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p38 Mitogen-Activated Protein Kinase Mediates Synergistic Induction of Inducible Nitric-Oxide Synthase by Lipopolysaccharide and Interferon-γ through Signal Transducer and Activator of Transcription 1 Ser727 Phosphorylation in Murine Aortic Endothelial Cells

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ABSTRACT

Nitric oxide (NO) can be produced in large amounts by up-regulation of inducible NO synthase (iNOS). iNOS is induced in many cell types by pro-inflammatory agents, such as bacterial lipopolysaccharide (LPS) and cytokines. Overproduction by endothelial cells (EC) may contribute to vascular diseases. In contrast to macrophages, murine aortic endothelial cells (MAEC) produced no NO in response to either LPS or interferon γ (IFNγ), whereas combined treatment was highly synergistic. In this study, we investigated the mechanisms of synergy in MAEC. LPS activated p38 mitogen-activated protein kinase (MAPK), whereas IFNγ activated Janus kinase and signal transducer and activator of transcription-1 (STAT1). Both pathways were required for iNOS induction because herbimycin A, a tyrosine kinase inhibitor, and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole · HCl (SB202190), a p38 MAPKα/β inhibitor, each blocked induction. LPS increased the phosphorylation of STAT1α at serine 727 in IFNγ-treated MAEC. SB202190, but not 2′-amino-3′-methoxyflavone (PD98059), an inhibitor of p44/p42 MAPK activation, abolished the phosphorylation and induction of iNOS. SB202190 did not affect tyrosine 701 phosphorylation or nuclear translocation of STAT1. However, STAT1-DNA binding activity was reduced by SB202190. Although LPS stimulated the DNA binding activity of nuclear factor κB and activating protein-1, combined treatment with IFNγ did not enhance activation, and SB202190 did not inhibit it. The results indicate that p38 MAPKα and/or β are required for the synergistic induction of iNOS by LPS and IFNγ in MAEC. Furthermore, the synergistic induction is associated with phosphorylation of STAT1α serine 727 in MAEC. This observation may explain potentially beneficial effects of p38 MAPK inhibitors in vascular inflammatory diseases.

Nitric oxide (NO) participates in a number of important biological functions, including vasodilation. Its overproduction, however, may contribute to the pathophysiology of inflammatory diseases. Large amounts of NO are produced from L-arginine by inducible NO synthase (iNOS), which is expressed in many cell types treated with inflammatory cytokines and bacterial products, such as lipopolysaccharide (LPS) (MacMicking et al., 1997). A previous study using a murine model of endotoxic shock showed that LPS-induced iNOS expression occurred mainly around blood vessels (Morikawa et al., 1999), suggesting that vascular endothelial cells play a critical role in LPS-induced NO production, and perhaps tissue injury.

The expression of iNOS can be induced by transcriptional activation (Xie et al., 1993). The murine iNOS promoter contains consensus sequences for the binding of nuclear factor-κB (NFκB) and activating protein-1 (AP-1), which can be activated by LPS. The promoter also has binding sites for the IFNγ activated factors, interferon regulatory factor-1, and signal transducer and activator of transcription-1 (STAT1) (Xie et al., 1993). We recently demonstrated that LPS was

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ABBREVIATIONS: NO, nitric oxide; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; NFκB, nuclear factor-κB; AP-1, activating protein-1; STAT1, signal transducer and activator of transcription-1; MAEC, murine aortic endothelial cells; IFNγ, interferon-γ; MAPK, mitogen-activated protein kinase; GAS, γ-activating sequences; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole · HCl; KN93, 2-[*N*-(2-hydroxyethyl)]-*N*-4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine); H89, *N*-[2-(4-bromocinnamylamino) ethyl]-5-isoquinoline; PCR, polymerase chain reaction; PD98059, 2′-amino-3′-methoxyflavone.

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unable to induce NO production by cultured murine aortic endothelial cells (MAEC). However, the expression of iNOS was greatly induced when LPS was combined with the T cell cytokine interferon-γ (IFNγ) (Huang et al., 2003). This requirement for LPS and IFNγ differs from inflammatory cells, such as macrophages, where either LPS or IFNγ alone were sufficient for induction (Hammermann et al., 2000). Varied regulation of iNOS expression may be caused by different regulation of transcription factors in different cell types.

LPS, a component of Gram-negative bacterial cell walls, acts through Toll-like receptors to activate NFκB and AP-1. LPS also activates mitogen-activated protein kinase (MAPK) family members that may participate in the activation of transcription factors (Hwang et al., 1997). At least three MAPK cascades are well described: extracellular signal-regulated kinase (p44/p42), p38, and Jun N-terminal kinase/stress-activated protein kinase (Kyriakis and Avruch, 1996). Recent data indicate that inhibitors of these various MAPKs can inhibit inflammation (Jackson et al., 1998), demonstrating their physiological and pathological significance. p38 MAPK is particularly responsive to LPS (Han et al., 1994; Schumann et al., 1996). Activation of p38 is strictly dependent on dual phosphorylation of Thr180 and Tyr182 (Raingeaud et al., 1995).

The signaling pathway in macrophages for iNOS induction by IFNγ is well elucidated. IFNγ activates Janus kinase, causing the rapid phosphorylation of Tyr701 of the latent cytoplasmic transcription factor STAT1. Phospho-Tyr701 STAT1 forms homodimers that translocate to the nucleus and bind to γ-activating sequences (GAS) in IFNγ-responsive promoters. One form of STAT1, STAT1α, contains a second phosphorylation site, Ser727, in its carboxyl-terminal domain. An alternatively spliced version of STAT1, STAT1β, lacks 38 carboxyl-terminal residues, including Ser727. Only STAT1α is able to activate transcription of IFNγ-responsive genes, indicating that the carboxyl terminus of STAT1α mediates transactivation (Horvath and Darnell, 1996). Furthermore, the phosphorylation of Ser727 in the carboxyl terminal domain is critical for the full activity of STAT1α, because its mutation to alanine is inhibitory (Wen et al., 1995; Wen and Darnell, 1997).

The mechanistic basis for STAT1 Tyr701 phosphorylation by JAK1/2 has been established for some time (Stark et al., 1998). However, the mechanisms through which STAT1α is phosphorylated on Ser727 are more diverse than Tyr701 phosphorylation and are not yet well understood in endothelial cells. In different cell types, different serine kinases can phosphorylate Ser727. These include phosphatidylinositol 3-kinase/Akt (Nguyen et al., 2001), Ca^{2+}/calmodulin-dependent kinase II (Nair et al., 2002), and protein kinase Cδ (Deb et al., 2003). Ser727 is located in the carboxyl terminus of STAT1α in the sequence PMS^{727}P, which fits the consensus motif for the proline-directed MAPKs [P*X*n(S/T)P, where S/T is serine or threonine and n is 1 or 2] (Decker and Kovarik, 2000). p38 has been implicated in LPS- and UV light-induced STAT1α Ser727 phosphorylation in macrophages (Kovarik et al., 1998, 1999) and in HeLa cells treated with type I IFNα or IFNγ (Goh et al., 1999). In contrast, inhibition of p38 did not reduce Ser727 phosphorylation in IFNα-treated leukemia cell lines (Uddin et al., 2000). Thus, the particular kinase that phosphorylates Ser727 of STAT1α seems to vary with the stimulus and cell type.

The mechanisms regulating the phosphorylation of STAT1α on Ser727 in LPS and IFNγ-treated endothelial cells have not been elucidated. In this study, we hypothesized that p38 activation by LPS would lead to phosphorylation of STAT1α Ser727 and allow for the synergistic effect of LPS and IFNγ on iNOS gene expression in MAEC.

