Both p38αMAPK and JNK/SAPK Pathways Are Important for Induction of Nitric-oxide Synthase by Interleukin-1β in Rat Glomerular Mesangial Cells*

(Received for publication, June 14, 1999, and in revised form, August 24, 1999)

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Interleukin 1β (IL-1β) 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 NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38MAPK. 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-1β. Similarly, overexpression of the kinase-dead mutant form of p38αMAPK also inhibits IL-1β-induced iNOS expression and NO production. In previous studies we demonstrated that IL-1β 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-1β. Overexpression of the dominant negative form of MKK4/SEK1 decreases IL-1β-induced iNOS expression and NO production with inhibition of both SAPK/JNK and p38MAPK phosphorylation. Overexpression of the kinase-dead mutant form of MKK3 or MKK6 demonstrated that either of these two mutant kinases inhibited IL-1β-induced p38MAPK (but not JNK/SAPK) phosphorylation and iNOS expression. Interestingly overexpression of wild type MKK3/6 was associated with phosphorylation of p38MAPK; however, in the absence of IL-1β, iNOS expression was not enhanced. This study suggests that the activation of both SAPK/JNK and p38MAPK signaling cascades are necessary for the IL-1β-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)3 and tumor necrosis factor α (TNF-α) 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-α, 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-1β 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 NH2-terminal kinases (JNKS), which include p54 SAPK (SAPKα/β, JNK2) and p45 SAPK (SAPKγ, JNK1); and the p38MAPK kinases (α, β, γ, and δ) (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 (MEKK1–5 or Raf in the case of ERK) phosphorolyses and activates SAPK/ERK kinase/MAP kinase kinases which include MKK7, which in turn phosphorylate and activate ERKs, JNKS, and p38MAPKs (9).

Previous work has demonstrated that both SAPK/JNK and p38MAPK cascades are activated in many cell types including renal mesangial cells, by the inflammatory cytokines IL-1 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). These findings strongly suggest a role for these two kinase pathways as important signal...

* This work was supported in part by United States Public Health Award DK 50606. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Missouri Kidney Foundation award.

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1 The abbreviations used are: IL-1, interleukin 1; iNOS, inducible nitric-oxide synthase; MAPK, mitogen-activated protein kinase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activated protein kinase; ERK, extracellular signal-regulated kinase; MEKK, MAP kinase kinase; MKK, MAP kinase kinase; MBP, myelin basic protein; TBS-T, Tris-buffered saline with 0.05% Tween 20; wt, wild type; mut, mutant; DTT, dithiothreitol; GST, glutathione S-transferase; PGE2, prostaglandin E2; PAGE, polyacrylamide gel electrophoresis; WCE, whole cell extract; SEK, SAPK activator.
ing mechanisms underlying the inflammatory process. We and others have previously demonstrated that p38MAPK 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 p38MAPK and ERK (12).

The data presented in this report suggest a requirement for both p38MAPK 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 p38MAPK and JNK to facilitate full expression of iNOS in response to interleukin-1β 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. Polyclonal or monoclonal rabbit or mouse antibodies against iNOS were from Transduction Laboratories; from Life Technologies, Inc. Polyclonal or monoclonal rabbit or mouse (MBP) was purchased from Sigma. Fetal bovine serum was purchased from Life Technologies, Inc. 15% heat-inactivated fetal calf serum, 0.3 IU/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 15 μm HEPES, pH 7.4. All experiments were performed with confluent cells grown in 25-cm² or 75-cm² flasks and used at passages 10–18. For all experiments, cells were grown in serum and serum reduced from 15% to 5% on the day of the experiment. Cells were treated with IL-1β at 50 μunits/ml for 24 h as indicated.

