The mitogen-activated protein kinase (MAPK) cascade is believed to function as an important regulator of prostaglandin biosynthesis. Previously we reported that interleukin-1β induces activation of JNK/SAPK and p38 MAPK with concomitant up-regulation of cyclooxygenase (Cox)-2 expression and prostaglandin E2 (PGE2) synthesis. Our experiments demonstrate that overexpression of MEKK1 (a constitutively active truncation mutant of MEK1 containing the C-terminal 324 amino acids) increases Cox-2 expression and PGE2 production which is completely blocked by SC68376, a pharmacologic inhibitor of p38 MAPK. MEKK1 overexpression results in activation of both c-Jun N-terminal kinases/extracellular signal-regulated kinases (JNK/SAPK) and p38 MAPK. Furthermore, activation of MEKK1 increases SEK1/MKK4 but not MKK3 or MKK6 activity. These findings suggest that MEKK1 → SEK1/MKK4 may function as an upstream kinase capable of activating both p38 MAPK and JNK/SAPK with subsequent induction of Cox-2 expression and PGE2 production. We also found that overexpression of the constitutively active form of SEK1 (SEK1-ED) increases both p38 MAPK and JNK/SAPK phosphorylation, and increases PGE2 production and Cox-2 expression. By comparison, overexpression of the dominant negative form of SEK1 (SEK1-AL) decreases the phosphorylation of both p38 MAPK and JNK/SAPK and reduces Cox-2 expression. Together, this data suggests a potential role for the MEKK1 → SEK1/MKK4 → p38 MAPK → Cox-2 cascade linking members of the MAPK pathway with prostaglandin biosynthesis.

Prostaglandins are ubiquitous compounds involved in various homeostatic and inflammatory processes throughout the body. They are formed by the combined action of a phospholipase A₂ (PLA₂) which liberates arachidonic acid from the sn-2 position of cellular membrane phospholipids and the cytochrome P450 (Cox) which converts arachidonic acid to the endoperoxide intermediate PGH₂. PGH₂ is subsequently converted to prostaglandins by the action of cell-specific synthases. There are two cytochrome P450 enzymes which have been identified (60% homology), Cox-1 and Cox-2. Cox-1 is constitutively expressed in most tissues and mediates physiologic responses such as regulation of renal and vascular homeostasis and cytoprotection of the stomach. By comparison Cox-2 is primarily considered an inducible immediate-early gene product whose synthesis can be up-regulated by mitogenic or inflammatory stimuli including: tumor promoters (2), IL-1β (3), endotoxins (4), platelet-derived growth factor (5), and serum (6).

The physiologic role of the cyclooxygenase has been the topic of much interest. Cyclooxygenases are the main therapeutic target for non-steroidal anti-inflammatory drugs which exhibit their antipyretic, analgesic, and anti-inflammatory effects in humans via inhibition of prostaglandin biosynthesis (7). Non-steroidal anti-inflammatory drugs have been effective in the reduction of inflammatory symptoms in carrageenan-induced rat paw inflammation models (8) and in the reduced incidence of colon cancer (9, 10). Moreover, rat intestinal epithelial cells overexpressing Cox-2 demonstrate resistance to apoptosis (11) and Caco-2 human colon cancer cells demonstrate increased metastatic potential when transfected with Cox-2 (12). Perhaps, the most striking evidence for the permissive role of Cox-2 is obtained from targeted gene disruption models. Mice which are homozygously deficient for Cox-2 develop severe renal pathology and die prematurely of renal failure (13).

Recently, a novel class of cytokine-suppressive anti-inflammatory drugs have been shown to be inhibitors of endotoxin-stimulated tumor necrosis factor and IL-1 induction (14). These cytokine-suppressive anti-inflammatory drugs have been further shown to inhibit the catalytic activity of p38 MAPK. These and other recent findings serve to potentially link prostaglandin biosynthetic pathways which mediate inflammatory responses, with activation of the mitogen-activated protein kinase (MAPK) signaling cascade.

