereas hyperosmolarity produced little change in NF- κ B activity, hypo-osmolarity induced sustained activation of this transcription factor (Michalke et al., 2000).

NF- κ B plays a pivotal role in communicating the signals from cytokines, toxins, and various stressors to regulation of gene expression. Activation of NF- κ B is initiated by phosphorylation of serine-32 and serine-36 residues of I κ B by I κ B kinases (IKKs), leading to its ubiquitination and proteasome-mediated degradation. The free NF- κ B then translocates to the nucleus, where it interacts with the consensus DNA binding sequences present in regulatory regions of numerous target genes (Karin and Ben-Neriah, 2000). Stimulation of inducible nitric-oxide synthase (iNOS) expression by bacterial lipopolysaccharide (LPS) involves activation and nuclear translocation of NF- κ B, where it interacts with κ B sequences in the promoter of the *iNOS* gene (Xie et al., 1994). The *iNOS* gene is predominantly regulated at the level of transcription (de Vera et al., 1998) via several putative NF- κ B response elements (Spitsin et al., 1997; Taylor et al., 1998). However, this gene also contains the transcription factor binding sites for the AP-1, as well as for members of CCAAT/enhancer-binding protein, activating transcription factor/cAMP response element-binding protein, and signal transducer and activator of transcription family of transcription factors (Hecker et al., 1997). The generation of nitric oxide (NO) via iNOS is the basis of the profound vasodilation observed during septic shock (Rackow and Astiz, 1991; Titheradge, 1999). As such, drugs that inhibit the production of or increase the elimination of NO could be useful in the management of septic shock (Kilbourn et al., 1990).

The observations linking hypertonicity to regulation of NF- κ B activation prompted our current interest in examining the effect of hypertonicity on LPS- and cytokine-induced activation of NF- κ B-dependent signal transduction pathway in smooth muscle cells in culture. Specifically, the goals of this study were to determine whether hypertonicity alters LPS- and cytokine-dependent activation of NF- κ B and induction of iNOS expression and to delineate the mechanism(s) underlying this action. We show that hypertonicity inhibits LPS- and cytokine-stimulated iNOS expression through inhibition of I κ B- α phosphorylation.

Materials and Methods

Cell Culture. Porcine aortic smooth muscle cells and hamster vas deferens smooth muscle cells (DDT₁MF-2) were cultured in minimum essential medium supplemented with 5% fetal bovine serum. Cells were maintained as a monolayer and detached in ice-cold phosphate-buffered saline (PBS) containing 5 mM EDTA. For the measurement of iNOS expression using Western blotting, the cells were lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA, 10 mg/ml soybean trypsin inhibitor, 10 mg/ml benzamidine, and 2 mg/ml pepstatin and homogenized briefly using a Polytron homogenizer. The homogenates obtained were centrifuged at 40,000g for 15 min, and the supernatants were used for Western blotting experiments.

Preparation of Nuclear Extracts. Nuclear extracts were prepared from the cells as described previously (Schreiber et al., 1989). Briefly, the cells were suspended in buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% Nonidet P-40, 1 mM dithiothreitol

(DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF)]. The mixtures were centrifuged at 5000g for 30 s, and the cytosolic extract was separated. The nuclear pellet was washed with excess volume of buffer A and then resuspended in buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF). After incubating for 5 min at 4°C with rotation, the extracts were centrifuged (5000g, 1 min), and the supernatants were used for DNA binding activity analyses.

SDS Polyacrylamide Gel Electrophoresis/Western Blotting.

For quantitation of iNOS isozymes, I κ B- α , and α -actin, supernatants were resolved by SDS polyacrylamide gel electrophoresis as described by Laemmli (1970). Proteins were transferred to nitrocellulose membranes, blocked in a solution containing 130 mM NaCl, 2.7 mM KCl, 1.8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.1% NaN₃, 0.1% Triton X-100, and 5% low-fat skim milk for 2 h, and then incubated at 4°C overnight with the primary polyclonal antibody. After five washes in blocking solution, blots were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) for 2 h at room temperature, washed three times with Tris-buffered saline/Tween 20 (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6 at 25°C), followed by three washes with Tris-buffered saline without 0.1% Tween 20, treated with ECL Plus reagents (Amersham Biosciences) and visualized by exposure to Kodak XAR film or by using a charge-coupled device camera (Hitachi Genetic Systems, MiraiBio Inc., Alameda, CA). All antibodies used in this study for NOS isozymes, I κ B- α , NF- κ B and subunits and α -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunocytochemistry for iNOS. DDT₁MF-2 cells and porcine aortic smooth muscle cells were cultured and treated as described in the figure legends. After specific treatments, cultures were washed twice with warm PBS and fixed with 4% paraformaldehyde for 10 min. After two more washes with PBS, nonspecific binding was reduced by exposing cover slips for 5 min with a solution containing 5% normal goat serum and 0.5% Triton X-100. The cells were treated with rabbit NOS antibody (Santa Cruz Biotechnology) to detect NOS isozymes, diluted 1:200 in 5% normal goat serum along with 0.05% Triton X-100 in PBS, and incubated overnight at 4°C. After rinsing four times in PBS, cells were treated for 1 h with goat anti-rabbit IgG labeled with rhodamine (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:100 in 5% normal goat serum and 0.05% Triton X-100 in PBS. After four rinses in PBS, the coverslips were mounted on glass microscope slides using Aquamount. The cells were observed using an Olympus confocal microscope using a 40 \times objective. In some experiments, localization of cells was facilitated using 0.5 μ M Sytox (Molecular Probes, Eugene, OR) to stain cell nuclei in 0.05% Triton X-100 permeabilized cells.

Electrophoretic Mobility Shift Assay. EMSAs were performed by incubating nuclear extracts with ³²P-radiolabeled double-stranded oligonucleotide probes suspended in reaction buffer (12 mM HEPES, pH 7.9, 100 mM NaCl, 0.25 mM EDTA, 1 mM DTT, and 1 mM PMSF) at room temperature for 10 min. The protein-DNA complexes were electrophoresed using 4% nondenaturing polyacrylamide gels, dried, and exposed to X-ray films (Amersham Biosciences) or to phosphor screen imaging (Cyclone Storage Phosphor System; PerkinElmer, Boston, MA). The -fold increase in the expression of the transcription factors was determined using background subtract. The probes used in these assays were as follows. 1) NF- κ B, 5'-CAACGGCAGGGGAATTCCTCTCCTT-3'; 2) AP-1, 5' TGTCGAATGCAAATCACTAGAA-3'; and 3) OCT-1, 5'-TGTCGAATGCAAATCACTAGAA-3'.