Materials and Methods

Reagents and Chemicals. Endothelial cell growth supplement, heparin, bovine serum albumin, sodium nitrite, *Escherichia coli* LPS, serotype 0111:B4, herbimycin A, fludarabine, actinomycin D, cycloheximide, sulfanilamide, naphthalene-ethylenediamine dihydrochloride, phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and Bradford reagent were from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IFNγ was from R&D systems (Minneapolis, MN). SB202190, a p38 inhibitor; KN93, a Ca^{2+}/calmodulin kinase II inhibitor; bisindolylmaleimide 1, a protein kinase C inhibitor; H89, a protein kinase A inhibitor, and a specific peptide inhibitor of protein kinase G (RKRARKE) were from Calbiochem (San Diego, CA). PD98059 was from New England Biolabs Inc. (Beverly, MA). Triton X-100 was from Pierce (Rockford, IL). Tris-base, sodium chloride, EDTA, EGTA, sodium orthovanadate, sodium fluoride, Tween 20, agarose, and ethidium bromide were from Fisher Scientific (Fair Lawn, NJ). Aprotinin, leupeptin, and pepstatin A were from Roche Molecular Biochemicals (Indianapolis, IN). Tris-glycine gels, TRIzol reagent, and cDNA cycle kit were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone (Logan, UT). Chemiluminescence reagents were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). BD Mercury TransFactor Kit for measuring STAT1-GAS binding was from BD Biosciences Clontech (Palo Alto, CA). TransAM NFκB p65/NFκB p50 and AP-1 c-Jun transcription factor assay kits were from Active Motif North America (Carlsbad, CA). Rabbit anti-iNOS polyclonal antibody was obtained from BD Transduction Laboratories (Lexington, KY). Rabbit antibody to unphosphorylated STAT1, anti-phospho-STAT1 (Tyr701), anti-p38, and anti-phospho-p38 (Thr180/Tyr182) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit anti-phospho-STAT1 (Ser727) antibody was from Upstate Biotechnologies, Inc. (Charlottesville, VA). Horseradish peroxidase-conjugated goat anti-mouse, goat anti-rat, goat anti-rabbit, and rabbit anti-goat secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Culture. MAEC were isolated from normal C57BL/6 mice as described before (Huang et al., 2003). The cells were cultured in medium containing 40% Dulbecco's modified Eagle's medium, 40% Ham's F-12 medium, 20% fetal bovine serum (heat-inactivated, 30 min at 56°C), 30 μg of endothelial cell growth supplement/ml, 10 U/ml heparin, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Treatment of Cells. MAEC were stimulated with 0 to 10 μg/ml LPS for 0 to 24 h, with or without IFNγ (1–20 ng/ml). In the sequential experiment, MAEC were preincubated with either 10 μg/ml LPS or 20 ng/ml IFNγ for 0, 1, 2, and 4 h before a 6-, 10-, or 24-h cotreatment of the two drugs. In some experiments, the cells were coincubated with actinomycin D, cycloheximide, SB202190 (3.125–50 μM) or PD98059 (3.125–50 μM). In other experiments, MAEC were also pretreated for 30 min with KN93 (0–30 μM), bisindolylmaleimide 1 (0–25 μM), H89 (0–25 μM), or the protein kinase G inhibitor (0–500 μM), before the cotreatment (10 μg/ml LPS and 20 ng/ml IFNγ, 24 h).

Griess Reaction. The concentration of nitrite in cell culture supernatant, which reflects cumulative NO production, was measured by the Griess reaction (Finder et al., 1995). MAEC were treated in 96-well plates. Cultured supernatant (50 μl) was sampled and immediately mixed with 50 μl of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylenediamine dihydrochloride in 5% H_3PO_4). After incubation for 15 min at room temperature, the sam-

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ples were read at 550 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Solutions of sodium nitrite diluted in culture media served as the standard. A standard curve was made for every experiment carried out. The levels of nitrite were further normalized to the protein level in each sample. Protein was measured after washing cells three times with phosphate-buffered saline and extraction with 1% Triton X-100. The samples were centrifuged 3000 rpm for 10 min. Protein levels were then measured as described previously (Huang et al., 2003).

Cell Lysis and Nuclear Extract. After treatment with LPS and/or IFNγ, cells were washed with phosphate-buffered saline at room temperature and lysed in lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM Na_3VO_4, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. For nuclear and cytosol extracts, the cells were lysed in buffer containing 0.34 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.5, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Igepal. The extracts were centrifuged at 10,000*g* at 4°C for 10 min. The supernatant was collected as cytosol extract, and the pellet was resuspended in lysis buffer as nuclear extract.

Immunoblotting. Extracts were sonicated and protein concentrations were determined by the Bradford assay. Ten μg of protein from each sample were heated at 95°C for 10 min and separated on a 4 to 20% Tris-glycine gel. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. After transfer, the membranes were blocked with 5% nonfat milk in Tris-buffered saline buffer-Tween 20 (0.1% Tween 20, 10 mM Tris, pH 7.5, and 150 mM NaCl), and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. Then the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody. The blots were developed with an enhanced chemiluminescence system (PerkinElmer Life and Analytical Sciences). The chemiluminescent signals were captured on X-ray film, scanned and quantified by digital image analysis (ImageJ ver. 1.22d; http://rsb.info.nih.gov/ij/).

STAT1, NFκB, and AP-1 DNA Binding Assay. MAEC were treated with IFNγ (0–50 ng/ml, 0–24 h), with or without LPS (10 μg/ml), and nuclear extracts were prepared as mentioned above. The activation of STAT1 and its DNA binding activity was measured by an enzyme-linked immunoassay following the manufacturer's instructions. In brief, sample protein (50 μg/ml) was incubated for 1 h at room temperature in a 96-well plate precoated with a double-stranded GAS (5′-attcctgtaag-3′), NFκB (5′-gggactttcc-3′), or AP-1 (5′-tgagtca-3′) DNA binding elements. After washing three times with blocking buffer, mouse anti-STAT1, anti-NFκB, or anti-AP-1 antibody was added to the appropriate wells and incubated for another hour at room temperature. A horseradish peroxidase-conjugated anti-mouse antibody was added after washing out unbound primary antibody and incubated for 30 min. Then the wells were washed and tetramethyl benzidine was added to each well and incubated for 10 min for color development. The reaction was stopped by adding sodium azide and 1.0 M sulfuric acid to each well. The samples were mixed and the absorbance was read at 450 nm within 30 min using the microplate spectrophotometer.

RNA Extraction and Reverse Transcription and Polymerase Chain Reaction (PCR) for iNOS and β-Actin mRNA. MAEC were treated with 0, 12.5, or 50 μM SB202190 30 min before treatment with 10 μg of LPS and 20 ng of IFNγ. After 8 h, cells were rinsed with PBS and lysed with TRIzol reagent, and RNA was extracted and recovered by ethanol precipitation. RNA was dissolved in water and its concentration was determined by absorbance of an aliquot at 260 nm. Three micrograms of RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase and deoxynucleotides at 42°C for 1 h. Five percentage of the cDNA was used for PCR. The following primers were used: iNOS, 5′-CCT GGA CAA GCT GCA TGT GA-3′ and 5′-GCT GTG TGG TGG TCC ATG AT-3′, to produce an 1104-bp product, designed from GenBank accession number NM_010927; β-actin, 5′-ATG GAT GAC GAT ATC GCT-3′ and 5′-ATG AGG TAG TCT GTC AGG T-3′ to produce a 538-bp product, designed from GenBank accession number X03672. PCR was performed for 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for each primer set. Fifty percent of the reaction was subjected to agarose electrophoresis and ethidium bromide staining. Gels were digitally photographed and signal intensities were quantified by image analysis (ImageJ). The ratio of iNOS signal to β-actin for each sample was calculated.

Statistical Testing. All data were evaluated by analysis of variance, with Bonferroni correction for multiple comparisons (Snedecor and Cochran, 1980). A p value less than 0.05 was considered significant.

Results

NO Production in IFNγ and LPS-Treated MAEC. We found previously that LPS or IFNγ alone had no effect on MAEC iNOS or NO production, measured as nitrite. However, their simultaneous combination synergistically induced iNOS in a time- and concentration-dependent manner (Huang et al., 2003). In this study, we found that pretreatment with actinomycin D (1 μg/ml) or cycloheximide (30 μg/ml) inhibited the induced accumulation of nitrite, indicating that RNA and protein synthesis are required for the induction (Fig. 1A).