Components of the MAPK pathway have been implicated as mediators of phosphorylation of intracellular substrates such as protein kinases and transcription factors (15) as well as regulators of cell growth and differentiation (16). In mammalian cells, at least three different subfamilies of MAPK have been identified each having several isoforms. They include the extracellular signal-regulated kinases (ERKs), p44 MAPK (ERK1) and p42 MAPK (ERK2); stress-activated protein kinases; MBP, myelin basic protein; GST, glutathione S-transferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MEK, mitogen-activated protein/Erk kinases.
nases (SAPKs), also called c-Jun N-terminal kinases (JNKs); which include JNK2 (p54 SAPKα/β) and JNK1 (p46 SAPKα/β) and the p38 MAPKs (α, β, γ, and δ). These kinases are in turn activated by distinct upstream MAPK/ERK kinases (MEKs, MKKs) which recognize and phosphorylate threonine and tyrosine residues within a tripeptide motif (Thr-X-Tyr) required for MAPK activation (17). Once phosphorylated, these MAPKs then activate their specific substrates on serine and/or threonine residues to produce their effects on downstream targets. Recent work has demonstrated that both SAPK/JNK and p38 MAPK cascades are activated preferentially by the inflammatory cytokines IL-1β and tumor necrosis factor-α, as well as by a wide variety of cellular stresses such as ultraviolet light, ionizing radiation, hyperosmolarity, heat shock, oxidative stress, etc. (17). These findings further implicate the role of these two kinase pathways as important signaling mechanisms underlying the inflammatory process.

Recent work has linked activation of the prostaglandin generative pathway with the MAPK pathway. Lin et al. (18) have demonstrated that the activation of cytosolic phospholipase A₂ (cPLA₂) is mediated by MAPK. Furthermore, Kramer et al. (19) have demonstrated that thrombin activates both ERK and p38 MAPK in human platelets which may utilize cPLA₂ as a downstream target. We have shown that IL-1 stimulation of renal mesangial cells mediates prostaglandin E₂ production and Cox-2 expression with concomitant activation of p38 MAPK and SAPK-mediated signaling pathways (20, 21). Furthermore, we have demonstrated that inhibition of p38 MAPK with a pyridyl oxazole results in the down-regulation of IL-1-mediated Cox-2 expression and PGE₂ production, suggesting a role for p38 in the modulation of prostaglandin synthesis (20). Previous work by Templeton et al. (22) has shown that cells induced to overexpress a constitutively active form of ΔMEKK1 directly activate the SAPK pathway. The availability of this model system encouraged exploration of possible SAPK-mediated effects on prostaglandin production suggested by our previous cytokine stimulation data. In our current study, we describe the ability of ΔMEKK1 expressing cells to induce Cox-2 expression and PGE₂ production and analyze the intermediate kinases involved in the regulation of prostaglandin biosynthesis.

**EXPERIMENTAL PROCEDURES**

Reagents—Myelin basic protein (MBP) was purchased from Sigma. The p38 MAPK inhibitor SC88736 was kindly provided by Dr. Joe Portnova (G. D. Searle Corp.) and dissolved in dimethyl sulfoxide. Fetal bovine serum was purchased from Life Technologies, Inc. Polyclonal or monoclonal rabbit or mouse IgG antibodies against Cox-2 and Cox-1 were from Cayman Chemical Co. Inc.; cPLA₂, MEKK1, MKK3, MKK4, MKK6, JNK, phospho-specific JNK, ERK, and p38 MAPK antibodies were from Santa Cruz Biotechnology Inc. Phospho-specific p38 MAPK, SEK/MKK4, and MKK3/MKK6 were from New England BioLabs. Phospho-specific ERK antibody was from Promega. pET28-c-Jun, a histidine-tagged fusion protein expression plasmid that encodes c-Jun-(1–79), His-c-Jun-(1–79, S63A/S73A), or 400 μM MBP. After electrophoresis, SDS was removed by incubation in 50% isopropyl alcohol in 50 mM Tris-HCl (pH 8.0) for 1 h. The gel was then washed for 1 h with 1 mM DTT, 50 mM Tris-HCl (pH 8.0). To denature the proteins, gels were incubated in 6 M guanidine-HCl, 20 mM DTT, 2 mM EDTA, and 50 mM Tris-HCl (pH 8.0) for 1 h. The proteins were then renatured by overnight incubation in 1 mM DTT, 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 mM DTT, 0.1 mM EGTA, 20 mM MgCl₂, 40 μM HEPS-NaOH (pH 8.0), 100 μM NaVO₄. The kinase reaction was carried out for 1 h in kinase buffer with 30 μM ATP and 5 μM/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. PET28-c-Jun was expressed in E. coli and purified by His-binding resin (Pharmacia Biotech Inc.).

