Both p38\textsuperscript{MAPK} and JNK/SAPK Pathways Are Important for Induction of Nitric-oxide Synthase by Interleukin-1\textbeta in Rat Glomerular Mesangial Cells*

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Interleukin 1\textbeta (IL-1\textbeta) induces expression of the inducible nitric-oxide synthase (iNOS) with concomitant release of nitric oxide (NO) from glomerular mesangial cells. These events are preceded by activation of the c-Jun NH\textsubscript{2}-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38\textsuperscript{MAPK}. Our current study demonstrates that overexpression of the dominant negative form of JNK1 or p54 SAPK/JNK2 significantly reduces the iNOS protein expression and NO production induced by IL-1\textbeta. Similarly, overexpression of the kinase-dead mutant form of p38\textsuperscript{MAPK} also inhibits IL-1\textbeta-induced iNOS expression and NO production. In previous studies we demonstrated that IL-1\textbeta can activate MKK4/SEK1, MKK3, and MKK6 in renal mesangial cells; therefore, we examined the role of these MAPK kinases in the modulation of iNOS induced by IL-1\textbeta. Overexpression of the dominant negative form of MKK4/SEK1 decreases IL-1\textbeta-induced iNOS expression and NO production with inhibition of both SAPK/JNK and p38\textsuperscript{MAPK} phosphorylation. Overexpression of the kinase-dead mutant form of MKK3 or MKK6 demonstrated that either of these two mutant kinases inhibited IL-1\textbeta-induced p38\textsuperscript{MAPK} (but not JNK/SAPK) phosphorylation and iNOS expression. Interestingly overexpression of wild type MKK3/6 was associated with phosphorylation of p38\textsuperscript{MAPK}; however, in the absence of IL-1\textbeta, iNOS expression was not enhanced. This study suggests that the activation of both SAPK/JNK and p38\textsuperscript{MAPK} signaling cascades are necessary for the IL-1\textbeta-induced expression of iNOS and production of NO in renal mesangial cells.

Resting mesangial cells produce low basal levels of inflammatory mediators such as eicosanoids or NO, but soluble factors secreted by inflammatory cells such as macrophages or neutrophils that invade the glomerulus or by factors present in blood can up-regulate these products. Interleukin 1 (IL-1)\textsuperscript{3} and tumor necrosis factor \alpha (TNF-\alpha) are two such molecules produced by “activated” mesangial cells and other inflammation related cells that help to perpetuate the formation of inflammatory mediators such as eicosanoids, growth factors, or NO.

NO, synthesized from L-arginine, is an important molecule with diverse biological functions in the cardiovascular system, exerting effects such as vasodilatation, inhibition of adhesion and aggregation of platelets, and inhibition of vascular smooth muscle cell growth. NO synthesis is increased in the synovial fluid of patients with rheumatoid arthritis (1), in the colon of the patients with ulcerative colitis (2), and in the glomerulus in experimental nephritis (3). The inducible nitric-oxide synthase (iNOS) is found in several cell types including macrophages, vascular smooth muscle cells, endothelial cells, and mesangial cells. It is highly regulated by cytokines such as IL-1 and TNF-\alpha, which increase iNOS mRNA and protein expression. Once iNOS is induced, it produces large amounts of NO that can influence cell and tissue function and damage. However, iNOS gene expression, mRNA stability, and protein synthesis and degradation are all amenable to regulation by cytokines and growth factors. We previously reported that pro-inflammatory cytokines such as IL-1\textbeta induce iNOS in rat mesangial cells (4). However, the cellular mechanisms that signal this up-regulation are not fully understood. Recent studies have suggested that iNOS expression may be modulated by the MAPK pathway (5, 6). In mammalian cells, several different subfamilies of MAPK have been identified. These MAPK family members include: the extracellular signal-regulated kinases (ERKs), p44 MAPK (ERK1) and p42 MAPK (ERK2); stress-activated protein kinases (SAPKs), also referred to as c-Jun NH\textsubscript{2}-terminal kinases (JNKs), which include p54 SAPK (SAPK\textalpha/\beta, JNK2) and p45 SAPK (SAPK\textgamma, JNK1); and the p38\textsuperscript{MAPK} kinases (\alpha, \beta, \gamma, and \delta) (7, 8). Phosphorylated and activated MAPKs phosphorylate and activate downstream targets such as transcription factors and regulators of cell growth and differentiation. Activation of these kinases involve a cascade in which the upstream activator MAP kinase kinase kinase (MEKK\textsubscript{1-5} or Raf in the case of ERK) phosphorylates and activates SAPK/ERK kinase/MAP kinase kinases which include MKK\textsubscript{1-4} which in turn phosphorylate and activate ERKs, JNKs, and p38\textsuperscript{MAPK} (9).