Assay for Nitric Oxide Production. Intracellular nitric oxide production was detected in smooth muscle cells using 4,5-diaminofluorescein diacetate (DAF-2 DA; Calbiochem, San Diego, CA) based on methods described previously (Kojima et al., 1998; Nakatsubo et al., 1998). Cells were plated on sterile 12-mm glass coverslips at 400 cells/mm² in individual wells of 24-well tissue culture plates. The cells were treated with 50 mM mannitol (Sigma Chemical Co., St. Louis, MO), in the absence and presence of 10 μ g/ml LPS (Sigma

Chemical) for 24 h. Coverslips were washed with PBS and the cells loaded with DAF-2 DA by incubating in 5 mM DAF-2 DA for 20 min at 37°C and washed with PBS. The cultures were analyzed for green fluorescence 1 h later using an Olympus FluoView laser-scanning confocal microscope using an argon laser and a 40× objective.

Infection of Cultures with Adenovirus. DDT₁MF-2 cells were infected with recombinant adenovirus vectors, coexpressing either humanized version of jellyfish green fluorescent protein (GFP) and full-length RelA (RelA_{FL}), a transcriptionally inert RelA mutant (RelA₁₋₃₀₀) (Ramirez et al., 2001), or a mutant form of IκB-α, which acts as a super-repressor of NF-κB (mIκB-α, kindly provided by Dr. E. M. Schwarz, University of Rochester Medical Center, Rochester, NY). Twenty-four hours after infection, cells were either left untreated or treated with mannitol (50 mM) and/or LPS (10 μg/ml).

Immune Complex Kinase Assays. DDT₁MF-2 cells were pretreated with mannitol for 30 min, followed by the addition of LPS 10 μg/ml for 15 min. Whole-cell lysates were then collected in 1 ml of buffer containing 50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, and 50 μM ZnCl₂, supplemented with 0.1 mM PMSF, 1 mM DTT, and a mixture of protease and phosphatase inhibitors, and cellular debris was removed by high-speed centrifugation. Lysates were precleared by incubation with nonimmune serum for 1 h at 4°C followed by incubation with protein A agarose beads (Santa Cruz) and centrifugation. Lysates were then incubated with 1 μg of anti-IKKα (Santa Cruz Biotechnology) overnight at 4°C, after which IKKα was precipitated using protein A agarose beads. After several washes, beads containing IKKα were incubated with 0.25 μg of recombinant GST-IκBα as an exogenous substrate (Santa Cruz Biotechnology) and 10 μCi of ³²P-γ-ATP in 20 μl of kinase buffer (containing 50 mM Tris-Cl, pH 8, 10 mM MgCl₂, 5 mM DTT) at 30°C for 30 min. Kinase reactions were stopped by addition of SDS sample buffer. Incorporation of ³²P into IκBα was analyzed by performing SDS-PAGE and autoradiography. The membranes were subsequently probed with anti-IKKα (Santa Cruz Biotechnology) antibodies to determine the levels of immunoprecipitated IKKα kinase.

Reverse Transcription and Polymerase Chain Reaction. Isolation of total RNA was performed using TRIzol reagent kit (Invitrogen, Carlsbad, CA). Samples of total RNA (1 μg each), were reverse-transcribed using a first-strand cDNA synthesis kit (Amersham Biosciences) in a total volume of 15 μl. Five microliters of the reaction volume was then used for PCR amplification. Primers used included sequences GCCTCGCTCTGGAAAGA (sense) and TCCATGCAGACAACCTT (antisense) for human iNOS (Geller et al., 1993) and TGAAGGTCGGTGTCAACGGATTGGC (sense) and CATGTAGCCATGAGGTCCACCAC (antisense) for GAPDH. PCR reactions were performed in a total volume of 50 μl and contained 2.5 mM MgCl₂. The sequences of interest were amplified over 30 cycles. The amplified products were resolved on 1.2% agarose gels. Bands of interest were visualized using a charge-coupled device camera and intensities quantitated by densitometry using GAPDH for normalization.

Results

LPS-Mediated iNOS Expression in Smooth Muscle Cells Is Dependent on NF-κB. Under normal cell culture conditions, both DDT₁MF-2 and porcine aortic smooth muscle cells express low levels of iNOS, as detected by Western blotting assays using a polyclonal antibody specific for iNOS. Basal expression of 130-kDa iNOS protein was very low and was detectable only after 30 min exposure to X-OMAT film. Neither endothelial (eNOS) nor neuronal NOS (nNOS) were detectable in DDT₁MF-2 cells (Fig. 1). The expression of iNOS was significantly elevated after exposure to LPS (10 μg/ml) for 24 h and was associated with an increase in the steady-state levels of iNOS mRNA (see Fig. 2E.) However, no

induction of eNOS or nNOS was detected (data not shown). This induction of iNOS was abrogated after inhibition of NF-κB by overexpressing a mutant form of IκB-α (mIκB-α) that acts as a super-repressor of NF-κB, implying an integral role of this transcription factor in the induction of iNOS by LPS (data not shown). Under the conditions used, we achieved approximately 70 to 80% infectivity with the viral vector, as detected by indirect immunofluorescence.

Hypertonicity Inhibits LPS-Induced iNOS mRNA and Protein Expression. To test the effect of hypertonicity on iNOS expression, DDT₁MF-2 smooth muscle cells were pretreated with mannitol (50 mM) for 30 min, followed by the addition of LPS (10 μg/ml) for 24 h. Cells exposed to LPS showed a significant increase (~3.4-fold) in iNOS expression, compared with untreated control cells (Fig. 2A). The addition of mannitol (50 mM) alone produced no change in basal iNOS expression but completely abolished the induction by LPS. This effect of mannitol on inhibition of iNOS expression was observed at mannitol concentrations of 30, 50, and 100 mM, with maximum inhibition observed at 50 mM mannitol (Fig. 2B). The higher concentration of mannitol did not produce any greater inhibition of iNOS expression but in fact showed a slight stimulation above that observed for 50 mM mannitol. The addition of TNF-α (20 ng/ml) or IL-1β (20 ng/ml) to DDT₁MF-2 cultures also induced iNOS expression (Fig. 2C). Preincubation of cells with mannitol (50 mM) resulted in significant reduction in iNOS expression induced by these cytokines. To test whether this response to mannitol could be mimicked in vascular smooth muscle cells, we exposed primary cultures of porcine aortic smooth muscle cells to similar concentrations of mannitol (50 mM) and LPS (10 μg/ml). In these cells, a ~2.2-fold induction in iNOS was observed with LPS. Pretreatment with mannitol (50 mM) also significantly suppressed this induction (Fig. 2D). In these cells, we observed a small but statistically insignificant increase in iNOS expression produced by mannitol alone. The reason for this increase is not known. To determine whether the changes in iNOS expression were associated with concomitant changes in iNOS RNA, we quantitated the steady-state levels of mRNA by PCR. The product obtained by PCR was ~477 base pairs long, as predicted from the primers used. As shown in Fig. 2E, LPS produced a significant increase in iNOS mRNA