**Cell Culture and Transfection**—All experiments were performed with NIH 3T3 cells as well as NIH 3T3 cells stably transfected with an IPTG inducible EE-epitope-tagged MEKK1 truncation mutant (containing the C-terminal 324 amino acids, ΔMEKK1) subcloned into the lac switch promoter construct (Stratagene) (22, 23). Normal NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml HEPES. MEKK1 3T3 cells were maintained in the same media with 500 μg/ml of G418 and 200 μg/ml of hygromycin. ΔMEKK1 expression was induced using 1 mM IPTG for various time periods.

SEK1/MKK4 wild type (SEK1-WT), a constitutively active mutation form of SEK1 (SEK1-ED, serine 220 and threonine 224 mutated to glutamic acid and aspartic acid, respectively) or the dominant negative mutation (SEK1-AL, serine 220 and threonine 224 mutated to alanine and aspartic acid, respectively) were subcloned into the ppopRSV1 mammalian expression vector (Stratagene) and stably transfected in NIH 3T3 cells. Cells were plated and transfected at 50–80% confluence using 20 μg of DNA per 75-cm² flask using LipofectAMINE (Life Technologies, Inc.). Stably transfected isolates were selected in 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 buffer (25 mM HEPES-NaOH (pH 7.7), 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiorethithiol (DTT), 20 mM β-glycerophosphate, 100 μM NaVO₄, 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 resolved on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and developed with antibodies against Cox-2 and Cox-1, MAPK, and phospho-specific JNK, ERK, and p38 MAPK antibodies. The membranes were washed extensively with 5% fat-free dry milk in Tris-buffered saline (50 mM Tris, 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 (Amer sham) at 1:3000 dilution in TBS-T. Blots were then washed five times in TBS-T before visualization. Enhanced chemiluminescence (ECL) kit (Amer sham) was used for detection.

**In-gel Protein Kinase Assay**—Harvested cells were solubilized in whole cell extract buffer. Protein kinase assays were performed using our previously described methods (20). Briefly, SDS-polyacrylamide gel was polymerized in the presence or absence of 200 μg/ml His-c-Jun-(1–79), His-c-Jun-(1–79, S63A/S73A), or 400 μg/ml MBP. After electrophoresis, SDS was removed by incubation in 20% isopropyl alcohol in 50 mM Tris-HCl (pH 8.0) for 1 h. The gel was then washed for 1 h with 1 mM DTT, 50 mM Tris-HCl (pH 8.0). To denature the proteins, gels were incubated in 6 M guanidine-HCl, 20 mM DTT, 2 mM EDTA, and 50 mM Tris-HCl (pH 8.0) for 1 h. The proteins were then renatured by overnight incubation in 1 mM DTT, 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 mM DTT, 0.1 mM EGTA, 20 mM MgCl₂, 40 μM HEPES-NaOH (pH 8.0), 100 μM NaVO₄. The kinase reaction was carried out for 1 h in kinase buffer with 30 μM ATP and 5 μM/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. PET28-c-Jun was expressed in E. coli and purified by His-binding resin (Pharmacia Biotech Inc.).