Previous work has demonstrated that both SAPK/JNK and p38\textsuperscript{MAPK} cascades are activated in many cell types including renal mesangial cells, by the inflammatory cytokines IL-1 and TNF-\alpha, as well as by a wide variety of cellular stresses such as ultraviolet light, ionizing radiation, hyperosmolarity, heat shock, oxidative stress, etc. (10). These findings strongly suggest a role for these two kinase pathways as important signal-
ing mechanisms underlying the inflammatory process. We and others have previously demonstrated that p38 MAPK activation is linked to IL-1β-induced NO biosynthesis in renal mesangial cells (5, 11). In addition, recent data also have demonstrated that IL-1β-induced rat pancreatic islet nitric oxide synthesis requires both p38 MAPK and ERK (12).

The data presented in this report suggest a requirement for both p38 MAPK and JNK activity for cytokine-induced iNOS expression in glomerular mesangial cells. These observations suggest a potential mechanism for transcriptional regulation of iNOS expression and activation, which involves the activation and binding of intermediate transcription factors induced by both p38 MAPK and JNK to facilitate full expression of iNOS in response to interleukin-1ß stimulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IL-1β and restriction enzymes were purchased from Roche Molecular Biochemicals. Myelin basic protein (MBP) was purchased from Sigma. Fetal bovine serum was purchased from Life Technologies, Inc. Polyomonal or monoclonal rabbit or mouse IgG antibodies against SEK1 were purchased from Transduction Laboratories. MKK3, MKK4, MKK6, JNK, phosphospecific JNK, ERK, and p38 MAPK antibodies were from Santa Cruz Biotechnology Inc. Phosphospecific p38 MAPK, SEK/MKK4, and MKK3/MKK6 antibodies were from New England Biolabs. Phosphospecific ERK antibody was from Promega. pET28δβ, a histidine-tagged fusion protein expression plasmid that encodes c-Jun-(1–79), which contains the NH2-terminal activation domain of c-Jun, and a mutant c-Jun-(1–79, Ala63/73), in which serines 63 and 73 of c-Jun-(1–79) were mutated to alanine, were generously provided by Dr. Mayrnn Gruda (Department of Molecular Biology, Bristol Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). Both wild type His-c-Jun-(1–79) and mutant His-c-Jun-(1–79, Ala63/73) in which serines 63 and 73 of c-Jun-(1–79) were mutated to alanine, were generously provided by Dr. Dennis Templeton, Institute of Molecular and Pharmaceutical Biology, University of Georgia, and by Dr. Garry Londone (Molecular Signaling Unit, Laboratory of Cellular Development and Oncology, NIH). SEK1/MKK4 wild type (pCMV SEK1-WT), a constitutively active mutant form of SEK1 (pCMV SEK1-ED, serine 220 and threonine 224 mutated to glutamic acid and aspartic acid, respectively), the dominant negative mutation (pCMV SEK1-AL, serine 220 and threonine 224 mutated to alanine and leucine, respectively), were from Dr. Dennis Templeton, Institute of Molecular and Pharmaceutical Biology, University of Georgia, Reserve University School of Medicine. Wild type or dominants negative mutants of p54 SAPKβ (Lys8α → Ala) in pGEX, MKK3 in pCMV, and MKK6 (Ser207→Ala/Thr208→Ala) or Leu in pcDNA3 were kindly provided by Dr. Jim Woodgett, Ontario Cancer Institute, Princess Margaret Hospital. Wild type or dominant negative mutant of JNK1 (Thr183/185Ser) was kindly donated from Dr. J. Silvio Gutkind (Molecular Signaling Research Center). Recombinant p38 MAPK or JNK1 was subcloned into the pcDNA3 mammalian expression vector (Stratagene). Wild type or dominant negative mutant form of p38 MAPK or JNK1 was subcloned into the pcDNA mammalian expression vector. Primary cultured rat mesangial cells were plated and transfected at 50–80% confluence using 20 μg of DNA/75-cm² flask by using LipofectAMINE (Life Technologies, Inc). Stably transfected isolates were selected in 500 μg/ml G418 for several weeks.

