Original Article

Cytokine-Activated p42/p44 MAP Kinase Is Involved in Inducible Nitric Oxide Synthase Gene Expression Independent from NF- κ B Activation in Vascular Smooth Muscle Cells

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Recent evidence suggests the possible involvement of inducible nitric oxide synthase (iNOS) in the development and maintenance of hypertension in certain animal models. Inflammatory cytokines activate nuclear factor (NF)- κ B, which plays a major role in transactivation of the inducible nitric oxide synthase (iNOS) gene. However, it remains unknown whether cytokine-mediated iNOS expression in vascular smooth muscle cells (VSMCs) requires signaling pathway(s) other than NF- κ B activation. The purpose of this study was to determine whether the p42/p44 MAP kinase pathway is involved in cytokine-induced NF- κ B activation and/or iNOS expression in cultured rat VSMCs. Nitrite/nitrate (NO_x) production stimulated by interleukin (IL)-1 β or tumor necrosis factor (TNF)- α in VSMCs was markedly suppressed by inhibiting MAP kinase by pretreatment with a p42/p44 MAP kinase kinase (MAPKK)-1 inhibitor (PD98059) or by transfecting the dominant-interfering form of the nonphosphorylated MAPKK-1 expressing construct (MAPKK S222A). Inhibition of p42/p44 MAP kinase also antagonized the upregulation of iNOS mRNA and protein, as demonstrated by the quantitative RT-PCR method and Western blot analysis, respectively. Furthermore, rat iNOS promoter activity using an iNOS-luciferase construct stimulated by cytokines was inhibited by MAPKK-1 inhibition. However, κ B-dependent transcription analysis revealed that cytokine-stimulated NF- κ B activity was unaffected by MAP kinase inhibition. Western blot analysis using anti-I κ B- α and anti-phospho-I κ B- α antibodies showed that PD98059 had no effect on transient phosphorylation or degradation of I κ B- α by cytokines. An electrophoretic mobility shift assay using synthetic oligonucleotide corresponding to the downstream NF- κ B site of rat iNOS promoter as a probe showed that MAP kinase inhibition did not block cytokine-stimulated activation of NF- κ B. These data suggest that the MAP kinase pathway is in part involved in cytokine-induced iNOS expression independent from NF- κ B activation in rat VSMCs.

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Key Words: p42/p44 MAP kinase, inducible nitric oxide synthase, NF- κ B, cytokine, vascular smooth muscle cells

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Introduction

Nitric oxide (NO) plays an important role in the basal vascular tonus and blood flow (1). It is well recognized that NO derived from endothelial NO synthase (NOS) mediates the maintenance of blood pressure, inhibition of platelet aggregation, leukocyte adhesion, and so forth (1, 2). By contrast, inducible NOS (iNOS) produces large amounts of NO in response to bacterial lipopolysaccharides (LPS) and proinflammatory cytokines (IL-1 β and TNF- α). High-output NO derived from augmented iNOS expression in vascular smooth muscle cells (VSMCs) profoundly relaxes vascular tonus, inhibits cell proliferation, and induces apoptosis (3, 4). It has recently been demonstrated that reduced iNOS expression is involved in the development and/or worsening of hypertension in Dahl salt-sensitive hypertensive rats (5). Chronic inhibition of NO production has been shown to aggravate the rise in blood pressure in dogs with two-kidney, one-clip renovascular hypertension (6). Furthermore, high blood pressure itself has been shown to increase basal and cytokine-stimulated iNOS expression in the aorta of spontaneously hypertensive rats (SHR) (7). Therefore, altered iNOS gene expression in the vasculature may be implicated in the development and/or maintenance of hypertension, as well as in vascular remodeling in atherosclerosis.

NF- κ B is a heterodimeric complex, usually consisting of a p50/p65 subunit, and forming an inactive ternary complex by associating with a cytoplasmic inhibitor, I κ B- α (8). NF- κ B is essential for the cytokine-induced iNOS gene expression in a variety of cell types, including VSMCs (9-11). Stimulation with cytokines causes a rapid phosphorylation of I κ B- α by serine/threonine protein kinases, termed I κ B kinases (IKK), and a transient proteasome-mediated degradation of I κ B- α , which subsequently induces the NF- κ B heterodimer to translocate into the nucleus and bind to the κ B sites of the genes (12-14). Blockade of I κ B- α degradation and activation of NF- κ B by proteasome inhibitors suppress the cytokine-induced iNOS expression in rat VSMCs (9). However, the presence of yet-unspecified pathways mediating iNOS expression distinct from the proteasome-mediated I κ B- α degradation has been suggested (9). Recently accumulating lines of evidence suggest that PD98059, a selective inhibitor of p42/p44 mitogen-activated protein (MAP) kinase kinase (MAPKK)-1, affects cytokines- and LPS-induced iNOS expression in several cell types (15-17).