**Immunocomplex p38 MAPK Activity Assay**—The cell extracts were immunoprecipitated by incubation overnight with anti-p38 MAPK antibody and then with protein A-Sepharose beads for 3 h at 4 °C. The beads were washed 3 times with 1 ml of ice-cold whole cell extract buffer. The immunocomplex p38 MAPK activity assay using MBP or GST-ATP-2 (1–96) as the substrate was performed at 30 °C for 20 min in 30 μl of kinase reaction buffer (5 μg of MBP or GST-ATP-2 (1–96), 20 μM ATP, 10 μCi of [γ-32P]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. The phosphorylated MBP or GST-ATP-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 antibodies and then incubated with protein A-Sepharose beads for 3 h at 4 °C. The beads were washed 3 times with 1 ml of ice-cold whole cell extract buffer. The immunocomplex MKK3, MKK4, or MKK6 activity assay using GST-p38 MAPK (10 μg/reaction) and GST-p38 MAPK were expressed as a GST fusion protein in E. coli and purified by GST-binding resin (Pharmacia Biotech Inc.).
**RESULTS**

**ΔMEKK1 Activation Induces PGE₂ Production and Cox-2 Expression**—A MEKK1 inducible model system was utilized in which NIH 3T3 cells were stably transfected with a constitutively active truncated fragment of MEKK1 (the C-terminal 324 amino acids, ΔMEKK-1) under the control of an IPTG inducible promoter. Confluent normal NIH 3T3 cells as well as ΔMEKK1 3T3 cells were incubated for varying periods of time with 1 mM IPTG to confirm overexpression of MEKK1 protein by Western blot assay using an anti-MEKK1 antibody. Normal NIH 3T3 cells were virtually unaffected by IPTG stimulation. By comparison, in MEKK1 3T3 cells, ΔMEKK1 expression was minimally detected under basal conditions and was maximally induced at 12–36 h following IPTG stimulation (Fig. 1). Notably, the MEKK1 antibody detected duplicate bands of equal intensity following IPTG stimulation. Previous data suggests that the lower band represents untagged ΔMEKK1 (resulting from an internal initiation product), whereas the upper band represents the EE epitope-tagged ΔMEKK1 (24). In this study, we examined whether activation of ΔMEKK1 expression induces prostaglandin biosynthesis. Comparison of untreated MEKK1 3T3 cells with cells induced by IPTG demonstrates an approximately 4-fold enhancement in PGE₂ production (Fig. 2B). Since cyclooxygenase is a key rate-limiting enzyme which mediates prostaglandin biosynthesis, we further examined whether the enhancement of PGE₂ production was correlated with changes in the expression of these enzymes. As shown in Fig. 2A, induction of ΔMEKK1 by IPTG resulted in a significant increase in Cox-2 expression. However, induction of ΔMEKK1 did not increase either Cox-1 or cPLA₂ protein expression (data not shown).

To demonstrate whether MEKK1-induced PGE₂ synthesis is mediated by the activation of Cox-2, a Cox-2 specific inhibitor, NS-398, was utilized. As shown in Table I, NS-398, at a concentration of 0.1 μM, completely blocked PGE₂ production induced by the activation of MEKK1. These findings suggest that Cox-2 plays an important role in MEKK1-induced PGE₂ biosynthesis.

**ΔMEKK1 induces p46 SAPK and p54 SAPK Activity**—To investigate the signal transduction pathway responsible for mediating induction of COX-2 expression in MEKK1 cells, in-gel kinase assays using His-c-Jun-(1–79) as a substrate were performed to measure SAPK activity following ΔMEKK1 induction by IPTG. Fig. 3A demonstrates that two bands corresponding to the molecular weights of p46 SAPK and p54/55 SAPK were maximally induced in MEKK1 3T3 cells by 24 h after IPTG induction. Similar experiments utilizing mutated His-c-Jun-(1–79, S63A/S73A) as a substrate demonstrated no phosphorylation of this substrate (Fig. 3B). Similarly, Western blot analysis using an anti-phospho-specific JNK which recognizes both phosphorylated (and presumably active) p46 SAPK and p54/55 SAPK further illustrated that ΔMEKK-1 induction could increase phosphorylation of both p46 SAPK and p54/55 SAPK, respectively (Fig. 3C). Western blot analysis employing a pan-JNK antibody (which also recognizes murine SAPK) indicated that ΔMEKK1 induction did not influence total protein expression of p46 SAPK or p54/55 SAPK (Fig. 3D).