**Western Blot Analysis**—At the time of harvest, cells were washed with ice-cold phosphate buffer and lysed in whole cell extract (WCE) buffer (25 mM HEPES-NaOH (pH 7.1), 0.3 mM Na3VO4, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiobitartrate (DTT), 20 mM β-glycerophosphate, 100 μM Na3VO4, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) to which 6× Laemmli sample buffer was added before heating. After boiling for 5 min, equal amounts of protein were run on 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon BF; Millipore Corp., Bedford, MA). The membranes were saturated with 5% fat-free dry milk in Tris-buffered saline (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 0.05% Tween 20 (TBS-T) for 1 h at room temperature. Blots were then incubated overnight with primary antibodies at 1:1000 dilution in 5% bovine serum albumin TBS-T. After washing with 5% milk TBS-T solution, blots were further incubated for 1 h at room temperature with goat anti-rabbit or mouse IgG antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech) at 1:3000 dilution in TBS-T. Blots were then washed five times in TBS-T before visualization. Enhanced chemiluminescence (ECL) kit (Amerham Pharmacia Biotech) was used for detection.

**In-gel Protein Kinase Assay**—Harvested cells were solubilized in WCE buffer. Protein kinase assays were performed using our previous procedures. SDS–polyacrylamide was polymerized in the presence or absence of 200 μg/ml His-c-Jun-(1–79), His-c-Jun-(1–79, Ala63/73), or 400 μg/ml MBP. After electrophoresis, SDS was removed by incubation in 20% isopropanol in 50 mM Tris-HCl (pH 8.0) for 1 h. The gel was then washed for 1 h with 1× TDTT, 50 mM Tris-HCl (pH 8.0). To denature the proteins, gels were incubated in 6× guanidine-HCl, 20 mM TDT, 2 mM EDTA, 50 mM Tris-HCl (pH 8.0) for 1 h. Gels were then rewashed by overnight incubation in 6× TDTT, 2 mM EDTA, 0.04% Tween 20, 50 mM Tris-HCl (pH 8.0). For the protein kinase assays, gels were equilibrated for 1 h in kinase buffer containing 1× TDTT, 0.1× MGTA, 20 μg/ml MBPNaO, 40 μg/ml HEPES-NaOH (pH 8.0), 100 μM Na3VO4. The kinase reaction was carried out for 1 h in kinase buffer with 50 μM MBP and 3 μCi/ml [γ-32P]ATP. Finally, the gels were washed extensively in 5% trichloroacetic acid and 1% sodium pyrophosphate until washes were free of radioactivity. Autoradiography of dried gel was performed at ~80 °C.

**Immunocomplex p38 MAPK or JNK Activity Assay**—The cell extracts were immunoprecipitated by incubation overnight with anti-p38 MAPK or anti-JNK antibody and then with protein A-Sepharose beads for 3 h at 4 °C. The beads were washed three times with 1 ml of ice-cold WCE buffer and p38 MAPK activity assayed using MBP or GST-ATF-2(1–96) as the substrate at 30 °C for 20 min in 30 ml of kinase reaction buffer (5 μg of MBP or GST-ATF-2(1–96) for p38 activity assay or His-c-Jun-(1–79) for JNK activity assay, 20 μM ATP, 10 μM of γ-32P]ATP, 25 mM HEPES and 20 mg/ml MgCl2). The reaction was terminated with Laemmli sample buffer and the products were resolved by 10% SDS-PAGE. The phosphorylated His-c-Jun, MBP, or GST-ATF-2 was visualized by autoradiography.