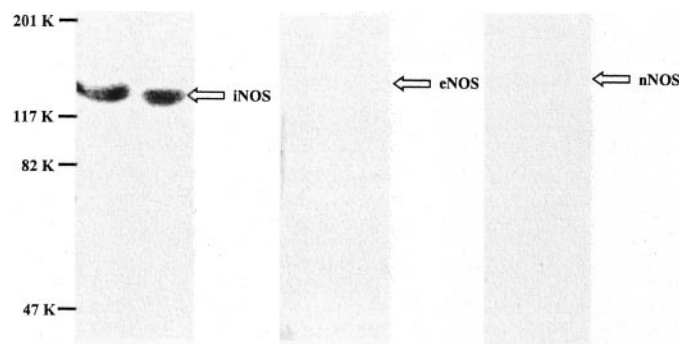


Fig. 1. DDT₁MF-2 smooth muscle cells express iNOS. DDT₁MF-2 cells were sonicated and cell lysates (50 μg of protein per lane) were resolved by SDS-PAGE and used in Western blotting experiments. Blots were probed with antibodies against iNOS, eNOS, and nNOS to determine the basal levels of expression of these proteins. Bands were visualized by enhanced chemiluminescence followed by exposure to X-OMAT film for 30 min. Molecular mass markers in kilodaltons (K) are shown on the left.

(~5-fold increase) which was attenuated in cells that were pretreated with mannitol ($64 \pm 22\%$ inhibition).

The ability of mannitol to suppress LPS-induced iNOS

expression was further confirmed by immunocytochemistry using the same polyclonal antibody for iNOS (as above) and a rhodamine (tetramethylrhodamine B isothiocyanate)-con-

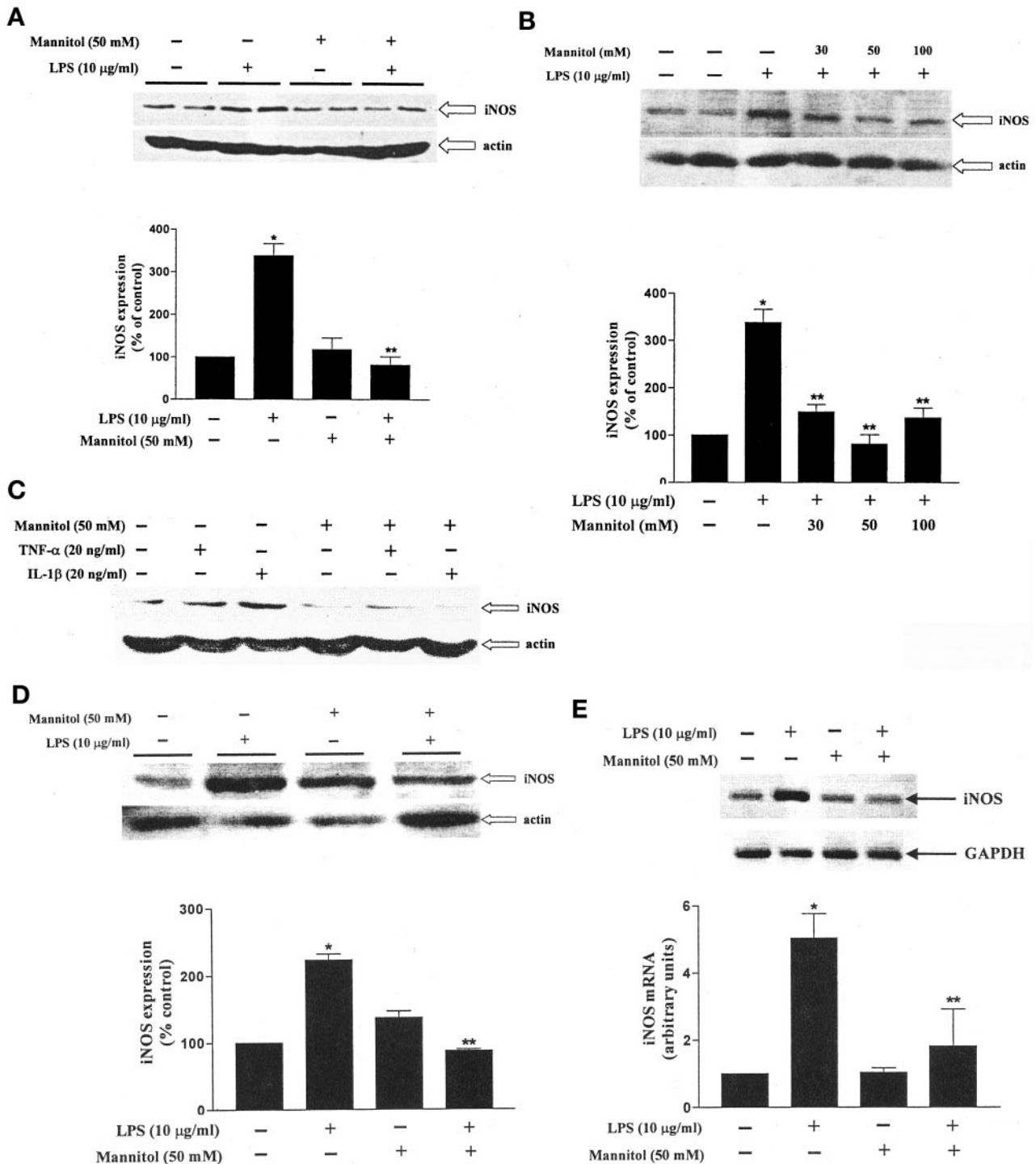


Fig. 2. Mannitol inhibits LPS-induced iNOS expression. A, DDT₁MF-2 cells were exposed to LPS (10 µg/ml) for 24 h with or without 50 mM mannitol. Cells were then lysed and cytosolic fractions (50 µg protein per lane) were used in Western blotting assays to quantitate iNOS expression. Blots were normalized to α -actin levels and were expressed as a percentage of the basal (control) expression, as depicted in the histograms. Data are expressed as the mean \pm S.E. B, the LPS-stimulated iNOS expression was reduced in the dose range of 30 and 50 mM, with partial reversal at 100 mM. C, mannitol reduced cytokine-induced iNOS expression. Cells were pretreated with mannitol, followed by the addition of either vehicle, TNF- α (20 ng/ml) or IL-1 β (20 ng/ml) for 24 h. D, primary cultures from pig aortic vascular smooth muscle cells were pretreated with either vehicle or mannitol as above, followed by vehicle or LPS (10 µg/ml) for an additional 24 h. E, DDT₁MF-2 smooth muscle cells were treated with either LPS (10 µg/ml) alone or with 50 mM mannitol for 12 h, after which total RNA was extracted, reverse transcribed, and used for PCR using specific primers for iNOS and GAPDH. The PCR products were resolved using 1.2% agarose gels. The band intensities were quantitated by densitometry and iNOS band was normalized to GAPDH mRNA. *, $p < 0.05$, statistically significant difference from control cells. **, $p < 0.05$, statistically significant difference from LPS-treated cells.