**Infection of Rat Mesangial Cells by Retroviral Vector—**p54 SAPKβ was subcloned into retroviral vector, pLXSN, and 10 μg of plasmid DNA was purified and used to transfect PA317 retroviral packaging cells (American Type Culture Collection CRL 9076) by LipofectAMINE (Life Technologies, Inc.). Transfected clones were selected in Dulbecco’s modifed Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418 (Life Technologies, Inc.) and then isolated by sterile glass cloning rings. Virus was harvested by placing 5 ml of mesangial medium (RPMM 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 0.3 units/ml insulin, 15 μm HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin) on confluent, 10-cm plates of transfected PA317 cultures. Twelve to 24 h later, the culture supernatant was removed and filtered through a 0.45-μm membrane (Gelman Sciences) and diluted 1:3 with mesangial medium. Hexamethyldimethrolydine (Polybrene) was then added to a final concentration of 8 μg/ml (14). Primary rat mesangial cells were obtained from adult male Harlan Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN). One ml of the culture containing primary rat mesangial cells was plated and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air to primary rat mesangial cells at 50–60% confluence. This procedure was repeated at 12 h. At 24 h in the virus-containing medium was removed and replaced by normal mesangial medium. At 48–72 h G418 was added to the medium at a concentration of 500 μg/ml. Medium was subsequently changed every 72 h. After two passages G418 was reduced to 250 μg/ml.

**Western Blot Analysis—**At the time of harvest, cells were washed with ice-cold phosphate buffer and lysed in whole cell extract (WCE) medium (15). 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.). Blotted transfected isolates were selected in 500 μg/ml G418 for several weeks.

**In-cell Protein Kinase Assay—**Harvested cells were solubilized in WCE buffer. Protein kinase assays were performed using our previously described method (15). SDS-PAGE and phosphorylation of SEK1 (pCMV SEK1-ED, serine 220 and threonine 224 mutated to alanine), wild type form of SEK1 (pCMV SEK1-ED, serine 220 and threonine 224 mutated to glutamic acid and aspartic acid, respectively), the dominant negative mutation of p54 SAPKβ (Lys55→Ala, Lys65→Ala, Gln68→Ala/Ser211→Leu) in pDNA3 were kindly provided by Dr. Jim Woodgett, Ontario Cancer Institute, Princess Margaret Hospital. Wild type or dominant negative mutant of JNK1 (Thr183→Ala/Ser368→Ser, 100 μm EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT), 20 μM β-glycerophosphate, 100 μM NaF, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) to which 6× Laemmli sample buffer was added before boiling. 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 (Amersham Pharmacia Biotech) was used for detection.
**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 addition, 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 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 p38α MAPK in the regulation of iNOS protein expression, we analyzed the effects of overexpression of the kinase-inactive p38α MAPK mutant on IL-1β-induced iNOS expression and NO production. Fig. 3A shows wild type and mutant p38α MAPK 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 p38α MAPK functioning as a molecular inhibitor blocked iNOS expression and NO production following IL-1β stimulation. These results clearly demonstrate a physiologic function of p38α MAPK 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 p38α MAPK. We first analyzed MKK3 and MKK6 activity by an immunocomplex kinase assay using GST-p38α MAPK as the substrate and measurement of phosphorylated p38α MAPK with an anti-phosphospecific p38α MAPK 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 p38α MAPK 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-p38α MAPK but was not associated with an increase in iNOS in the absence of IL-1β (Fig. 5). This suggested that, while p38α MAPK was necessary, by itself it was insufficient for induction of iNOS. These data verify that MKK3 and MKK6 are upstream kinases that can activate p38α MAPK 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 p38α MAPK activation as well as iNOS protein expression and NO production.