**Immunocomplex MKK3, MKK4, and MKK6 Activity Assay**—The cell extracts were immunoprecipitated by incubation overnight with anti-MKK3, -MKK4, or -MKK6 antibody and then incubated with protein A-Sepharose beads for 3 h at 4 °C. The beads were washed three times with 1 ml of ice-cold WCE buffer. The immune complex MKK3, MKK4, or MKK6 activity assay using GST-p38 MAPK (10 μg/reaction) as the
substrate was performed at 30 °C for 20 min in 30 μl of kinase reaction buffer (10 μg of GST-p38MAPK, 100 μM ATP, 25 mM HEPES, and 20 mM MgCl2). The reaction was terminated with Laemmli sample buffer, and the products were resolved by 10% SDS-PAGE. Phosphorylated p38αMAPK was analyzed by Western blot using anti-phosphospecific p38MAPK antibody and detected by enhanced chemiluminescence. The phosphorylation level of p38αMAPK was used to reflect MKK3, MKK6, or MKK4/SEK1 activity.

PGE₂ Determination—PGE₂ in the overlying culture medium was measured with a PGE₂ enzyme-linked immunosorbent assay kit (Cayman Chemical).

Statistical Analysis—Data were expressed as the mean ± S.E. Statistical analysis was performed using paired or unpaired Student's t test. A difference with a P value of 0.05 was considered statistically significant.

RESULTS

JNK/SAPK Mediates IL-1β-induced iNOS Expression—To determine whether the activation of JNK/SAPK in response to IL-1β is required for induction of iNOS protein expression and NO biosynthesis, stably transfected cells overexpressing JNK/SAPK in rat glomerular mesangial cells were used. We first evaluated whether a catalytically inactive form of JNK1 would function as a dominant inhibitor of IL-1β induction of iNOS expression. Overexpression of both wild type and dominant negative mutant JNK1 was verified by a Western blot assay using an anti-JNK antibody as previously demonstrated. Immunocomplex JNK activity assay demonstrated that overexpression of the kinase-dead form of JNK1 resulted in decreased IL-1β-induced JNK activity (data not shown). As shown in Fig. 1 A and B), the kinase-dead mutant JNK1 inhibited iNOS protein expression and NO production in response to IL-1β stimulation. In additional experiments we also evaluated whether the kinase-negative mutant of JNK2/p54 SAPKβ (Lys₅₅ → Ala) could inhibit iNOS expression and NO production after IL-1β stimulation. Rat mesangial cells transfected with either wild type JNK2/p54 SAPKβ or the JNK2/p54 SAPKβ kinase-inactive mutant were stimulated with IL-1β. Overexpression of JNK2/p54 SAPKβ was again verified by the Western blot analysis, followed by immunocomplex JNK activity assays which revealed that the kinase negative form of p54 SAPKβ inhibited total JNK activity induced by IL-1β. Similar to JNK1, the dominant negative JNK2/p54 SAPKβ blocked IL-1β-induced iNOS expression and NO production in renal mesangial cells (Fig. 2, A and B). It should be noted that the basal levels of both iNOS protein and NO were increased with infection of empty retrovirus pLXSN. These results nevertheless demonstrate that JNK/SAPK is important for IL-1β activation of iNOS protein expression and that the activation of JNK/SAPK is necessary for IL-1β-induced iNOS expression and NO production.

p38MAPK Is Involved in the Regulation of iNOS Expression Induced by IL-1β—We previously demonstrated that IL-1β increases p38MAPK phosphorylation and activation in rat renal mesangial cells. Pharmacological inhibition of p38MAPK with SC 68376 (2-methyl-4-phenyl-(4-pyridyl)oxazole), demonstrated an increase in iNOS expression and NO release in mesangial cells when stimulated with IL-1β (5). However, SE 203580, another p38MAPK inhibitor, was found to inhibit iNOS expression and NO production stimulated by bacterial lipopolysaccharide in glial cells (15) but have no influence on iNOS expression in human DLD-1 cells (16). A potential explanation for these differing results may be the relative tissue distribution and expression of the four isoforms of p38MAPK and the
relative selectivity of the pharmacological tools for the isoforms. To further assess the physiological function of p38αMAPK in the regulation of iNOS protein expression, we analyzed the effects of overexpression of the kinase-inactive p38αMAPK mutant on IL-1β-induced iNOS expression and NO production. Fig. 3A shows wild type and mutant p38αMAPK expressed in stably transfected mesangial cells as a fusion protein with the Flag epitope. As shown in Fig. 3 (C and D), the dominant negative mutant form of p38αMAPK functioning as a molecular inhibitor blocked iNOS expression and NO production following IL-1β stimulation. These results clearly demonstrate a physiologic function of p38αMAPK in the regulation of IL-1β-stimulated iNOS induction and NO synthesis.