jugated secondary antibody. Immunoreactivity for iNOS appeared as a reddish-orange cytoplasmic stain by confocal microscopy, surrounding the yellow nuclear staining provided by the nuclear stain (Sytox). Cells exposed to LPS showed substantially elevated expression of iNOS compared with untreated control cells (Fig. 3A). Pretreatment with mannitol resulted in no demonstrable effect on iNOS expression but attenuated the induction of iNOS expression by LPS. Additional experiments were performed to test whether a similar response to mannitol could be produced in primary

cultures of porcine aortic smooth muscle cells. As observed above for DDT₁MF-2 smooth muscle cells, mannitol significantly suppressed iNOS expression upon stimulation by LPS in the porcine aortic smooth muscle cells (Fig. 3B). No nuclear staining (with Sytox) was used in the latter cell line. In both DDT₁MF-2 and porcine aortic smooth muscle cells, the responses observed with mannitol could be mimicked by exposing cells to hypertonic saline, produced by addition of 50 mM NaCl to the cell culture medium (data not shown).

Mannitol Inhibits LPS-Induced Production of NO.

Additional experiments were performed to test whether inhibition of LPS-induced iNOS immunoreactivity by mannitol was reflected in a decrease in NO production. NO production was assessed using a fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA), which binds NO in the cells and produces triazolofluoresceins, detectable by confocal microscopy at ~488 nm (Nakatsubo et al., 1998). The addition of mannitol alone to DDT₁MF-2 cells had little effect on NO release compared with untreated controls. LPS treatment for 24 h led to a significant increase in NO release, which was attenuated by pretreatment of these cells with mannitol 30 min before the addition of LPS (Fig. 4A). LPS also produced a substantial increase in NO production in porcine aortic smooth muscle cells. This response was significantly reduced by mannitol (Fig. 4B). Induction of hypertonic stress using NaCl (50 mM) in the culture medium was also able to suppress LPS-induced NO release, suggesting that this might be a common property of hypertonic solutions (data not shown).

Mannitol Suppresses LPS-Induced iNOS Expression by Inhibiting NF- κ B.

To determine the mechanism(s) underlying inhibition of LPS-induced expression and activity of iNOS, the effect of mannitol on transcription factors known to regulate the level of this protein was determined. In this regard, the principal focus was on NF- κ B, which has been extensively studied concerning the induction of iNOS (Nishiyama et al., 2000). DDT₁MF-2 cells were pretreated with mannitol (50 mM), followed by addition of either LPS (10 μ g/ml), TNF- α (20 ng/ml), or IL-1 β (20 ng/ml) for the indicated periods. Next, nuclear fractions were isolated and used to perform EMSAs. Incubation of DDT₁MF-2 cells with TNF- α or IL-1 β substantially increased the activity of NF- κ B, as detected by a time-dependent increase in its binding to the radiolabeled oligonucleotide that contained *cis*-acting NF- κ B responsive element (Fig. 5A). Pretreatment of cells with mannitol (50 mM) for 30 min resulted in a dramatic reduction in the activation of NF- κ B by TNF- α and IL-1 β at all time points tested, indicating that NF- κ B could be the target of action of mannitol. Pretreatment with mannitol and NaCl also inhibited LPS-induced NF- κ B activation in DDT₁MF-2 cells (Fig. 5B). Supershift assays were performed using selective antibodies for NF- κ B subunits to identify the subunit composition of the NF- κ B complex. Supershifted bands were observed in samples pretreated with antibodies against p50, p65, and c-Rel, indicating that the NF- κ B complex was composed predominantly of these proteins. There was no differential effect of mannitol or NaCl on the particular composition of NF- κ B dimers. The antibody against p52 seemed ineffective in inducing supershift, suggesting that p52 is not involved in the activation of NF- κ B by LPS in these cells (Fig. 5C). Finally, we observed a similar pattern of NF- κ B activity in porcine aortic smooth muscle cells treated with LPS, in presence of mannitol or NaCl. As shown in Fig. 5D, the

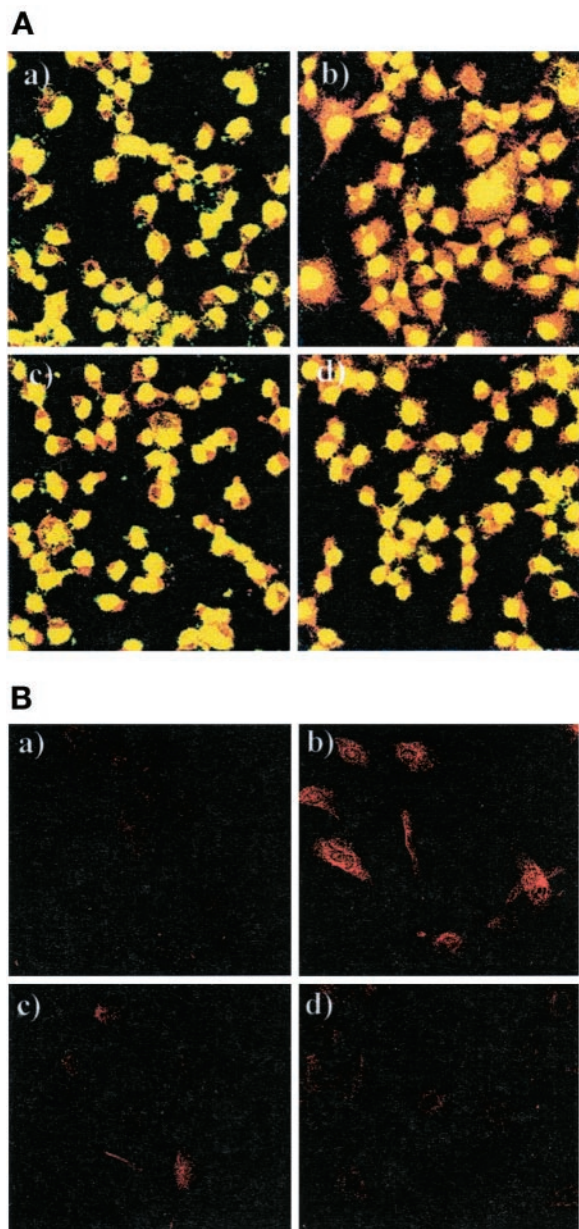


Fig. 3. Mannitol inhibits iNOS expression, as determined by immunocytochemistry. Cells were pretreated with mannitol (50 mM), followed by the addition of LPS (10 μ g/ml) for 24 h. Immunocytochemistry was performed using a polyclonal antibody against iNOS as the primary antibody and a rhodamine-tagged secondary antibody. Nuclear staining was performed using Sytox and was visualized as bright yellow fluorescence. Visualization of the labeled proteins (red) was performed using confocal microscopy with a 200-fold magnification. A, shown are control (a), LPS (b), mannitol (c), and mannitol + LPS (d). B, porcine aortic smooth muscle cells with identical treatments and labels as in A. No Sytox staining was performed in these experiments.

addition of either mannitol or NaCl resulted in a dose-dependent inhibition of NF- κ B activation. Interestingly, the effect of hypertonicity seemed specific to NF- κ B activation, because preincubation of DDT₁MF-2 cells with mannitol or NaCl had no effect on AP-1 or Oct-1 transcription factors in cells exposed to 10 μ g/ml LPS, 20 ng/ml TNF- α , or 20 ng/ml IL-1 β (data not shown).