These observations led us 1) to determine whether p42/p44 MAP kinase activation is involved in IL-1 β - and TNF- α -induced iNOS expression in rat VSMCs and 2) to delineate the relationship of the p42/p44 pathway to NF- κ B activation in mediating transcriptional regulation of iNOS.

Methods

Materials

Murine recombinant tumor necrosis factor (TNF)- α was purchased from Life Technologies (Grand Island, USA), and rat recombinant interleukin (IL)-1 β from R&D Systems (Minneapolis, USA). Sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), Triton X-100, and sodium orthovanadate were purchased from Wako Pure Chemical (Osaka, Japan). NADPH was purchased from Oriental Chemical (Tokyo, Japan); tetrahydrobiopterin from Research Biochemical International (Natick, USA); phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), poly (dI-dC), ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid (EGTA) and β -mercaptoethanol from Sigma Chemical (St. Louis, USA); (32 P) dCTP and sheep anti-mouse Ig conjugated to horseradish peroxidase from Amersham International (Tokyo, Japan); protein A/G plus-agarose and rabbit polyclonal antibody for human I κ B- α from Santa Cruz Biotechnology (Santa Cruz, USA); dNTP and a Klenow fragment of DNA polymerase I from Takara Shuzo (Shiga, Japan); mouse monoclonal antibody for murine iNOS and anti-phosphotyrosine (PY20) from Transduction Laboratories (Lexington, USA); PD98059 and phospho-I κ B- α antibody from New England Biolabs (Beverly, USA); and pNF- κ B-Luc plasmid containing four NF- κ B binding consensus elements (GGGAATTTCC) upstream from the luciferase reporter gene, from Clontech Laboratories (Palo Alto, USA). The plasmids MAPKK1 WT, an unphosphorylated wild-type MAPKK1 expressed from the SV40 early promoter, and MAPKK1 S222A, a dominant-interfering form, were kindly provided by Drs. A. Brunet and J. Pouyssegur (18).

Cell Culture

VSMCs prepared by the explant method from the thoracic aorta of male Wistar rats were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air-5% CO₂, as described (19). Subcultured VSMCs (7-10th passages) were used in the experiments.

Transfection

Transient transfection was performed using synthetic cationic liposome, (+)-N, N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide according to instructions provided by the supplier (Promega) with the following modification using transferrin-receptor operated transfer (20). In brief, 3 μ l of liposome was added to 100 μ l HEPES-buffered saline

(HBS, 20 mmol/l HEPES, pH 7.4, and 100 mmol/l NaCl) containing 16 μ g human holo-transferrin (Sigma Chemical Co., St. Louis, USA), incubated for 20 min at room temperature, mixed with 100 μ l HBS containing various amounts of DNA mixtures, and further incubated for 15 min. DNA-liposome-transferrin complex was then overlaid on cells that had been covered with serum-free DMEM and incubated for 2 h (21). The introduction of β -galactosidase-expressing construct in the same manner as indicated above resulted in a high transfection efficiency in VSMCs: regardless of the DNA concentrations used (0.05–1 μ g/well), nearly 100% of the total number of cells were transfected, and the intensity of the reporter signal correlated well with the DNA concentrations used (22).

Determination of Nitrite/Nitrate (NO_x)

Quiescent VSMCs (10^6 cells/well) were stimulated with IL-1 β (10 ng/ml) or TNF- α (100 ng/ml) for 15 h; NO_x concentrations in the conditioned media were measured by a nitric oxide analyzer (MODEL 280 NOATM, Sievers Instruments, Boulder, USA). After premixing with 0.13 U nitrate reductase (Sigma Chemical, St. Louis, USA) and 66.7 μ M β -NADPH-4Na (Oriental Yeast, Osaka, Japan) to reduce NO_3^- to NO_2^- , each sample was reduced with nitrite oxide gas from solution using iodide and acetic acid. The gas phase chemiluminescent reaction between NO and ozone was measured by NOATM, and the real-time signal was plotted and analyzed by the NO analysis program (Sievers Instruments, Boulder, USA). NO_3^- was used as a standard.