**ΔMEKK1 Induces p38 MAPK Activity**—Recent data gener-
activated in our laboratory has suggested that the p38 MAPK activated pathway may contribute to the signaling mechanism for IL-1β-induced Cox-2 expression and prostaglandin synthesis in renal mesangial cells (20). With this data in mind and the above observations linking Cox-2 expression with MEKK-1 activation, we assessed IPTG-induced ΔMEKK1 3T3 cells for p38 MAPK activity. As shown in Fig. 4, p38 MAPK activity increased within 24–36 h after ΔMEKK1 induction which was examined by immunocomplex kinase assay using MBP (Fig. 4A) or GST-ATF-2(1–96) (Fig. 4B) as the substrate. This increase in activity was further correlated with Western blot analysis illustrating increased phosphorylation of p38 MAPK at 24 h following IPTG stimulation (Fig. 4D). Western blot analysis using anti-p38 MAPK indicated that total protein expression was unaffected by ΔMEKK1 induction (Fig. 4C). Together, these results demonstrate a role for MEKK1, a kinase heretofore considered upstream of the SAPK pathway, as an activator of p38 MAPK in an intact cell system.

Previous in vitro and overexpression studies suggest that MEKK1 is able to activate and phosphorylate MEK which in turn, can phosphorylate and activate ERK (23, 25). However, similar to observations by Yan et al. (22), we found that overexpression of ΔMEKK1 at the functional time points failed to activate ERK (data not shown) although SAPK and p38 MAPK were phosphorylated and activated.

p38 MAPK Mediates MEKK1-induced COX-2 Expression—To ascertain whether p38 MAPK signaling pathways mediate PGE₂ production and cyclooxygenase expression induced by ΔMEKK1 induction, we studied the effects of SC68376, an inhibitor of p38 MAPK. SC68376 directly inhibits p38 MAPK catalytic activity (IC₅₀ of 2–5 μM) without influencing ERK and JNK activity. When ΔMEKK1 3T3 cells were treated with 1 mM IPTG and 10 μM SC68376 for 24 h, the inhibitor completely eliminated Cox-2 expression and concomitant PGE₂ production normally induced by ΔMEKK1 induction. As shown in Fig. 5, SC68376 exhibits selectivity for inhibition of Cox-2 protein expression. There was negligible influence on MEKK1, SAPK, and cPLA₂ expression (data not shown). Similarly, PGE₂ production induced by ΔMEKK1 was completely inhibited by the presence of 10 μM SC68376 (Fig. 5). The above observations confirm that the activation of p38 MAPK provides a crucial signaling mechanism which promotes Cox-2 expression and prostaglandin biosynthesis.

SEK1/MKK4 Mediates MEKK1-induced COX-2 Expression—Previous data has suggested that SEK1/MKK4 is the immediate upstream kinase of SAPK/JNK (26, 27) whereas MKK3 and MKK6 are immediately upstream of p38 MAPK (28–30). Therefore, we decided to investigate whether this proposed signaling cascade was responsible for expression of Cox-2 and activation of p38 MAPK in ΔMEKK1 inducible cells. In accordance with previous findings, IPTG induction of ΔMEKK1 produced phosphorylation of SEK1/MKK4 (Fig. 6A). Furthermore, our experiments also demonstrated that the active ΔMEKK1 resulted in an increase of SEK1/MKK4 activity which was demonstrated by its ability to enhance p38 MAPK phosphorylation (Fig. 6B). However, phosphorylation of both MKK3 and MKK6 (as evidenced using an anti-phospho-MKK3/ MKK6 antibody) was not induced by IPTG stimulation (Fig. 7A). Furthermore, these findings were reinforced by immunocomplex kinase assays using p38 MAPK as the substrate for either MKK3 (Fig. 7B) or MKK6 activities (Fig. 7C). As a positive control, anisomycin (31) activated MKK3/MKK6 phosphorylation and activation in NIH 3T3 cells (Fig. 7A, right panel). Thus in both circumstances, either via kinase activity assay or direct phosphorylation analysis, MKK3 and MKK6 were not activated with IPTG induction of p38 MAPK activity. These findings suggest a signaling mechanism for activation of p38 MAPK involving activation of SEK1/MKK4 induced by MEKK1.