We found previously that significant increases in iNOS protein and NO production occurred by 8 to 10 h after cotreatment with LPS and IFNγ, which progressively increased over 24 h (Huang et al., 2003). In this study, we further determined the effect of the order of addition of LPS or IFNγ on NO production. MAEC were exposed to LPS or IFNγ for 0 to 4 h, at which time the other agent, IFNγ or LPS, respectively, was added for an additional 6, 10, or 24 h. Thus, the total incubation time for the first agent was 6, 10, or 24 h, plus the preincubation time, whereas that for the second agent was 6, 10, or 24 h. All MAEC that were pretreated for 0 to 4 h and then coincubated with both agents for 24 h produced similarly high levels of nitrite (averages of 5.28 to 6.06 nmol of nitrite/mg of protein were not significantly different from each other and were greater than the 0.21 ± 0.02 nmol/mg protein in untreated cells). This accumulation was far greater than the levels seen for the 6- or 10-h cotreatment groups shown in Fig. 1B. In addition, all cells allowed a 10-h cotreatment period accumulated higher levels of nitrite in medium than the corresponding 6-h groups. This is consistent with the progressive increase in nitrite noted previously (Huang et al., 2003).

In the 6-h cotreatment experiment, cells pretreated with IFNγ for 2 or 4 h accumulated more nitrite than those pretreated with LPS. With 10-h cotreatment, preincubation with IFNγ for 1 to 4 h induced more nitrite than LPS pretreatment. These results are consistent with an effect of LPS on pathways progressively activated, or primed, by IFNγ. When the order of addition was reversed, we found that 2-h pretreatment with LPS plus 6-h cotreatment and 4-h pretreatment plus 10-h cotreatment resulted in less nitrite than did no pretreatment. This may indicate slight tolerance or desensitization to LPS during pretreatment with this agent. Nevertheless, the mild inhibition was overcome within 10 to 24 h after addition of IFNγ, where large increases were observed. We next investigated the pathways involved in the synergistic effect of LPS and IFNγ in MAEC.

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IFNγ-Induced Activation of STAT1 in MAEC. The signal transduction pathway initiated by IFNγ for iNOS induction is well established in mouse macrophages and some other cell types. Less is known regarding endothelial cells. As in most other cell types, IFNγ activated the Janus kinase-STAT1 pathway in MAEC. IFNγ caused STAT1 Tyr701 phosphorylation within 5 min (Fig. 2A). Elevated phospho-Tyr701 STAT1 was maintained for 24 h. Tyrosine phosphorylated STAT1 accumulated in the nucleus over a similar time course, measured by immunoblotting (Fig. 2B) and immunocytochemistry (data not shown). IFNγ also activated STAT1 binding to a synthetic GAS DNA sequence (Fig. 2C). A unique phenomenon in this cell line was observed. In addition to STAT1 Tyr701 phosphorylation, unphosphorylated STAT1 protein levels progressively increased after treatment with IFNγ (Fig. 2, A and B). STAT1 Tyr701 phosphorylation was required for LPS/IFNγ-induced NO production because accumulation was blocked by both herbimycin A (Fig. 3A), a tyrosine kinase inhibitor, and fludarabine (Fig. 3B), which causes a selective depletion of STAT1 (Frank et al., 1999).

LPS-Induced Phosphorylation of p38 in MAEC. LPS is known to activate p38. Activation is strictly dependent on phosphorylation (Raingeaud et al., 1995; Goh et al., 1999). A phosphospecific antibody was used to measure phosphorylated p38. Blots were re-probed with a nonphosphospecific antibody to demonstrate that equivalent amounts of p38 were present in each sample (Fig. 4). Treatment with 10 to 200 μg LPS/ml for 30 min significantly increased p38 phosphorylation to a level comparable with that induced by UV light. SB202190, a p38-specific inhibitor, significantly inhibited LPS-induced phosphorylation, and it lowered the basal level of phosphorylated p38 (Fig. 4A). Whereas 10 μg/ml LPS increased phosphorylated p38 within 30 min (3.4-fold compared with basal level), IFNγ alone failed to increase p38 phosphorylation (Fig. 4B). In addition, the combination of LPS and IFNγ did not further increase phosphorylation.

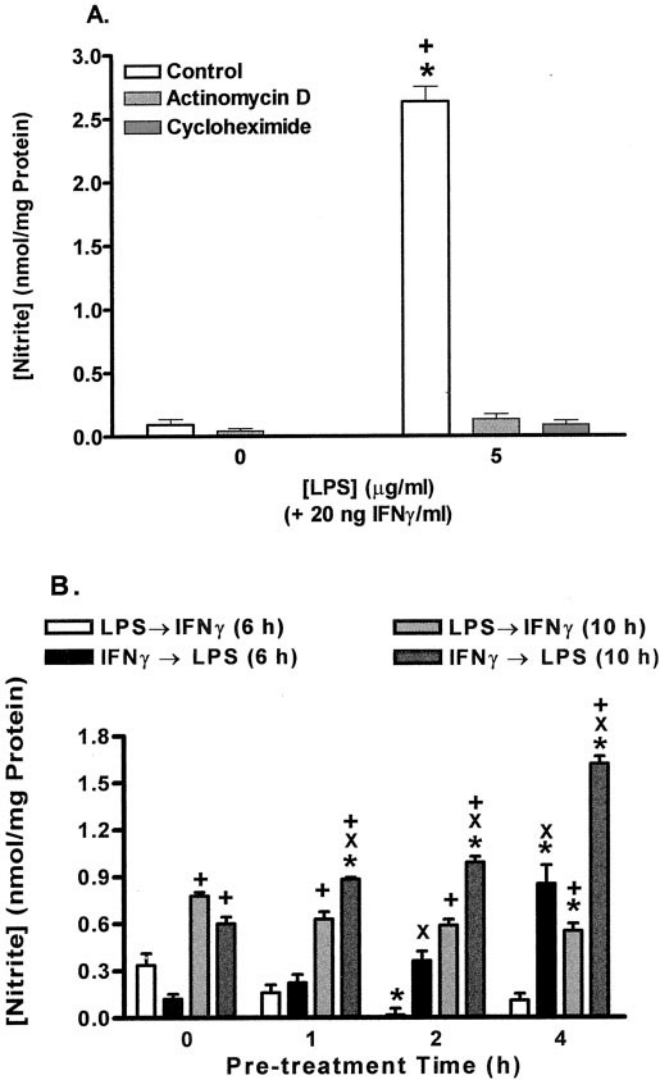

Fig. 1. Effect of sequential addition of LPS or IFNγ on NO production. A, MAEC were treated simultaneously with 0 or 5 μg/ml LPS plus 20 ng/ml IFNγ for 24 h. Actinomycin D (1 μg/ml) or cycloheximide (30 μg/ml) were added 30 min before LPS/IFNγ. *, $p < 0.001$ for comparison with cells incubated without LPS; +, $p < 0.001$ for comparison with actinomycin D and with cycloheximide-treated cells. B, mouse aortic endothelial cells (MAEC) were pretreated with either LPS (10 μg/ml, open bars) or IFNγ (20 ng/ml, solid bars) alone for 0 to 4 h. The cells were continuously treated with the combination of the two drugs for additional 6 or 10 h. Bars represent the mean + S.E. of six independent cultures per experimental group. Values for untreated cells incubated for 6 to 10 h were 0.0005 ± 0.0001 nmol of nitrite/mg of protein. *, $p < 0.05$ for comparison with 0 h pretreatment; x, $p < 0.05$ for comparison of between IFNγ-pretreated and LPS-pretreated groups; +, $p < 0.05$ for comparison between corresponding 6- and 10-h cotreatment groups.