Quantitative RT-PCR

Total RNA was extracted from a single dish (5×10^6 cells/dish) by the acid guanidinium thiocyanate-phenol-chloroform methods. First-strand cDNA from each mRNA was generated by a reverse-transcription method using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, USA). Rat iNOS mRNA was amplified using synthetic oligomer (forward: 5'-GAAGGCT-GGAAGTAAGTGC-3', complement: 5'-TGCCTTGCT-GAATGAAGTTG-3' as a probe) detected and quantitated in real time using Light CyclerTM (Roche Diagnostics, Basel, Switzerland), as described (23). The amplification mixture in the DNA amplification kit (DNA Master SYBR Green I: Roche Diagnostics) contained 50 nM template cDNA and 500 μ M primer DNA. Denaturation was carried out at 95°C, annealing at 52°C for 5 s, and extension at 72°C for 22 s. The reaction produced a 377-bp PCR product that was confirmed by agarose gel electrophoresis. Quantitation of the cDNA copy number was performed using control samples according to the manufacturer's protocol.

Western Blot Analysis

After confluent VSMCs (5×10^6 cells/dish) were stimulated with cytokines for the indicated times, cells were lysed in cell lysis buffer, and the extracted protein was subjected to Western blotting using mouse monoclonal antibody for murine iNOS, rabbit polyclonal antibody for human I κ B- α , and phospho-I κ B- α antibody as described (9–11).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein was extracted from VSMCs (5×10^6 cells/dish) and subjected to EMSA as described (9–11). Single-stranded oligonucleotides (forward: 5'-TGGGGACTCTC C-3', complement: 5'-AAGGGAGAGTCC-3') corresponding to the NF- κ B binding site (–107 to –97) in the 5'-flanking region of the rat iNOS gene were used.

Transcription Assay

A fragment of the murine iNOS gene promoter region (–1,588 to +161 bp) was amplified using a 5'-sense primer (5'-CAATGCCTCGAG GACTTTGATATGCT GAAATC-3', –1,588 to –1,569) and a 3'-anti-sense primer (5'-TCAGTTAAGCTTGACTAGGCTACTCCG TGGAG-3', +142 to +161) using a murine iNOS-CAT construct as a template (Oxford Biomedical Research Laboratories, Oxford, UK). The underlined nucleotides in the oligonucleotide sequences represent modified nucleotides not present in the iNOS promoter region. These nucleotides introduce *Xho*I and *Hind*III sites into the 5' and 3' ends of the PCR fragment, respectively. Direct sequencing of the amplified double-stranded PCR products was carried out from both directions by standard semi-automated methods on an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, USA). PCR-amplified DNA fragments consisting of the murine iNOS gene and additional modified nucleotides were digested with *Xho*I and *Hind*III, and the resulting 1,750 bp fragments spanning –1,588 to +161 with sticky ends were subcloned into the *Xho*I/*Hind*III site of the pGL3-Basic Vector (Promega, Madison, USA). The iNOS promoter construct, designated as piNOS (–1,588 to +161)-Luc and containing two NF- κ B-binding consensus sequences (–972 to –962 and –86 to –76), was co-transfected with pRL-TK vector (Promega), expressing Renilla luciferase as an internal control. Subconfluent cells (96-well plates) were transfected with a DNA mixture containing 1 μ g each of piNOS-luc and pRL-TK using the transferrin receptor-operated transfer method as described (24). After transfection, cells were incubated in DMEM with 10% FCS, deprived of serum for 24 h, and then stimulated with IL-1 β (10 ng/ml) or TNF- α (100 ng/ml) in serum-free DMEM for 15 h. Firefly and Renilla

Fig. 1. Effect of a MAPKK-1 inhibitor on cytokine-stimulated NO production and iNOS gene expression in rat VSMCs. (A) Quiescent cells pretreated for 60 min with PD98059 in the indicated concentrations were stimulated with IL-1 β (10 ng/ml) or TNF- α (100 ng/ml) for 15 h; concentrations of NO_x released into media were measured. Each point with a bar for IL-1 β shows the mean $\pm$ SE, and each point for TNF- α represents mean values; SE values were within each point (n=4). (B) Quiescent cells pretreated with or without PD98059 were stimulated with IL-1 β for 6 h. Extracted total RNAs were subjected to real-time quantitative PCR using the LightCyclerTM for the estimation of relative iNOS mRNA levels as described in Materials and Methods. Each column with a bar shows mean $\pm$ SE (n=8). *p<0.01, **p<0.005, treated vs. nontreated cells.

luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) in a single-tube assay format using MicroLumat^{Plus} (EG&G Berthold, Wildbad, Germany). The Firefly luciferase activity of each sample was normalized to an internal reference standard of Renilla luciferase activity. All transfections were repeated two to four times in octet, with at least three different plasmid preparations. Data from representative experiments are shown.