MKK3 and/or MKK6 Regulate iNOS Expression Stimulated by IL-1β—MKK3 and MKK6 are upstream kinases that activate and phosphorylate p38αMAPK. We first analyzed MKK3 and MKK6 activity by an immunocomplex kinase assay using GST-p38αMAPK as the substrate and measurement of phosphorylated p38αMAPK with an anti-phosphospecific p38αMAPK antibody to verify whether MKK3 and MKK6 are involved in IL-1β signaling. We found that IL-1β increases MKK3 and MKK6 activity, as described previously, suggesting that MKK3/6 may function as an important intermediates in IL-1β signaling. Mesangial cells carrying mammalian expression plasmids MKK3 or MKK6 wild type or the kinase negative mutant stably transfected, were assessed by Western blot analysis using anti-Flag tag antibody, as described previously. Transfection of cells with dominant negative MKK3 or MKK6 inhibited p38αMAPK phosphorylation following IL-1β stimulation (Figs. 4 and 5). In these experiments, JNK phosphorylation was unaffected (data not shown). Of some significance was that transfection of wild type Flag-MKK6 into mesangial cells led to a high basal level of phosphorylation of GST-p38αMAPK but was not associated with an increase in iNOS in the absence of IL-1β (Fig. 5). This suggested that, while p38αMAPK was necessary, by itself it was insufficient for induction of iNOS. These data verify that MKK3 and MKK6 are upstream kinases that can activate p38αMAPK following IL-1β stimulation in renal mesangial cells. We examined the effects of the kinase-inactive mutant forms of MKK3 or MKK6 on iNOS expression and NO production stimulated by IL-1β. Overexpression of either kinase negative mutant (MKK3 or MKK6) resulted in the inhibition of IL-1β-induced iNOS expression and NO synthesis in renal mesangial cells (Figs. 4 and 5). These results demonstrate that both MKK3 and MKK6 may mediate IL-1β-induced p38αMAPK activation as well as iNOS protein expression and NO production.

MKK4/SEK1 Mediates IL-1β-induced iNOS Expression through Both JNK/SAPK and p38αMAPK Mechanisms—Our previous studies have demonstrated that MKK4/SEK1 activates and phosphorylates both JNK/SAPK and p38αMAPK. We analyzed the MKK4 activity by an immunocomplex kinase assay using GST-p38αMAPK as the substrate to confirm that IL-1β can enhance MKK4/SEK1 activity in mesangial cells (data not shown). Stably transfected mesangial cells containing wild type (SEK-WT), dominant negative mutant form (SEK-AL), or the constitutively active mutant form (SEK-ED) of MKK4/SEK1 were stimulated with IL-1β. We found that SEK-AL inhibited both JNK/SAPK and p38αMAPK phosphorylation. In contrast, SEK-ED enhanced IL-1β-induced JNK/SAPK and p38αMAPK phosphorylation (Fig. 6, A and B). These results suggest that MKK4/SEK1 can mediate IL-1β-induced JNK/SAPK and p38αMAPK activation in the intact mesangial cell. More importantly, our experiments show that the kinase negative mutant form of MKK4/SEK1 (SEK-AL) inhibits IL-1β-induced iNOS expression and NO production. Fig. 6 (A and B) also suggests that the constitutively active mutant form of MKK4/SEK1 (SEK-ED) enhanced basal phosphorylation of both JNK and p38αMAPK but did not alter the expression of iNOS and NO production in the absence of IL-1β stimulation (Fig. 6C). Together, these results suggest a role for JNK/SAPK and p38αMAPK activation in IL-1β-induced and modulation of nitric oxide biosynthesis in renal mesangial cells. However, it also suggests that, while both JNK and p38αMAPK are necessary, there is a requirement for additional signaling pathways for iNOS induction.