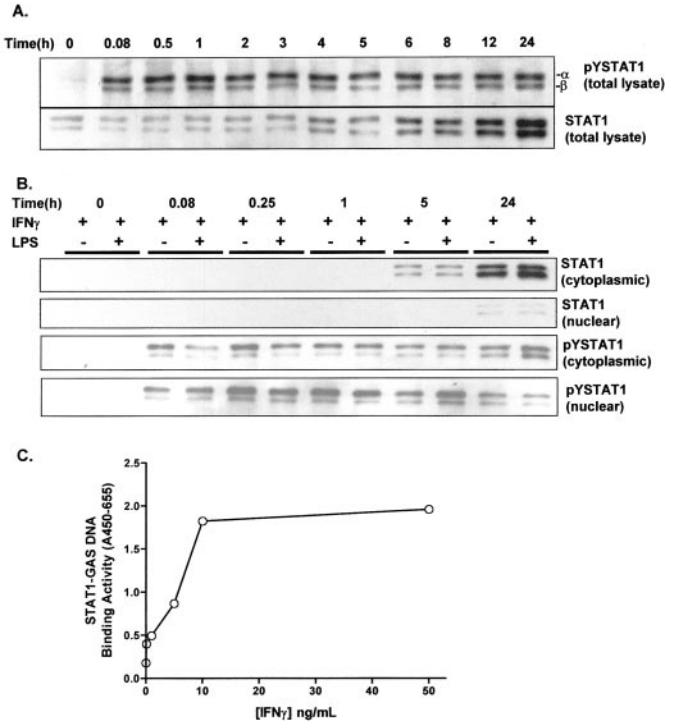

Fig. 2. Effect of IFNγ on tyrosine phosphorylation and nuclear translocation of STAT1. MAEC were treated with 20 ng/ml IFNγ for 0 to 24 h. Protein in total cellular extracts (A) and nuclear and cytoplasmic extracts (B) were subjected to Western blotting with phospho-Tyr701 STAT1-specific antibody (pYSTAT1), and with an antibody to STAT1 that is not sensitive to phosphorylation (STAT1). The upper band in each sample is STAT1α, and the lower band is STAT1β (α and β in A, for example). IFNγ caused rapid Tyr701 phosphorylation of STAT1, and a more delayed, time-dependent increase in unphosphorylated STAT1 protein levels. Tyrosine phosphorylated STAT1, but not unphosphorylated STAT1, appeared in the nucleus within 5 min (0.08 h) in response to IFNγ. LPS did not affect nuclear translocation of pYSTAT1. STAT1-GAS DNA binding activity was measured 0.5 h after treatment with 0 to 50 ng IFNγ/ml (C). The mean and S.E. for three independent cultures per treatment are depicted in the graph. STAT1-GAS binding activity was induced 15- to 20-fold by IFNγ, and it was concentration dependent.

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Requirement of p38 for iNOS Induction. To explore the role of p38 in LPS- and IFNγ-mediated iNOS induction, we treated MAEC with the p38 inhibitor SB202190 before stimulation with LPS and IFNγ. SB202190 inhibited NO production with an IC_{50} of 13.4 μM (Fig. 5A). This concentration is similar to the effective level in murine macrophages (Ajizian et al., 1999; Chen and Wang, 1999). The possibility that inhibition of NO production was caused by injury of MAEC was unlikely because there was no change in the morphology and growth rates of treated cells (data not shown). In contrast to macrophages (Chan and Riches, 2001), LPS/IFNγ-induced NO production in MAEC was not blocked by PD98059, an inhibitor of p42/44 MAPK activation, even at high concentrations (Fig. 5B).

Reverse transcription and PCR for 35 cycles failed to amplify iNOS mRNA in RNA samples from untreated MAEC, or from cells treated with either LPS or IFNγ alone (data not shown). In contrast, iNOS mRNA was readily detected within 8 h of simultaneous treatment with both agents (Fig. 6A). This result agrees with the sensitivity of nitrite accumulation to actinomycin D (Fig. 1A). As seen previously (Huang et al., 2003), untreated MAEC, or cells treated with either LPS or IFNγ alone, lacked iNOS protein (data not shown), whereas cotreatment with 10 μg of LPS and 20 ng of IFNγ/ml for 8 h greatly induced the enzyme (Fig. 6B). Pretreatment with 12.5 and 50 μM SB202190 reduced the relative level of iNOS mRNA by 42 and 70%, respectively (Fig. 6A). SB202190 at 12.5 and 50 μM reduced the levels of iNOS protein by 60 and 83%, respectively, after 8 h (Fig. 6B).

Requirement of p38 for LPS-Induced Ser727 Phosphorylation of STAT1α. Ser727 phosphorylation of STAT1α is required for IFNγ-mediated gene transactivation and antiviral activity (Wen et al., 1995; Horvath and Darnell, 1996). IFNγ alone, but not LPS, raised the level of phospho-Ser727 STAT1α. Combination of LPS and IFNγ doubled the level of phospho-Ser727 STAT1α (Fig. 7). We next examined whether p38 was required for serine phosphorylation. MAEC were treated with LPS and/or IFNγ, and SB202190 or PD98059 (50 μM, 30 min) and extracts were analyzed by blotting with a phospho-Ser727-specific antibody. SB202190 (Fig. 7, A and B), but not PD98059 (data not shown), greatly attenuated STAT1α serine phosphorylation in response to IFNγ alone and more dramatically in response to the combination of LPS and IFNγ. Taken together, these results indicate that p38 phosphorylation is required for the serine phosphorylation of STAT1α in MAEC, whereas p44/p42 MAPK is not required.

No Inhibitory Effects of Other Serine/Threonine Kinase Blockers on LPS/IFNγ-Induced NO Production in

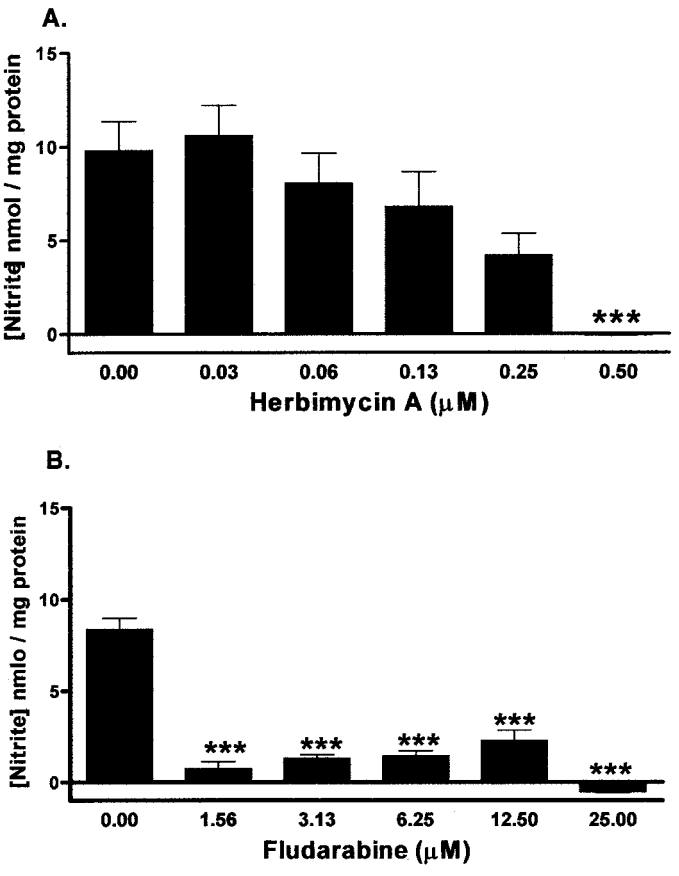

Fig. 3. Effect of herbimycin A and fludarabine on NO production. MAEC were preincubated with the indicated concentrations of the tyrosine kinase inhibitor, herbimycin A (A), or the nucleoside analog fludarabine, which selectively depletes STAT1 (B) for 60 min, and then treated with LPS (10 μg/ml) and IFNγ (20 ng/ml) for another 24 h. Both drugs inhibited NO production, as measured by the Griess reaction. Bars and symbols are as in Fig. 1.