Fig. 2. Effects of MAPKK-1 inhibition on cytokine-induced iNOS protein expression in rat VSMCs. Quiescent cells (A) pretreated with or without PD98059 (100 μM) or (B) transfected with or without dominant-negative MAPKK-1 were stimulated with IL-1 β (10 ng/ml) for 15 h for the detection of 130 kDa-iNOS protein by Western blotting. A representative from three separate experiments is shown. An arrow indicates the position of the 130-kDa iNOS protein.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical analysis was performed using analyses of variance for repeated measures. A p value less than 0.05 was considered statistically significant.

Results

MAPKK-1 Inhibitor Blocks Cytokine-Induced NO Production and iNOS Expression

Cultured rat VSMCs produce large amounts of NO following stimulation with IL-1 β or TNF- α (9-11). There were approximately ten- and five-fold increases in NO_x production in response to stimulation with IL-1 β (10 ng/ml) or TNF- α (100 ng/ml) for 15 h, respectively. The cytokine-stimulated NO_x production was inhibited dose-dependently (25-100 mM) by pretreatment with PD98059; the maximal inhibitions for IL-1 β and TNF- α were ~60% and ~80%, respectively (Fig. 1A). To quantitate changes in the iNOS mRNA transcripts, a real-time quantitative PCR method was utilized. The steady-state levels of iNOS mRNA were increased by IL-1 β , whose effect was suppressed by PD98059 in a dose-dependent manner (50-100 μM) (Fig. 1B). Similar results were obtained when TNF- α was used as an inducer (data not shown). Western blot analysis revealed that the amount of a 130-kDa iNOS protein induced by IL-1 β was markedly de-

Fig. 3. Effect of a MAPKK-1 inhibitor on cytokine-induced phosphorylation and degradation of I κ B- α in rat VSMCs. Quiescent cells pretreated without or with PD98059 (100 μ M) for 1 h were stimulated with IL-1 β (10 ng/ml) for 15 min for the detection of phospho-I κ B- α (upper panel) and I κ B- α protein (lower panel) by Western blot analysis using anti-phospho-I κ B- α antibody and anti-I κ B- α antibody, respectively.

Fig. 4. Effects of the dominant-negative MAPKK-1-expressing construct on cytokine-induced NO production in rat VSMCs. Subconfluent cells, transfected without or with the wild-type MAPKK-1 expressing construct (MAPKK1 WT) or the dominant-negative mutant (MAPKK1 S222A), were incubated in DMEM containing 10% FCS for 48 h, deprived of serum, and then stimulated with IL-1 β (10 ng/ml) and TNF- α (100 ng/ml) for 15 h; concentrations of NO $_x$ released into media were measured. Each column with bar shows the mean $\pm$ SE (n=4). *p<0.001, treated vs. nontreated cells.

increased by pretreatment with 50 μ M PD98059 (Fig. 2, upper panel). These results suggest that the MAPKK-1/MAP kinase pathway is partly involved in cytokine-induced iNOS expression. Since IL-1 β causes a rapid (15–30 min) and transient phosphorylation and degradation of the I κ B- α protein in rat VSMCs (9–11), we examined whether PD98059 affects cytokine-induced I κ B- α

Fig. 5. Effects of MAPKK-1 inhibition on cytokine-induced iNOS promoter activity. piNOS (–1,588 to +161)-Luc construct was transfected with or without MAPKK-1 dominant-negative mutant (MAPKK S222A) for 48 h or pretreated with or without PD98059 (100 μ M) for 1 h. Luciferase activities were measured after stimulation with IL-1 β (10 ng/ml) for 15 h as described in Materials and Methods. *p<0.01, treated vs. nontreated cells (n=6).