Our hypothesis that NF κ B is a potential target for mannitol was confirmed by Western blotting studies focusing on the degradation of the inhibitory I κ B- α complex. In DDT₁MF-2 cells, treatment with TNF- α or IL-1 β increased the degradation of cytosolic I κ B- α in a time-dependent manner, with maximum loss in protein levels observed 30 min after exposure to these cytokines. The level of I κ B- α quickly recovered

by 60 min, probably because of de novo protein synthesis (Sun et al., 1993). Pre-exposure of cells to mannitol (50 mM) attenuated this response to cytokines, leading to stabilization of I κ B- α (Fig. 6). Because phosphorylation of I κ B- α by IKK is a prerequisite for its proteasomal degradation, these results suggested that the site of action of mannitol is either upstream of or at the level of I κ B- α phosphorylation or the step leading to proteasomal degradation of this protein.

Analogous experiments were performed in HeLa cells to more specifically localize the site of action of mannitol. We decided to use these cells in the initial characterization of a potential effect of mannitol because these have been widely used in NF- κ B studies. As expected, exposure of HeLa cells to TNF- α (20 ng/ml) for 20 min resulted in a complete loss of I κ B- α protein in the cytosol, as determined by Western blotting analyses. This loss was attenuated in cells pretreated with either mannitol (200 mM) or NaCl (200 mM) for 2 h (Fig. 7A, top). These higher concentrations of mannitol and NaCl were required because we observed that HeLa cells were less sensitive to hypertonicity compared with DDT₁MF-2 smooth muscle cells or porcine aortic smooth muscle cells. Treatment of cells with the proteasome inhibitor MG-132 (Lee and Goldberg, 1996) resulted in stabilization of I κ B- α in its phosphorylated state, which was detected as a slowly migrating band, running just above the native protein. This band showed immunoreactivity with antiserum against I κ B- α phosphorylated at serine 32 (Fig. 7A, bottom). In the presence of mannitol or NaCl, the stabilized phosphorylated I κ B- α band disappeared, suggesting a direct role of hypertonicity in inhibiting phosphorylation of I κ B- α (serine 32). Similar responses were observed in other cell lines tested, which include DDT₁MF-2 cells (Fig. 7B, top and bottom) and primary cultures of porcine aortic smooth muscle cells (Fig. 7C) when these cells were incubated with LPS (10 μ g/ml). The concentrations of mannitol and NaCl used in these latter experiments were 50 mM each.

Suppression of I κ B- α degradation by MG-132 was reflected functionally as suppression of LPS-induced DNA binding activity of NF- κ B in DDT₁MF-2 cells (Fig. 7D). The addition of mannitol resulted in further suppression of DNA binding activity over that observed with MG-132 pretreatment, indicative of an additive effect, which suggests that these two agents inhibit NF- κ B activity at different steps in its activation cascade.

Overexpression of NF- κ B subunit RelA leads to the formation of active homodimers, which tend to override the inhibitory effects of endogenous I κ B- α . According to our hypothesis that the action of mannitol is mediated through inhibition of I κ B- α phosphorylation, we reasoned that overexpression of RelA would prevent the inhibitory effect of mannitol. For these experiments, DDT₁MF2 cells were infected with recombinant adenovirus vector that bicistronically expresses GFP and RelA (full-length RelA or mutant RelA₁₋₃₀₀). As shown in Fig. 8, cells overexpressing RelA_{FL}, but not the transcriptionally inactive mutant RelA₁₋₃₀₀, showed enhanced expression of iNOS. LPS was able to produce a small increase in iNOS expression in cells infected with viral vector containing full-length RelA, indicative of its activation of endogenous NF- κ B. Interestingly, exposure of cells to mannitol failed to block the induction of iNOS via RelA_{FL} (Fig. 8), indicating that the events downstream to the activation of NF- κ B are not probably sensitive to mannitol. To validate our argument, analo-

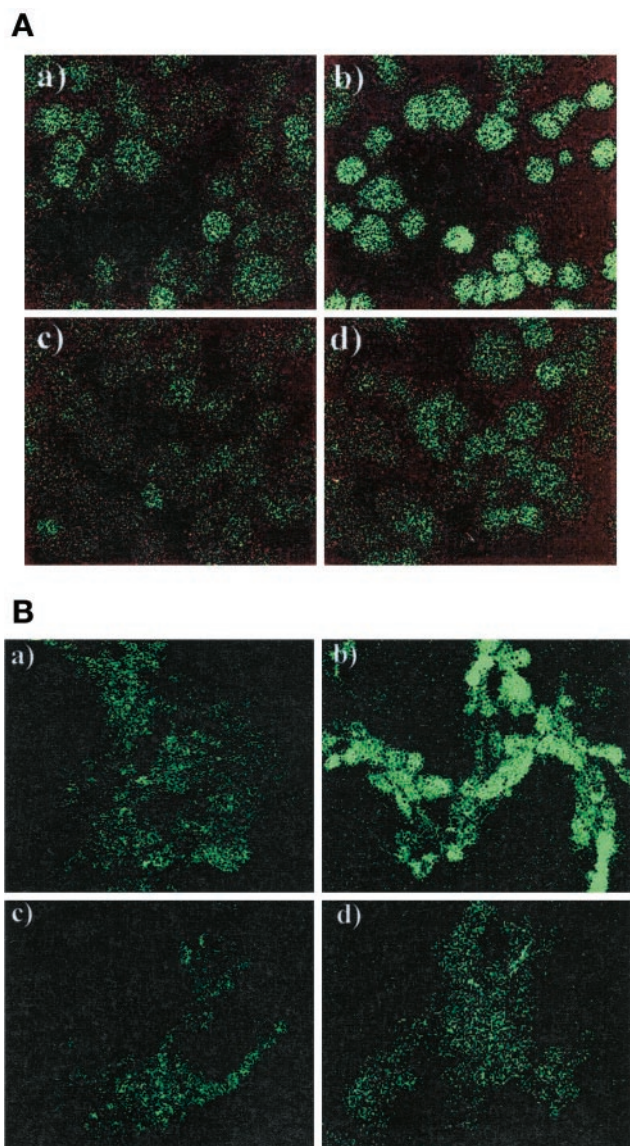


Fig. 4. Mannitol inhibits LPS-induced nitric oxide production. Cells were treated with either vehicle or mannitol (50 mM) for 30 min, followed by the addition of vehicle or LPS for 24 h. NO release was measured by confocal microscopy using the indicator DAF-2 DA (1 μ M), with wavelength setting of 488 nm. A, results obtained using DDT₁MF-2 cells. The represent control (a), LPS (b), mannitol (c), and mannitol + LPS (d). B, a representative experiment obtained using primary cultures of aortic smooth muscle cells. These experiments were repeated three times. The are labeled as in A.

gous treatments were performed followed by NF- κ B DNA binding assays. Significant DNA binding activity of NF- κ B was observed in RelA expressing untreated DDT₁MF2 cells, which was not affected by treatment with mannitol, whereas mannitol was able to inhibit basal NF- κ B DNA binding activity in cells expressing irrelevant control GFP (data not shown). This latter finding is consistent with a role of hypertonicity in the inhibition of NF- κ B activation.