DISCUSSION

Mesangial cells serve multiple functions within the glomerulus, including regulation of glomerular filtration, elaboration of extracellular matrix, and phagocytosis of immune complexes. Our laboratory has previously reported that IL-1β induces iNOS protein expression with concomitant synthesis of nitric oxide in renal mesangial cells (4, 5, 17). The induction of this key mediator may provide a critical intermediate involved in IL-1-induced renal inflammation. For example, NO release from the glomerulus increases cGMP in mesangial cells and inhibits angiotensin II-induced mesangial contraction. Although much effort has been made to identify the intracellular signaling pathways triggered by IL-1, the signal transduction mechanisms by which IL-1 induces iNOS protein expression and NO production are still unclear. Several recent reports indicate that the MAPKs may be involved in these signaling processes. The MAPK pathway is a mechanism by which some signals are transduced from the cell membrane to the nucleus in response to a variety of different stimuli and participate in intracellular processes by further inducing the phosphorylation of intracellular substrates such as other protein kinases and transcription factors. This signaling mechanism is believed to control a wide spectrum of cellular physiological and pathophysiological functions including cell growth,
induced by IL-1 expression and NO production.

B and p38MAPK) in the signaling mechanisms recruited by the inflammatory process. To elucidate the physiological function of JNK/SAPK, we overexpressed both wild type and kinase-dead forms of JNK1 and JNK2/p54 SAPK in the signaling mechanisms recruited by the JNK/SAPK pathway.

The MAPK pathway is also involved in regulating nitric oxide biosynthesis. For example, activation of iNOS by inflammatory cytokines IL-1β and TNF-α, as well as by a wide variety of cellular stresses such as ultraviolet light, ionizing radiation, hyperosmolarity, heat shock, oxidative stress, etc. (10). In the mesangial cell, IL-1β does not activate the ERK pathway (data not shown). These findings suggest an important role for the two kinase pathways (JNK and p38MAPK) in the signaling mechanisms recruited by the inflammatory process.

The MAPK pathway is also involved in regulating nitric oxide biosynthesis. For example, activation of iNOS by inflammatory cytokines or endotoxin involves activation of ERK since the ERK kinase (MEK) inhibitor PD98059 was demonstrated to reduce iNOS expression and NO synthesis in different cell systems. To elucidate the physiological function of JNK/SAPK, we overexpressed both wild type and kinase-dead forms of JNK1 and JNK2/p54 SAPKβ in renal mesangial cells. The kinase-dead form of both JNK constructs markedly inhibits IL-1β-induced iNOS expression and NO release, thus clearly confirming the requirement of JNK/SAPK activity for cytokine-induced nitric oxide biosynthesis.

Previous data demonstrated that IL-1β increases p38MAPK phosphorylation and activation suggesting that p38MAPK is another important signaling molecule involved in IL-1 signaling. However, using a pharmacological strategy, inhibition of p38MAPK shows disparate results on iNOS expression and NO release in various cell types. For example, we previously found that SC 68376, a p38MAPK inhibitor, increases iNOS expression induced by IL-1β in mesangial cells (5). By contrast, SE 203580, another p38MAPK inhibitor, was found to either inhibit iNOS expression and NO production stimulated by lipopolysaccharide in glial cells or have no influence on iNOS expression in human DLD-1 cells (15). One possible explanation for this inconsistency is the specificity of the p38MAPK inhibitors used in the studies. Since at least four different isoforms of p38MAPK have been identified recently, one would expect that different isoforms of p38MAPK may have different biological functions. The p38MAPK inhibitors used in previous studies may not be selective enough to inhibit one particular isoform of p38MAPK. Indeed, SC 68376 was only tested as a p38MAPK inhibitor on p38αMAPK. Second, there clearly is cell-specific expression of the various isoforms (18, 19), and this needs to be added to the equation. In our current study, overexpression of the kinase-inactive mutant p38αMAPK inhibits IL-1β-induced iNOS expression and NO production, thus confirming that activation of p38αMAPK is required for iNOS expression and NO production.

MKK4/SEK1 is an immediate upstream kinase activating the JNK pathway (20, 21). We recently reported that overexpression of a constitutively active mutant form of MKK4/SEK1 increases both JNK and p38MAPK activity and phosphorylation (22). Conversely, targeted disruption of SEK1 (23) or MKK4 (24) demonstrates defects in both pathways. Since IL-1β can activate MKK4/SEK1, we tested the effects of transfection of either constitutively active or dominant negative MKK4/SEK1. We observed that the dominant negative mutant SEK-AL inhibited IL-1β-induced JNK/SAPK and p38αMAPK activation, whereas the constitutively active form activated both JNK and p38αMAPK. Furthermore, overexpression of SEK-AL resulted in inhibition of IL-1β-induced iNOS expression and NO biosynthesis.