Fig. 6. Effects of MAPKK-1 inhibition on cytokine-induced κ B-dependent transcription. pNF- κ B-Luc construct was transfected with or without MAPKK-1 dominant-negative mutant (MAPKK S222A) for 48 h or pretreated with or without PD98059 (100 μ M) for 1 h. Luciferase activities were measured after stimulation with IL-1 β (10 ng/ml) for 15 h as in Fig. 5. *p<0.01, treated vs. nontreated cells (n=6).

phosphorylation and degradation by Western blotting by using anti-phospho-I κ B- α and anti-I κ B- α antibodies, respectively. PD98059 (100 μ M) had no effect on either the phosphorylation or degradation of I κ B- α by IL-1 β (Fig. 3). These data suggest that the cytokine-induced iNOS expression via the p42/p44 MAP kinase pathway does not involve the NF- κ B activation pathway consequent to phosphorylation and the subsequent degradation of I κ B- α .

Fig. 7. Effect of PD98059 on cytokine-induced NF- κ B activation by gel shift assay. Confluent cells pretreated with or without PD98059 (100 μ M) for 1 h were stimulated with IL-1 β (10 ng/ml) or TNF- α (100 ng/ml) for 2 h. Nuclear proteins (10 μ g) were extracted and subjected to EMSA using synthetic NF- κ B oligomer as a probe. An arrow denotes the position of the NF- κ B protein-DNA complex.

Dominant-Negative MAPKK-1 Blocks Cytokine-Induced NO Production

Wild-type unphosphorylated MAPKK-1 expressing the DNA construct (MAPKK1 WT) and its dominant-negative form (MAPKK1 S222A) can efficiently be transfected into rat VSMCs utilizing a transferrin receptor-operated transfer of synthetic cationic liposome/DNA complexes (22). Transfection of MAPKK1 S222A into rat VSMCs caused the blockade of massive NO_x production induced by IL-1 β and TNF- α , whereas that of MAPKK1 WT did not affect cytokine-stimulated NO_x production (Fig. 4). The transfection of MAPKK1 S222A also decreased IL-1 β -induced 130-kDa iNOS protein expression (Fig. 2, lower panel). These results indicate that cytokine-induced iNOS expression is mediated in part *via* the MAPKK-1/MAP kinase pathway.

Inhibition of MAPKK-1 Antagonizes Cytokine-Induced iNOS Promoter Activity, but not κ B-Binding Sites-Dependent Activation

Because PD98059 blocked cytokine-stimulated iNOS expression without affecting NF- κ B activation, we investigated whether the inhibition of MAPKK-1 affects the cytokine-induced transcriptional activation of piNOS (-1,588 to +161)-Luc and pNF- κ B-Luc (Fig. 5). IL-1 β (10 ng/ml) markedly increased iNOS promoter activity, whose effect was abolished by pretreatment with either PD98059 (100 μ M) or dominant-negative MAPKK-1 mutant (MAPKK1 S222A). In contrast, the increased κ B-dependent transcriptional activity by IL-1 β was not blocked by PD98059 or MAPKK1 S222A (Fig. 6). These results were corroborated by EMSA using synthetic oligonucleotides corresponding to the NF- κ B site (-107 to -97) of the rat iNOS promoter as a probe (Fig. 7). Stimulation with IL-1 β and TNF- α similarly caused a distinct shifted band, whereas there was little signal in the control cells. Pretreatment with PD98059 (100 μ M) did not affect the cytokine-stimulated shifted band. Thus, these data confirm that NF- κ B activation is not involved in the blockade of cytokine-induced iNOS expression by MAP kinase inhibition.

Discussion

The activation of NF- κ B is essential in cytokine-induced iNOS expression and subsequent NO production in a variety of cell types, including macrophages and VSMCs (9, 25). Bacterial LPS and proinflammatory cytokines (IL-1 β , TNF- α) activate the IKK complex that phosphorylates I κ B- α , a NF- κ B inhibitor, and triggers conjugation with ubiquitin and subsequent degradation of I κ B- α . This allows NF- κ B heterodimers to translocate to the nucleus and induce target genes such as the iNOS gene. We have previously shown that IL-1 β and TNF- α cause a rapid and transient phosphorylation and degradation of I κ B- α , NF- κ B activation, and iNOS gene expression, followed by massive NO generation in rat VSMCs (9-11). However, possible involvement of additional pathways other than NF- κ B activation by cytokines in the mediation of iNOS expression has been suggested (15, 26-28), but has remained elusive in VSMCs.