Hypertonicity Inhibits IKK Activity in Smooth Muscle Cells. To test the possibility that mannitol inhibits IKK activity, we used GST-tagged I κ B α to study activity of IKK. In cells treated with LPS, there was a significant (4.8-fold) increase in IKK activity that was inhibited by 25 and 50% by 50 and 100 mM mannitol, respectively (Fig. 9).

Discussion

LPS and inflammatory cytokines mediate their effects in vivo, in part through activation of NF- κ B (Xie et al., 1994). In the inactivated state, this transcription factor exists as a complex with the inhibitory protein I κ B (Karin and Ben-Neriah, 2000). Activation of NF- κ B by effectors such as inflammatory cytokines, oxidative stress, microbes, and viruses is preceded by phosphorylation and degradation of this inhibitory protein, freeing NF- κ B from its cytoplasmic localization and allowing entry into the nucleus (O'Connell et al., 1998; Hawiger et al., 1999; Karin and Ben-Neriah, 2000). Phosphorylation of I κ B is mediated by IKKs, which phos-

phorylate this protein on N-terminal serine residues (Karin and Ben-Neriah, 2000). The phosphorylated I κ B is then polyubiquitinated by a specific ubiquitin ligase and is then rapidly degraded via 26 S proteasomes (Yaron et al., 1998). In the nucleus, NF- κ B can regulate the transcription of a number of genes that possess the consensus κ B binding sequences in their promoters. An example of such a gene is *iNOS* (Xie et al., 1994; Nishiya et al., 2000).

NO serves an important second messenger role in cellular signal transduction processes. This second messenger, produced from a Ca²⁺-dependent nitric-oxide synthase, mediates endothelium-dependent relaxation of vascular smooth muscle (Waldman and Murad, 1988). NO released from activated macrophages mediates its cytostatic action (Bogdan et al., 2000). Moreover, NO plays an important role as a signaling molecule produced after activation of neuronal NMDA receptor (Doyle et al., 1996; Ayata et al., 1997). The induction of *iNOS* after exposure of vascular smooth muscle to LPS, a major component of bacterial cell wall (Morrison and Ryan, 1987), is believed to underlie the profound vasodilation observed in septic shock (Rackow and Astiz, 1991; Titheradge, 1999). The importance of NF- κ B in the induction of *iNOS* gene suggests that selective inhibitors of this transcription factor may prove beneficial in inhibiting LPS-mediated induction of *iNOS* and thereby reduce the vasodilation that accompanies septic shock. Various inhibitors of NF- κ B have been proposed in this regard. For example, treatment of