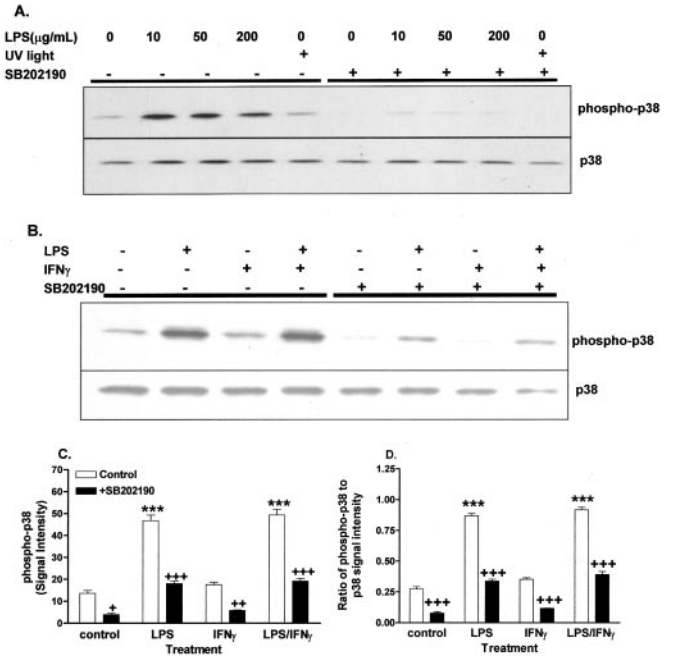

Fig. 4. Effect of LPS, IFNγ, UV light, and the p38 inhibitor SB202190 on p38 phosphorylation. MAEC were treated with LPS (0–200 μg/ml, 30 min) or UV light (30 min), with or without preincubation of SB202190, a p38 inhibitor, at 50 μM for 30 min (A), or LPS, IFNγ, and SB202190 (B). Proteins were extracted and Western-blotted with antibodies specific for phosphorylated p38 or for phosphorylated and unphosphorylated p38. Representative blots are shown in A and B. Densitometric analysis of signal intensities of images from three samples per group for phospho-p38 alone (C) and the ratio of phospho-p38 to total p38 (D) are shown. LPS treatment and UV light both induced p38 phosphorylation, without effect on total p38 levels. Phosphorylation was suppressed by SB202190 (A). IFNγ alone neither activated p38 nor potentiated the effect of LPS on p38 phosphorylation (B). Bars represent the mean + S.E. of three independent cultures per group. ***, $p < 0.001$ for comparison cells treated with medium alone or with LPS/IFNγ, in the absence of SB202190. +, $p < 0.05$; ++, $p < 0.01$; and +++, $p < 0.001$ for comparison between cells treated with LPS/IFNγ and SB202190 with cells treated with LPS/IFNγ only.

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MAEC. In other cell types and with different stimuli, several other protein kinases have been implicated in Ser727 phosphorylation. Several inhibitors were used to assess the contribution of these kinases in MAEC. Neither KN93 (a Ca^{2+}/calmodulin kinase II inhibitor), bisindolylmaleimide 1 (a protein kinase C inhibitor), H89 (a protein kinase A inhibitor), nor a specific peptide inhibitor of protein kinase G prevented LPS/IFNγ-induced NO production in MAEC (data not shown).

Requirement of p38-Sensitive STAT1α Ser727 Phosphorylation in STAT1-DNA Interactions. From the above data, p38 seems to facilitate iNOS gene expression in response to LPS plus IFNγ. We sought to determine the functional consequences of STAT1α serine phosphorylation. We examined whether inhibition of p38 has negative regulatory effects on 1) STAT1 protein level, 2) STAT1 tyrosine Tyr701 phosphorylation, 3) pY-STAT1 nuclear translocation, and 4) STAT1-GAS binding activity.

STAT1 protein levels, its Tyr701 phosphorylation, and pY-STAT1 nuclear translocation were measured by Western blotting. As expected, IFNγ greatly induced STAT1 Tyr701 phosphorylation. This effect was neither potentiated by LPS nor affected by inhibiting STAT1α Ser727 phosphorylation with SB202190 (Fig. 7, A, C, and D). Likewise, SB202190 did not block the nuclear translocation of pY-STAT1 (data not shown). LPS (2 h cotreatment), however, increased IFNγ-induced STAT1-GAS binding by approximately 2-fold (Fig. 8A). This in vitro DNA binding activation of STAT1 was significantly inhibited by SB202190 pretreatment (50 μM, 30 min) (Fig. 8B), suggesting that p38-sensitive Ser727 phosphorylation was important for STAT1α-GAS binding.

Effect of LPS, IFNγ, and SB202190 on NFκB and AP-1 Activation. The synergistic effect between LPS and IFNγ could result from the activation of multiple transcription factors that can bind to the iNOS promoter. LPS activates NFκB and AP-1 in many cell types (Deshpande et al., 1997), and NFκB is essential for the induction of iNOS in macrophages (Hammermann et al., 2000). We therefore investigated the role of NFκB and AP-1 in MAEC by measuring their DNA binding activity. The in vitro binding of MAEC NFκB and AP-1 to consensus DNA targets was increased 4- to 5-fold within 30 min of treatment with 10 μg/ml LPS (Fig. 9). The combination of LPS and IFNγ did not further increase this activation, and IFNγ alone had no effect on NFκB or AP-1 (data not shown). Furthermore, preincubation of MAEC

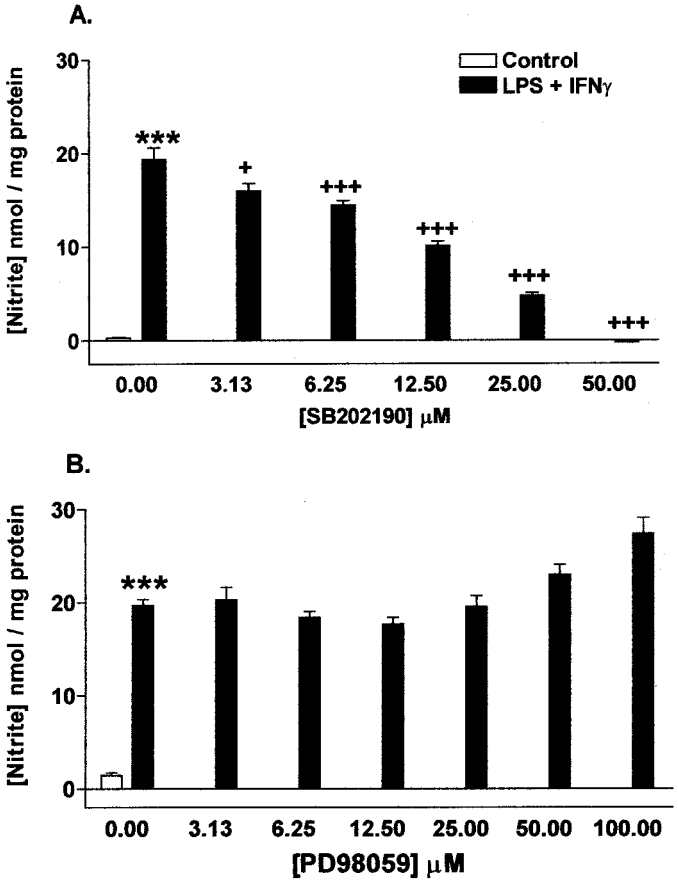

Fig. 5. Effect of the p38 MAPK inhibitor SB202190 and the inhibitor of p42/44 MAPK activation, PD98059, on NO production. MAEC were preincubated without or with SB202190 (A) or PD98059 (B) for 30 min, followed by treatment with 0 or 10 μg/ml LPS and 0 or 20 ng/ml IFNγ for 24 h. Nitrite, reflecting NO production, was measured by the Greiss reaction. Bars represent the mean + S.E. of six independent cultures per group. Symbols are as in Fig. 4. LPS and IFNγ again induced NO production, and SB202190 inhibited induction with an IC_{50} of 13.4 μM. PD98059 had no effect.