MKK4/SEK1 Mediates IL-1β-induced iNOS Expression through Both JNK/SAPK and p38α MAPK Mechanisms—Our previous studies have demonstrated that MKK4/SEK1 activates and phosphorylates both JNK/SAPK and p38α MAPK. We analyzed the MKK4 activity by an immunocomplex kinase assay using GST-p38α MAPK 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 p38α MAPK phosphorylation. In contrast, SEK-ED enhanced IL-1β-induced JNK/SAPK and p38α MAPK phosphorylation (Fig. 6, A and B). These results suggest that MKK4/SEK1 can mediate IL-1β-induced JNK/ SAPK and p38α MAPK 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 p38α MAPK 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 p38α MAPK activation in IL-1β-induced and modulation of nitric oxide biosynthesis in renal mesangial cells. However, it also suggests that, while both JNK and p38α MAPK 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 immune complex. Our laboratory has previously reported that IL-1β induces iNOS protein expression with concomitant synthesis of immune complex. Our laboratory has previously reported that IL-1β induces iNOS protein expression with concomitant synthesis of immune complex. Our laboratory has previously reported that IL-1β induces iNOS protein expression with concomitant synthesis of immune complex.
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 inhibited IL-1β-induced iNOS expression and NO release, thus 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 in the absence of IL-1β. 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 form of MKK1 can sustain iNOS expression and NO production induced by IL-1β.

Overall, our data suggest that MKK3, MKK4, and MKK6 are involved in cytokine-induced activation of p38MAPK and resultant iNOS expression. The observation that p38MAPK can be equally activated by MKK3, MKK4, or MKK6 (27) suggests that p38MAPK may function as a common substrate for these three MAPK kinases.
MEKK1 activates additional signaling pathways in addition to JNK and p38\(^{\alpha}\)MAPK. MEKK1 activates both IkB kinase \(\alpha\) and \(\beta\) (28–30) and, through this mechanism, activates NF\(\kappa\)B. Based on these observations and our current findings, Fig. 7 depicts a hypothetical model for the combined role of p38\(^{\alpha}\)MAPK, JNK and NF\(\kappa\)B activation in the modulation of iNOS expression. Interestingly, the converse also appears to be true, in that the binding of NF\(\kappa\)B to DNA is insufficient for TNF-\(\alpha\)-induced \(\kappa\)B-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 NF\(\kappa\)B (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 NF\(\kappa\)B 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 NF\(\kappa\)B, AP-1, and CAAT box cis-acting regions (39, 40). Furthermore, there is evidence that AP-1 and NF\(\kappa\)B 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\(^{\alpha}\)MAPK (41). Thus the evidence exists that JNK, through AP-1, and p38\(^{\alpha}\)MAPK could exert their effects through transcriptional mechanisms.

In summary, we demonstrate that activation of both SAPK/JNK and p38\(^{\alpha}\)MAPK are required for iNOS expression and NO

**Fig. 5.** Effects of wt and mut M KK6 on p38\(^{\alpha}\)MAPK phosphorylation, iNOS expression, and NO production. A, expression of epitope-tagged constructs in mesangial cells. B, effects of transfection on phosphorylation of p38\(^{\alpha}\)MAPK. C, effects of the constructs on iNOS expression in response to IL-1\(\beta\) (50 units/ml). D, effects on nitrite production.

**Fig. 6.** Effects of wt and mut SEK on phosphorylation of JNK and p38\(^{\alpha}\)MAPK, iNOS expression, and NO production. A, effects of SEK-AL, SEK-ED, and wt SEK on JNK phosphorylation. B, effects of the constructs on p38\(^{\alpha}\)MAPK phosphorylation. C, effects of the constructs on iNOS expression. D, effects of the constructs on nitrite production. IL-1\(\beta\) was used at a concentration of 50 units/ml.

**Fig. 7.** Model of the recruitment of transcription factors by IL-1\(\beta\) leading to activation and transcription of the iNOS gene.
MKK4/SEK1, MKK3, and MKK6 are all involved in IL-1β-induced nitric oxide biosynthesis. MKK3 and MKK6 function as upstream regulators of p38 MAPK, whereas MKK4/SEK1 can function as the upstream kinase of both p38 MAPK and SAPK/JNK. Together, we believe that the activation of both SAPK/JNK and p38 MAPK signaling cascades are crucial intracellular mechanisms that mediate iNOS expression and NO synthesis induced by cytokine stress.

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doi: 10.1074/jbc.274.51.36200

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