The present study has demonstrated that the blockade of MAPKK-1 by a selective inhibitor, PD98059, or transfection of a dominant-negative MAPKK-1 mutant inhibits iNOS expression and the massive NO production induced by IL-1 β and TNF- α . It should be noted that MAPKK-1 inhibition by pretreatment with PD98059 or by transfection of the dominant-negative MAPKK-1 construct suppressed cytokine-stimulated iNOS promoter activity, but not κ B-dependent transcription. Furthermore, the NF- κ B activation induced by IL-1 β and TNF- α was unaffected by

MAPKK-1 inhibition. These results suggest that the MAP kinase pathway is a prerequisite for iNOS expression in rat VSMCs, but not upstream to NF- κ B activation. The data are corroborated by the findings that MAPKK-1 inhibition does not affect the cytokine-induced phosphorylation and degradation of I κ B- α required for the activation and nuclear translocation of NF- κ B. Taken together, these results indicate that the MAP kinase pathway is required for iNOS expression independent from NF- κ B activation in rat VSMCs.

Proinflammatory cytokines have been shown to activate MAP kinase in many cells, including rat glomerular mesangial cells (18, 29), rat cardiac myocytes (30), and rat cardiac fibroblasts (31). PD98059 (10–50 μ M) has been shown to partly (30–60%) suppress LPS- or cytokine-induced iNOS expression in fibroblasts (31), glial cells (15), neonatal cardiac myocytes (30), and bovine retinal pigmented epithelial cells (17), whereas it is more effective (~95%) in rat astrocytes (16). The relatively high doses (50–100 μ M) of PD98059 needed to inhibit cytokine-induced iNOS expression in rat VSMCs, as demonstrated in the present study, suggest a differential response to the compound depending on species, cell type, and/or stimulants used. The failure of cytokine-stimulated phosphorylation and the degradation of I κ B- α by PD98059 in the present study also differs from a previous report that PD98059 suppresses sphingomyelinase-stimulated iNOS expression and NF- κ B activation in rat astrocytes (16). The apparent slight discrepancies of MAPKK-1/MAP kinase inhibition as observed in the present study in response to the dominant-negative mutant and PD98059 on iNOS promoter activity, its expression, and iNOS production, may be accounted for by the different transcription efficiencies, different doses of the compound, and/or different experimental conditions of each assay employed.

The molecular mechanism(s) by which the MAP kinase pathway activated by cytokines is linked to iNOS gene transcription remains obscure. The piNOS (–1,588 to +161)-Luc plasmid used in the present study contains several potential *cis*-elements for the binding of transcription factors other than NF- κ B sites (25, 32, 33). It is possible that NF- κ B-dependent iNOS gene transcription may require other cofactor(s) mediated by the MAP kinase pathway such as AP-1, interferon regulatory factor-1 (IRF-1), and so forth (17). Therefore, deletion and/or mutation analysis of potential *cis*-elements in the murine iNOS promoter should help to elucidate the molecular link of the MAP pathway to iNOS transcription in rat VSMCs. It also remains to be determined whether the MAP kinase signaling pathway may converge with and/or downstream from the NF- κ B pathway.

Several lines of evidence suggest an alteration of iNOS expression in the exacerbation of hypertension in certain experimental animal models (5, 7, 34, 35). However, the

basal and stimulated iNOS expression levels have been shown to vary from one animal model to another. For example, Dahl salt-sensitive hypertensive rats show reduced iNOS expression and produce less NO than Dahl salt-resistant rats (34, 35). By contrast, SHR have higher basal and LPS-stimulated iNOS activities than WKY; these, however, have been found to be reduced after blood pressure control, suggesting a regulatory role of blood pressure *per se* in iNOS expression (7). Pressor responses to various vasoconstrictors, such as angiotensin II, endothelin-1, and norepinephrin, which potently stimulate p42/p44 MAP kinase in VSMCs, are known to be enhanced in hypertension (36, 37). Furthermore, inflammatory cytokines are also produced in blood vessels in hypertension and atherosclerosis (7, 38). Thus, the p42/p44 MAP kinase pathway for efficient vascular iNOS gene expression, as demonstrated in the present study, may serve as an inherent mechanism to counteract the overall cell proliferative, promigratory, and vasoconstrictive actions exerted by mechanical and neurohumoral factors in hypertension.

In summary, inhibition of the MAP kinase pathway causes suppression of cytokine-stimulated iNOS gene expression without affecting NF- κ B activation, suggesting the possible involvement of the p42/p44 MAP kinase cascade in mediation of the transcriptional regulation of iNOS gene expression, independent from NF- κ B activation in VSMCs.

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