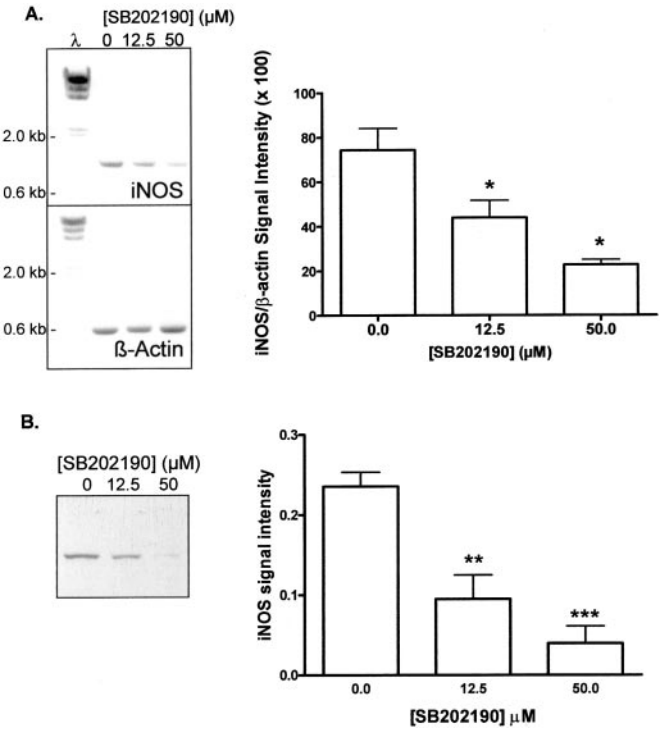

Fig. 6. Effect of SB202190 on iNOS mRNA and protein. A, MAEC were pretreated with 0, 12.5, or 50 μM SB202190 for 30 min and then with 10 μg of LPS and 20 ng of IFNγ/ml for 8 h. iNOS mRNA was determined by reverse transcription, PCR and agarose gel electrophoresis. Representative images of ethidium-stained gels of iNOS and β-actin PCR products are shown, with HindIII-digested λ phage DNA markers in the left lane (λ). No PCR product was detected in extracts of untreated MAEC or in MAEC treated with either LPS or IFNγ alone (data not shown). Results of image analysis are shown in the bar graph. B, MAEC were treated as described and proteins was extracted 24h later. INOS protein was measured by Western blotting and analyzed as described under *Materials and Methods*. A representative Western blot is shown above the graph. Graphs present the means + S.E. of 4 independent samples per treatment. *, $p < 0.05$; **; $p < 0.01$; and ***, $p < 0.001$ for comparison with cells incubated without SB202190.

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with SB202190 at concentrations that inhibit NO production did not affect NFκB or AP-1 activation by LPS (Fig. 9).

Discussion

INOS is induced by inflammatory stimuli in many cell types. The enzyme is induced by LPS and IFNγ in endothelial cells (Morikawa et al., 2000), where it may contribute to or limit inflammatory organ damage (Hickey et al., 1997). LPS and cytokine signaling pathways that regulate iNOS have been intensively studied in inflammatory cells, such as macrophages, but not in endothelial cells. The regulation of iNOS in endothelial cells differs from the more often studied macrophage: IFNγ and LPS are both required for induction of iNOS in MAEC (Huang et al., 2003), whereas IFNγ and LPS alone each induce iNOS in macrophages (Hammermann et al., 2000). Given that an understanding of signaling pathways involved in different cell types may lead to improved therapeutics for specific disorders, we examined the mechanisms of synergy between IFNγ and LPS in MAEC.

The induced accumulation of nitrite in MAEC depended on RNA and protein synthesis (Fig. 1A) and LPS plus IFNγ increased iNOS mRNA, and protein as seen previously (Huang et al., 2003) (Fig. 6). We determined whether the order of treatment and timing of exposure to LPS and IFNγ would affect the synergistic accumulation of nitrite (Fig. 1B). Nitrite accumulation was detected sooner with pre-exposure to IFNγ than to LPS. However, exposure to LPS before IFNγ still increased nitrite more than IFNγ alone within 10 to 24 h (Fig. 1A) (Huang et al., 2003). Necessary effects of IFNγ could take longer to develop than those of LPS. However, the degree of synergistic induction in LPS-pretreated MAEC decreased slightly as the duration of the LPS-pretreatment was extended to 4 h, suggesting that tolerance to LPS may occur in this time. Tolerance to LPS has been investigated extensively in macrophages and monocytic cells (reviewed in West and Heagy, 2002; Fujihara et al., 2003). In such cells, prolonged treatment with LPS depressed Toll 4 receptor expression and the recruitment or levels of intracellular signaling partners (Myd88 and interleukin-1 receptor activated kinase-1) and induced inhibitory κB proteins and NFκB p50

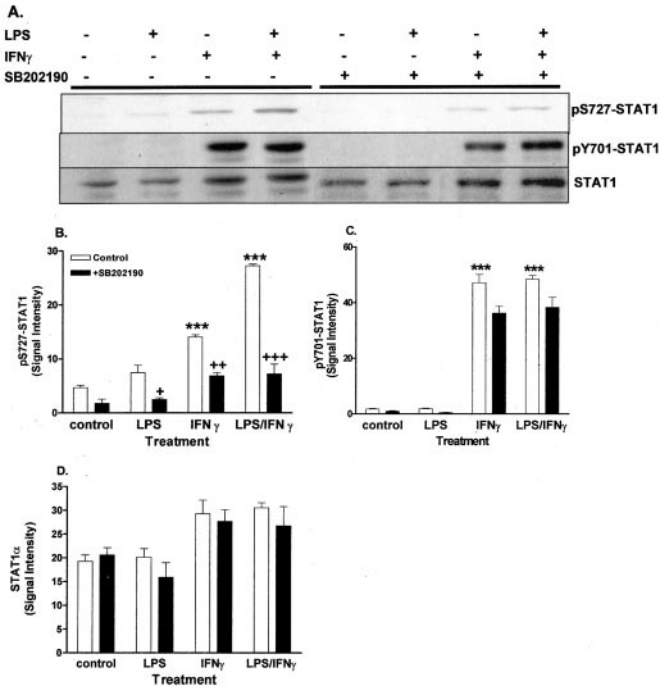

Fig. 7. Effect of SB202190 on STAT1α phosphorylation. MAEC were treated with LPS and/or IFNγ for 30 min, with or without preincubation of SB202190 (50 μM, 30 min). Unphosphorylated STAT1 (STAT1), Tyr701-phosphorylated STAT1 (pY701-STAT1), and Ser727-phosphorylated STAT1 (pS727-STAT1) were measured by Western blotting with specific antibodies. A representative image is shown in A. Note that blotting for phospho-Ser727 STAT1 detects the single STAT1α band, whereas anti-STAT1 or phospho-Tyr701 antibodies detect the doublet of STAT1α and β. Images of three samples per experimental group were analyzed densitometrically to determine the effect of treatments on pS727-STAT1α (B), pY701-STAT1α (upper band) (C), and unphosphorylated STAT1α (upper band) (D). LPS and IFNγ significantly induced both Ser727 and Tyr701 phosphorylation of STAT1α. However, only Ser727 phosphorylation was significantly blocked by SB202190. Bars and symbols are as in Fig. 4.