Both MKK3 and MKK6 are upstream kinases that can activate and phosphorylate p38MAPK (25, 26). Our experiments demonstrate that IL-1β increases the activity of both MKK3 and MKK6 in renal mesangial cells. In order to ascertain whether MKK3 and MKK6 function in the regulation of p38MAPK activation and iNOS expression induced by IL-1β, wild type and kinase-dead MKK3 or MKK6 constructs were utilized. The data presented demonstrate that activation of either MKK3 or MKK6 can activate p38αMAPK, and increase iNOS expression and NO synthesis with IL-1β stimulation. Overexpression of the dominant negative mutant forms of either MKK3 or MKK6 results in marked inhibition of p38αMAPK activation and iNOS expression induced by IL-1β.

Overall, our data suggest that MKK3, MKK4, and MKK6 are involved in cytokine-induced activation of p38αMAPK and resultant iNOS expression. The observation that p38αMAPK can be equally activated by MKK3, MKK4, or MKK6 (27) suggests that p38αMAPK may function as a common substrate for these three MAPK kinases.

The aforementioned results suggest that the activation of JNK/SAPK and p38αMAPK are both necessary for induction of iNOS protein expression and NO production in the renal mesangial cells when induced by IL-1β. This conclusion is based on the observation that the inhibition of either p38αMAPK or the JNK/SAPK pathway results in significant inhibition of IL-1β-induced iNOS expression and NO production. Furthermore, in the experiments with the stably transfected mesangial cells with wild type MKK6, there was clearly enhanced basal phosphorylation of p38αMAPK but in the absence of stimulation by IL-1β, there was no induction of iNOS. These observations suggest that, while p38αMAPK is necessary for induction of iNOS, by itself it is insufficient for full activation of iNOS expression. In addition, overexpression of SEK-ED was associated with increased basal phosphorylation of JNK and p38αMAPK but by themselves were unable to stimulate iNOS expression and NO production without IL-1β stimulation. These observations are intriguing and suggest the simultaneous requirement for additional signaling pathways for full expression of iNOS. In data not shown, expression of a constitutively active mutant MEKK1 can sustain iNOS expression and NO production in the absence of IL-1β. This suggests that
MEKK1 activates additional signaling pathways in addition to JNK and p38 MAPK. MEKK1 activates both IkB kinase α and β (28–30) and, through this mechanism, activates NFκB. Based on these observations and our current findings, Fig. 7 depicts a hypothetical model for the combined role of p38 MAPK, JNK and NFκB activation in the modulation of iNOS expression. Interestingly, the converse also appears to be true, in that the binding of NFκB to DNA is insufficient for TNF-α-induced κB-dependent transcription and requires additional activation pathways (31). This occurs despite the fact that cytokine-mediated transcriptional induction of human inducible nitric-oxide synthase requires NFκB (32). This mechanism for controlling gene transcription is analogous to the concept of “transcriptional activation by recruitment” as has been suggested by Ptashne et al. (33, 34). Thus, the recruitment of c-Jun, ATF2, or Elk1 or other Ets domain transcription factor (35, 36) and NFκB may be the minimal transcription factors required for the enhanceosome (37, 38) for iNOS, which interacts with the Pol II initiation complex required for iNOS expression. Mapping of the promoter for iNOS has confirmed the presence of NFκB, AP-1, and CAAT box cis-acting regions (39, 40). Furthermore, there is evidence that AP-1 and NFκB are both involved in cytokine-mediated induction of the human nitric-oxide synthase gene (32). Recently, there is evidence that RSK-B, a CREB kinase, is under dominant control of p38 MAPK (41). Thus the evidence exists that JNK, through AP-1, and p38α MAPK could exert their effects through transcriptional mechanisms.

In summary, we demonstrate that activation of both SAPK/JNK and p38α MAPK are required for iNOS expression and NO production.
production following IL-1β stimulation. Furthermore, we demonstrate that MKK4/SEK1, MKK3, and MKK6 are all involved in IL-1β-induced nitric oxide biosynthesis. MKK3 and MKK6 function as upstream regulators of p38MAPK, whereas MKK4/SEK1 can function as the upstream kinase of both p38MAPK and SAPK/JNK signaling cascades are crucial intracellular mechanisms that mediate iNOS expression and NO synthesis induced by cytokine stress.

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