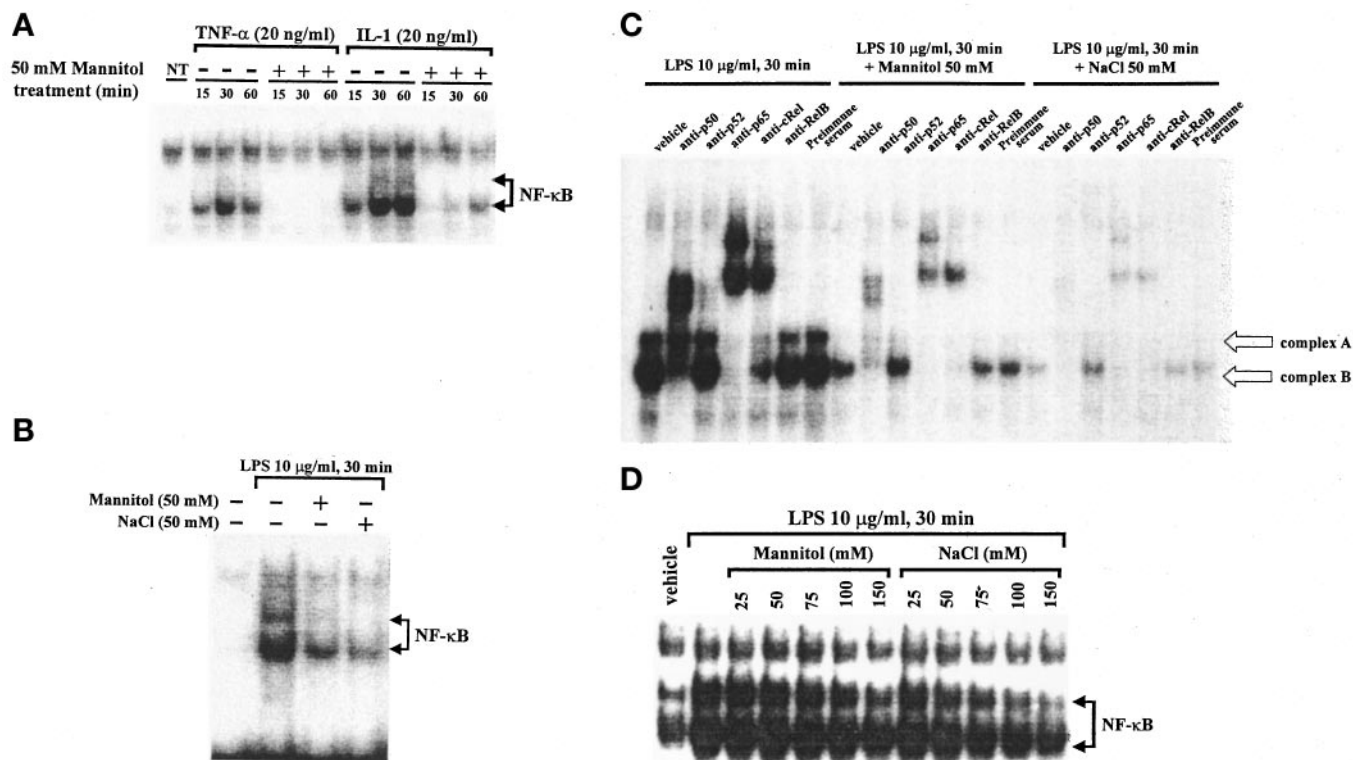


Fig. 5. Mannitol blocks the activation of NF- κ B by inflammatory cytokines and LPS. **A**, DDT₁MF-2 cells were pretreated with either vehicle or mannitol for 30 min, followed by the addition of TNF- α (20 ng/ml) or IL-1 β (20 ng/ml) for different time periods, as indicated. Nuclear extracts were prepared for EMSAs using a ³²P-labeled consensus oligonucleotide sequence for NF- κ B. **B**, suppression of LPS-induced NF- κ B activation by mannitol and NaCl. DDT₁MF-2 cells were pretreated with mannitol or NaCl, followed by the addition of LPS (10 μ g/ml) for 30 min. **C**, supershift assays were performed on extracts from LPS-stimulated DDT₁MF-2 cells, using specific antibodies against different subunits of NF- κ B and the composition of the complex includes p50, p65, and cRel. Complex A denotes p65/p65 homodimers or p65/cRel heterodimers whereas complex B denotes p65/p50 heterodimer or p50/cRel heterodimer. Neither mannitol nor NaCl affected the composition of the complex. **D**, EMSA was performed to measure DNA-binding activity of NF- κ B in porcine aortic smooth muscle cells pretreated with either mannitol or NaCl, followed by LPS.

endothelial cells with anisodamine, an inhibitor of NF- κ B, reduced LPS-mediated induction of plasminogen activator inhibitor-1 and tissue factor, markers of endothelial cell activation (Ruan et al., 2001). Direct inhibition of I κ B degradation by pyrrolidine dithiocarbamate reduced microvascular injury produced by LPS in rats (Liu et al., 1999). Furthermore, inhibition of I κ B degradation by calpain inhibitor I protected against endotoxin-mediated shock in rats (Ruetten and Thiernemann, 1997). Our study clearly indicates that hypertonicity reduced the activation of NF- κ B and thereby inhibited the induction of iNOS. Specifically, we show that hypertonicity inhibited the initial step in the activation of this transcription factor, the phosphorylation of I κ B- α , preventing the nuclear localization of NF- κ B. This conclusion is supported by several pieces of evidence. First, LPS failed to induce degradation of cytosolic I κ B- α in the presence of mannitol or NaCl, in sharp contrast to the substantial loss of I κ B- α observed during the normal course of LPS stimulation. Because phosphorylation of I κ B- α by IKK is a prerequisite for proteosomal degradation, a likely explanation for the action of mannitol is suppression of IKK activity. In addition, inhibition of the ubiquitin-proteasome pathway by MG-132, allowed for quantitation of the levels of phosphorylated I κ B- α produced. In cells pretreated with MG-132,

there was accumulation of phosphorylated I κ B- α in response to LPS. However, treatment with mannitol or NaCl along with LPS resulted in substantial reductions in the level of phosphorylated I κ B- α . Furthermore, the action of mannitol was abrogated in cells overexpressing the RelA protein. Because overexpression of RelA would probably overwhelm the endogenous inhibitory action of I κ B- α , the resulting induction of iNOS expression should seem unresponsive to the inhibition by mannitol if this represents the site of action of mannitol. Based on these observations, we conclude that the inhibitory effect of mannitol is mediated at the level of NF- κ B activation, probably at the level of IKK. Additional data from our laboratory demonstrated that mannitol inhibited LPS-stimulated IKK activity, which would explain its inhibitory action on downstream events. The inability of mannitol to produce full inhibition of IKK suggests that other factors may also contribute to the overall inhibition of iNOS in the intact cell and/or factors normally associated with IKK in the intact cells were lost during immunoprecipitation of the kinase.

Other studies have shown that the activation of NF- κ B can be modulated by different experimental conditions and by drugs. For example, heat stress attenuates NF- κ B activity by inhibiting I κ B- α phosphorylation (Curry et al., 1999; Shanley et al., 2000). The flavonoid resveratrol (Holmes-McNary and Baldwin, 2000), arsenic (Roussel and Barchowsky, 2000; Hershko et al., 2002), and the human papillomavirus oncoprotein E7 (Spitkovsky et al., 2002) stabilize I κ B- α by inhibiting IKK activity. The synthetic glucocorticoid dexamethasone inhibits TNF- α - and IL-1-induced nuclear translocation of NF- κ B by inducing transcription of the inhibitory protein I κ B- α (Scheinman et al., 1995). Furthermore, the anti-inflammatory agent sodium salicylate inhibits activation of NF- κ B by preventing degradation of I κ B (Kopp and Ghosh, 1994).

The concentrations of mannitol, which inhibit NF- κ B activation, are attained in the plasma after intravenous admin-

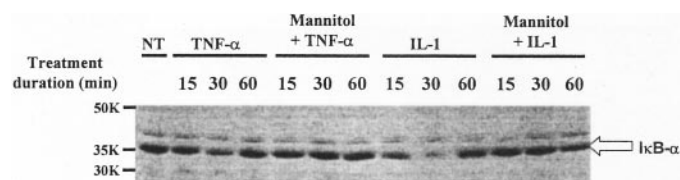


Fig. 6. Mannitol inhibits degradation of I κ B- α . DDT₁MF-2 cells were pretreated with either vehicle or mannitol (50 mM) for 30 min, followed by the addition of TNF- α (20 ng/ml) or IL-1 (20 ng/ml) for the indicated time periods. Cells lysates were prepared and used in Western blot studies to determine the levels of I κ B- α . Molecular mass markers (kilodaltons) are shown on the left side. NT indicates no treatment. Results indicate a representative experiment, which was repeated three times.