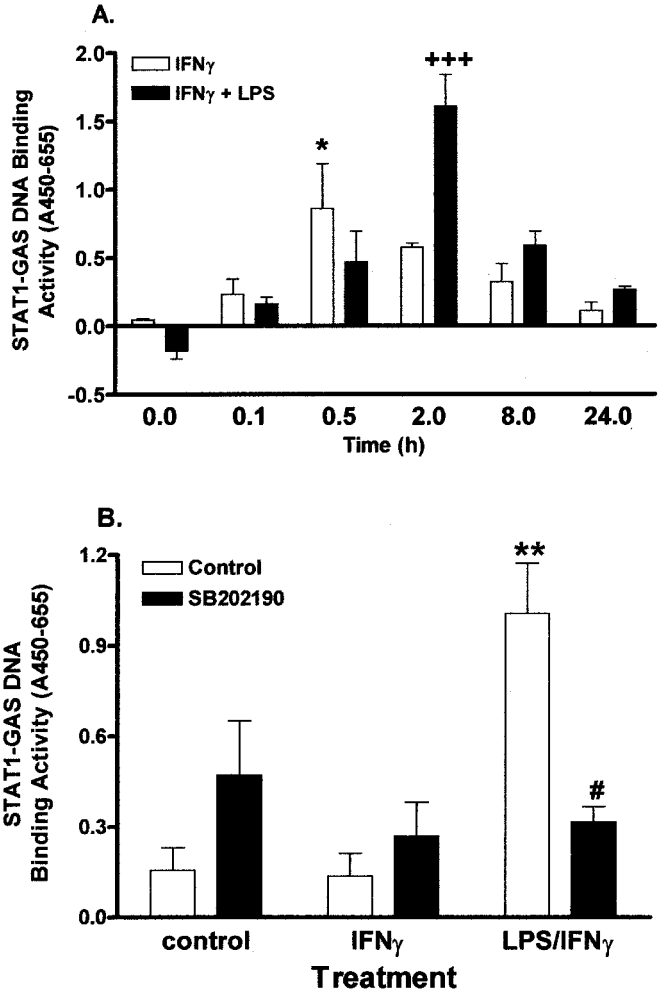

Fig. 8. Time course of induction of STAT1-GAS binding activity, and the effect of SB202190 on STAT activation. MAEC were treated with either IFNγ (20 ng/ml, open bars) alone or the combination of IFNγ and LPS (10 μg/ml, solid bars) for 0 to 24 h (A). STAT1 associated with GAS DNA bound to an assay well was measured by horseradish peroxidase-linked immunoassay with anti-STAT1. Cells treated with IFNγ alone had increased STAT1-GAS binding with a peak 30 min after treatment. LPS increased the effect of IFNγ at 2 h. Bars represent the mean + S.E. of three independent cultures per group. *, $p < 0.05$ for comparison with no incubation; +++, $p < 0.001$ for comparison with cells treated with IFNγ only. MAEC were treated with IFNγ alone or IFNγ/LPS for 2 h, with or without 30 min preincubation with 50 μM SB202190 (B). SB202190 significantly reduced IFNγ/LPS-induced STAT1-GAS binding activity. **, $p < 0.01$ for comparison with cells treated with medium alone; #, $p < 0.05$ for comparison between cells incubated with and without SB202190.

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homodimers that inhibit gene activation. A lesser induction of JunB, a component of AP-1, and less activation of p38 and p44/42 MAPKs and Jun-N terminal kinase was also seen in various cell lines. LPS may also increase the level or activity of inhibitors of IFNγ action, the suppressors of cytokine signaling proteins 1 and 3 (Crespo et al., 2002; Wormald and Hilton, 2004). Although the mechanisms remain to be determined in MAEC, the effect of LPS-pretreatment was overcome within 10 to 24 h after IFNγ was added, suggesting that synergistic interactions between LPS and IFNγ for iNOS induction outweighed negative actions of LPS here.

The murine iNOS promoter is regulated by NFκB, AP-1, and STAT1 (Xie et al., 1993). LPS is known to activate NFκB and AP-1 (Deshpande et al., 1997), whereas IFNγ is a powerful activator of STATs (Stark et al., 1998). Here IFNγ, but not LPS, increased STAT1 tyrosine phosphorylation, nuclear translocation, GAS DNA binding activity, and, later, protein level, in MAEC (Figs. 2 and 7). In contrast, LPS alone activated NFκB and AP-1 (Fig. 9), whereas IFNγ did not (data not shown). These results are in agreement with other studies indicating that NFκB activation and STAT1 tyrosine phosphorylation are separate effects of LPS and IFNγ in various cell types (Deng et al., 1996). Synergy between LPS and IFNγ could result from complimentary activation of NFκB, AP-1, STAT1α, and other factors. The combination of LPS and IFNγ failed to activate NFκB or AP-1 in MAEC beyond the response caused by LPS alone (Fig. 9). In contrast to NFκB or AP-1, STAT1-GAS DNA binding was further enhanced by combined treatment. The induction of iNOS in MAEC may require the activation of NFκB and AP-1, but synergy between LPS and IFNγ correlates with enhanced STAT1α activation.

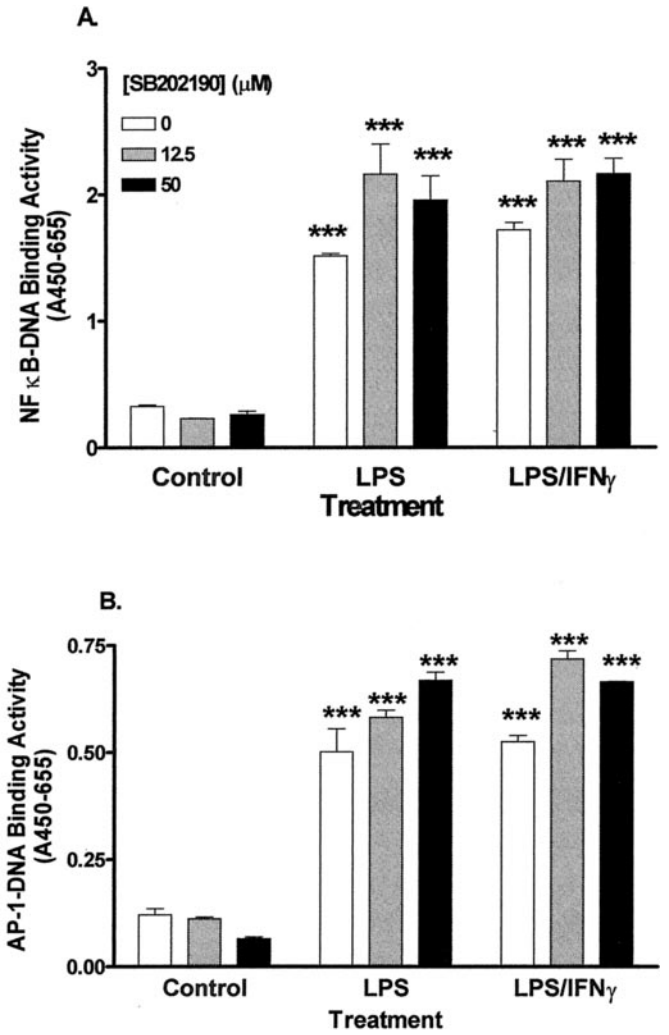

Fig. 9. Effect of LPS, IFNγ, and SB202190 on NFκB and AP-1 DNA binding activity. MAEC were treated 30 min with medium (Control), LPS (10 μg/ml), or the combination of LPS and IFNγ (10 and 20 ng/ml; LPS/IFNγ), with or without 30 min pretreatment with SB202190 at the indicated concentration. The amounts of NFκB p65 or AP-1 in nuclear extracts that bound to double-stranded oligonucleotide binding sequences attached to assay wells were measured by horseradish-peroxidase linked immunoassay. Bars represent the mean difference in A450-A655 + S.E. for three independent samples per group. ***, $p < 0.001$ for comparison with Controls incubated without LPS or IFNγ. NFκB-DNA binding (A) and AP-1-DNA binding (B) were both increased by LPS with or without IFNγ. IFNγ did not significantly increase activation caused by LPS, and alone it had no effect (data not shown). SB202190 did not block activation by LPS.

STAT1 must dimerize to translocate to the nucleus, bind to DNA and activate transcription. Dimerization requires tyrosine phosphorylation at position 701. Tyrosine phosphorylation occurs in response to activation of the IFNγ receptor, which recruits and activates JAK1 and -2 (Stark et al., 1998). This model seemed to operate in MAEC because herbimycin A, a tyrosine kinase inhibitor (Nishiya et al., 1995), inhibited NO production (Fig. 3A). Fludarabine, a nucleoside analog that reduces STAT1 levels (Frank et al., 1999), also inhibited NO production in response to LPS plus IFNγ (Fig. 3B).

The ability of STAT1α dimers to activate transcription is enhanced by a second phosphorylation at Ser727 (Wen et al., 1995). IFNγ alone elevated the level of Ser727 phosphorylation in MAEC (Fig. 7B). Although LPS alone did not induce serine phosphorylation, its addition with IFNγ doubled it. Thus, synergy between LPS and IFNγ may be caused by this extra modification of STAT1α. It is interesting that IFNγ alone increased Ser727 phosphorylation (Fig. 7) but did not induce iNOS (Huang et al., 2003). Activation by LPS of other factors that regulate the iNOS promoter, such as NFκB and AP-1 (Fig. 9), may be necessary for phospho-Ser727 STAT1α action. It is also possible that a threshold level of Ser727 phosphorylation is required for iNOS induction and that LPS pushes MAEC beyond the insufficient level induced by IFNγ. In any case, the results are consistent with previous findings that Ser727 phosphorylation occurs only with dimers of STAT1α, which form after IFNγ-induced tyrosine phosphorylation (Stark et al., 1998).

Ser727 of STAT1α is located in a MAPK target motif (PMS^{727}PEE) (Kovarik et al., 2001). Although STAT1α may be phosphorylated directly by p38 MAPKs in vitro, in vivo proof is lacking (Kovarik et al., 1999). LPS, UV light, and *Listeria monocytogenes* activate p38 and increase phosphorylation of Ser727 of STAT1α in macrophages (Kovarik et al., 1998; Kovarik et al., 1999), and LPS is a strong activator of p38 in many cell types, including endothelial cells (Schumann et al., 1996). Therefore we investigated the role of p38 in the synergy between LPS and IFNγ in MAEC.

p38 activity is strictly dependent on its phosphorylation (Raingeaud et al., 1995; Goh et al., 1999). LPS by itself induced the phosphorylation of p38 in MAEC. In contrast, IFNγ alone had no effect on p38 and did not enhance LPS-induced p38 phosphorylation (Fig. 4). The results are consistent with a model in which LPS, acting through p38, en-

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hances the phosphorylation of Ser727 in STAT1α dimers, which are separately formed in response to IFNγ in MAEC. We next used the p38 inhibitor, SB202190, to investigate the role of p38 MAPK in the iNOS response to LPS plus IFNγ. The reported IC_{50} for inhibition of p38α and -β MAPKs in vitro is 0.3 to 0.6 μM, whereas p38γ and -δ are not affected by even 100 μM SB202190 (Goedert et al., 1997). In MAEC, NO production in response to LPS plus IFNγ was suppressed with an IC_{50} of 13.4 μM (Fig. 5), and p38 phosphorylation was inhibited 60% with 50 μM SB202190 (Fig. 4). Although p42/44 MAPK participates in STAT activation in some cell types (Wen et al., 1995), PD98059, which inhibits activation of p42/44 MAPKs [IC_{50}, 2–7 μM (Alessi et al., 1995)], had no effect on NO production in response to LPS and IFNγ in MAEC (Fig. 5B). Furthermore, inhibitors of several other serine/threonine kinases, including protein kinase A, protein kinase C, protein kinase G, and Ca^{2+}/calmodulin kinase II, did not inhibit NO production in LPS/IFNγ-treated MAEC. The results implicate p38α and/or β in the synergy between LPS and IFNγ for iNOS gene activation in MAEC.

Although SB202190 had no affect on STAT1 Tyr701 phosphorylation or nuclear translocation (Fig. 7), Ser727 phosphorylation caused by LPS plus IFNγ was reduced by 74% (Fig. 7). SB202190 also inhibited STAT1α DNA binding (Fig. 8). It is not known exactly how Ser727 phosphorylation enhances the function of STAT1α, particularly because wild-type STAT1α and a mutant with alanine substituted for Ser727 had equal in vitro DNA binding activities (Wen and Darnell, 1997). MAEC may contain inhibitor(s) or facilitator(s) of phospho-Ser727 STAT1α DNA binding that are extractable from MAEC nuclei, which affect binding to GAS DNA in vitro. Ser727 itself may interact directly or indirectly with these hypothetical modulators of in vitro DNA binding. In any case, the results implicate p38α and/or β in regulation of STAT1α Ser727 phosphorylation and transcriptional activity in MAEC.

INOS protein levels at 8 h were suppressed by 60% with 12.5 μM SB202190 and 83% with 50 μM (Fig. 6). However, accumulation of nitrite over 24 h was completely suppressed by 50 μM SB202190 (Fig. 5). A threshold level of iNOS expression may be necessary to accumulate nitrite. High levels of SB202190 may have additional effects that result in the complete suppression of nitrite accumulation. In any case, SB202190 inhibited increases in iNOS mRNA and protein caused by LPS and IFNγ.

SB202190 at 50 μM lowered STAT1α Ser727 phosphorylation by 74% (Fig. 7) and reduced p38 phosphorylation by 60% (Fig. 4) 30 min after treatment with LPS and IFNγ, a time at which STAT1 tyrosine phosphorylation was high (Fig. 2). p38 MAPKs are phosphorylated by upstream MAPK kinases, which are insensitive to SB202190 (Davies et al., 2000). Thus, the reduction in p38 phosphorylation by SB202190 was not actually expected. However, intramolecular autophosphorylation of p38α, but not β, has been described in response to Toll-like receptor 4 activation by LPS in cultured cell lines. Furthermore, this p38α autophosphorylation was suppressed by another p38α/β inhibitor, SB203580 (Ge et al., 2002). A combination of reduction in p38α autophosphorylation and inhibition of other active molecules of p38α and β, reducing STAT1α Ser727 phosphorylation, may contribute to the inhibitory effects of SB202190. p38α and/or β may affect other mediators that contribute to iNOS induction in MAEC. p38 MAPKs may also affect gene activation independently of STAT1 phosphorylation. It is interesting that SB202190 did not inhibit the activation of NFκB and AP-1 by LPS (Fig. 9), suggesting that these transcription factors do not mediate such p38-dependent processes in MAEC. Further investigation of the specific roles of p38α and β in LPS/IFNγ-treated MAEC is warranted.

In conclusion, the results are consistent with the model in Fig. 10. It seems that IFNγ causes phosphorylation of Tyr701 of STAT1, which allows dimerization and nuclear translocation. LPS separately activates p38α/β and leads to phosphorylation of Ser727 of STAT1α dimers. The increase in Ser727 phosphorylation leads to increased DNA binding activity of STAT1α dimers in MAEC, and increased activation of the iNOS promoter. Activation of NFκB and AP-1 by LPS seems to be permissive for iNOS induction, because synergy between LPS and IFNγ was apparent only with respect to STAT1α phosphorylation and activity. Independent of the involvement of p38α or β or their precise mechanisms of activation, the results indicate the essential role of p38 MAPK and STAT1α in the synergy between LPS and IFNγ for activation of the iNOS gene in MAEC. The present study suggests that p38 inhibitors should be useful in acute or chronic vascular inflammatory diseases. Additional studies of the role of p38 isoforms in sepsis and inflammation are needed.

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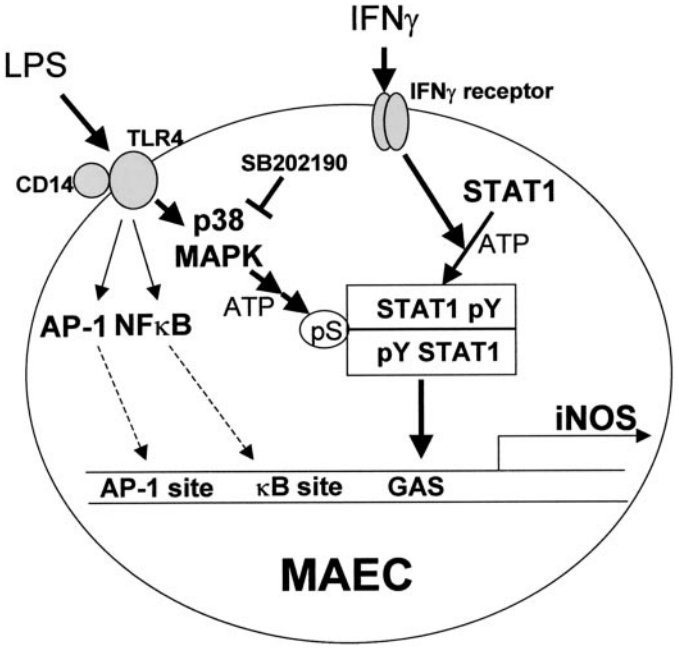

Fig. 10. A model for the induction of iNOS by LPS and IFNγ in MAEC. IFNγ activated STAT1 by causing its Tyr701 phosphorylation, nuclear translocation, and GAS binding. At same time, p38 was activated by LPS, which phosphorylated STAT1 on Ser727. STAT1α Ser727 phosphorylation had no effect on STAT1 Tyr701 phosphorylation and its translocation. However, it was required for STAT1-GAS binding, which induced iNOS gene transcription (solid arrows). LPS also activated NFκB and AP-1, which may be necessary for iNOS induction but do not account for synergy between LPS and IFNγ, or the sensitivity of synergy to p38 (dashed arrows), in MAEC.

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