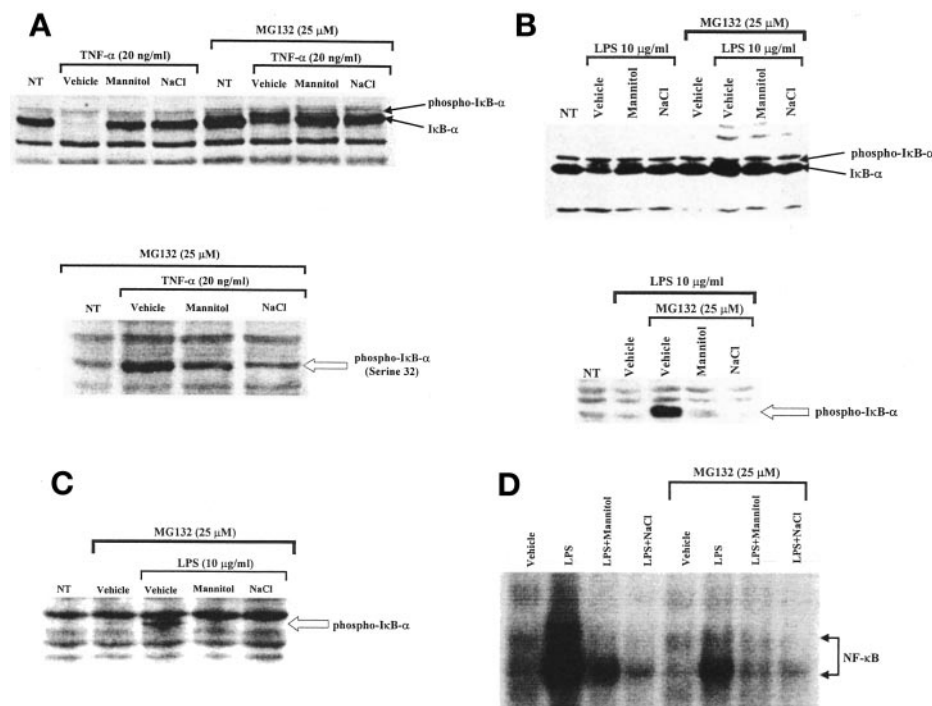


Fig. 7. Mannitol inhibits LPS-induced phosphorylation of I κ B- α . A, HeLa cells were pretreated with vehicle, mannitol (200 mM), or NaCl (200 mM), followed by TNF- α (20 ng/ml) for 30 min. To detect phosphorylated I κ B- α , cells were pretreated with MG-132 (25 μ M), a proteasome inhibitor. Top, the cytosolic fractions from these different treatments were examined for I κ B- α expression by Western blotting. Phosphorylated I κ B- α was detected as a slowly migrating band, running just above the native protein, as indicated. Bottom, the phosphorylated I κ B- α protein was detected using a phosphospecific antibody. Treatment of DDT₁MF-2 cells (B) or porcine aortic smooth muscle cells (C) with LPS yielded results similar to those in A. Note that the concentrations of mannitol and NaCl used in B and C were each 50 mM. D, DDT₁MF-2 cells were pretreated with mannitol (50 mM) and/or MG-132 (25 μ M) for 30 min, followed by the addition of LPS (10 μ g/ml) for 30 min. Nuclear fractions were obtained and used in EMSAs. NT, no treatment.

istration of mannitol. In treating renal failure and head trauma, mannitol is normally administered by infusion of a 20% solution to attain a concentration of 0.5 to 2 g/kg body weight, which results in a plasma concentration of 15 to 60 mM (Malek et al., 1998). Significant reductions in the activity of NF- κ B and iNOS expression were observed at 50 mM mannitol in smooth muscle cultures and it is likely that similar inhibition of iNOS gene expression could be achieved in vivo after administration of mannitol.

In summary, we have demonstrated that hypertonicity regulates iNOS expression and that this pathway involves inhibition of the NF- κ B transcription factor complex through

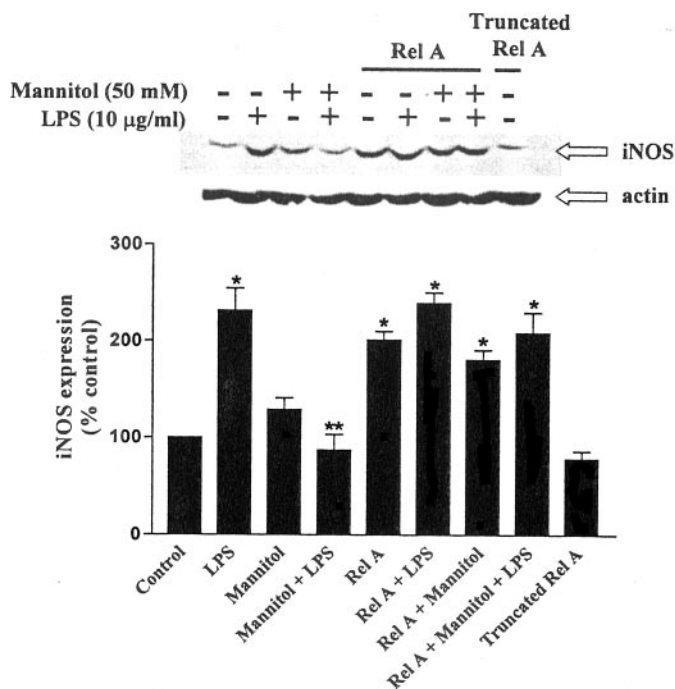


Fig. 8. Mannitol does not inhibit iNOS expression produced by overexpression of RelA. DDT₁MF-2 cells were infected with viral vectors containing a wild-type RelA (RelA_{FL}) or a truncated RelA (1–300) and incubated for 24 h to enable expression of the protein. Cells were then pretreated with either vehicle or mannitol, followed by either vehicle or LPS (10 μ g/ml) for 24 h and cytosolic fractions were used to determine iNOS expression by Western blotting. The band intensities were normalized to that of α -actin and plotted as a percentage of control (no mannitol, no LPS treatment). Results are presented as the mean \pm S.E. of three independent experiments. *, $p < 0.05$, statistically significant difference from control cells. **, $p < 0.05$, statistically significant difference from LPS-treated cells.

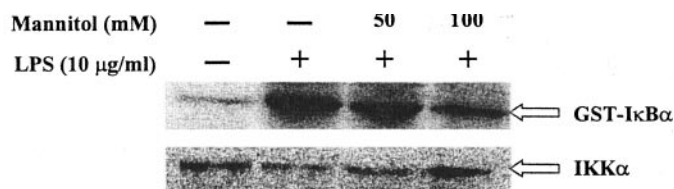


Fig. 9. Mannitol inhibits IkB phosphorylation by inhibiting activity of IKK. DDT₁MF2 cells were pretreated with mannitol at the indicated concentrations for 30 min, followed by the addition of LPS (10 μ g/ml) for 15 min. IKK α was immunoprecipitated from whole-cell lysates using anti-IKK α and protein A agarose beads. IKK activity was measured using GST-IkBa as an exogenous substrate and 32 P- γ -ATP in kinase buffer. Incorporation of 32 P into IkBa was analyzed by performing SDS-PAGE and autoradiography. Total IKK α was estimated by Western blotting and used to normalize IKK α activity. The data are a representative of two independent experiments showing similar results.

inhibition of IKK activity and reduction in IkB phosphorylation and degradation. Because iNOS plays an important role in manifestation of inflammatory processes, in addition to septic shock, these data suggest that mannitol may also be beneficial against these inflammatory processes. Furthermore, we propose that hypertonic solutions may be useful in suppressing the expression of other NF- κ B-dependent mediators of the inflammatory response, such as TNF- α , IL-1, and COX-2. This inhibitory effect of hypertonic solutions is selective to cells of the vasculature and is not observed in cells of the renal or hepatic systems (Hao et al., 2000; Michalke et al., 2000). Hence, the use of hypertonic agents may be particularly beneficial in septic shock, where it would selectively counter the effect of LPS and cytokines on the vascular smooth muscles without reducing renal or hepatic blood flow.

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Address correspondence to: Dr. Vickram Ramkumar, SIU School of Medicine, Box 19230, Springfield, IL 62974-1222. E-mail: vramkumar@siumed.edu