To further demonstrate whether SEK1/MKK4 mediates ΔMEKK1-induced p38 MAPK activation and Cox-2 expression, SEK1-WT (wild type), SEK1-ED (constitutively active form),
and SEK1-AL (kinase dead form) were stably transfected in NIH 3T3 cells, and protein expression was verified by Western blot assay using an anti-MKK4 antibody (Fig. 8A). In comparison to empty vector and SEK1-WT, expression of the constitutively active SEK1-ED increased both p38 MAPK (Fig. 8B) and JNK/SAPK phosphorylation. Notably, SEK1-ED expression enhanced Cox-2 expression (Fig. 8C) and PGE$_2$ production (Fig. 8D) which were completely blocked by p38 inhibitor, SC68376 at the dose of 10 $\mu$M (data not shown).

![Image](image-url)
Cox-2 Regulation by MEKK1

DISCUSSION

The MAPK family consists of at least three different subgroups which include: ERKs, JNKs (SAPKs), and p38 MAPK kinases. Among these groups, the JNK/SAPK and p38 MAPK pathways share activation by inflammatory cytokines, bacterial endotoxins, and environmental stress (17, 32–34), whereas the ERKs are preferentially activated by mitogens of the receptor tyrosine kinase family. Once activated, these MAPKs can phosphorylate and activate transcription factors which regulate gene expression. However, the physiological consequences for the cell following activation of these kinases remain unclear. Investigators have implicated the role of the JNK/SAPK and p38 MAPK pathway in the regulation of inflammatory mediators, cytokine production (14), platelet aggregation (35), and neuronal apoptosis (36). These kinases are in turn activated by upstream mitogen-activated protein/Erk kinases (MEKs) such as SAPK/ERK kinase-1 (SEK1, also known as MKK4) which is upstream of JNK/SAPK and MKK3/MKK6 which are upstream of p38 MAPK. These MEKKs are in turn activated by MEK kinases of which MEKK 1, 2, 3, and 5 have been characterized to date (25, 37). Previous data has implicated MEKK1 and MEKK2 as preferential activators of the JNK/SAPK pathway. MEKK 3 exhibits preference for activation of the ERK pathway in vivo (37). Currently, the identity of the MEKK (or MEKKs) upstream of the p38 MAPK pathway is unknown. Although SEK1/MKK4 can activate p38 in vitro (26), previous studies failed to demonstrate that inducible expression of MEKK1 can activate p38 in vivo.

The data presented in this paper provides evidence for the role of MEKK1 as an upstream activator of both p46 and p54 SAPK as well as p38 MAPK. More importantly, we provide evidence that cells overexpressing ∆MEKK1 can result in phosphorylation and activation of p38 MAPK. The activation and phosphorylation of both p46 and p54 SAPK as well as p38 MAPK peak within 24 h of IPTG exposure. ∆MEKK1 induction does not affect the phosphorylation and activation of ERK. These results provide an important indication that intact maminalian cells induced to overexpress ∆MEKK1 can phosphorylate and activate p38 MAPK, suggesting that MEKK1 is one of the upstream kinases regulating the p38 MAPK pathway.

Interestingly, our experiments also demonstrate that ∆MEKK1 induction by IPTG results in increased activity of SEK1/MKK4, but not MKK3 or MKK6. Co-transfection of 293 cells with MKK4 and p38 MAPK demonstrated activation of the p38 MAPK (38). Similarly, our results indicate that overexpression of the constitutively active form of SEK1 not only increases JNK/SAPK activity but also enhances p38 MAPK activity. However, overexpression of the kinase dead form of SEK1 decreases both JNK/SAPK and p38 MAPK activity. The above results clearly suggest that the MEKK1 → SEK1/MKK4 kinase cascade not only activates JNK/SAPK, but may also function as an upstream activator p38 MAPK pathway in vivo. Since several reports suggest that MKK3 and MKK6 are the immediate upstream kinases which activate p38 MAPK, it remains a possibility that MKK3/MKK6 utilizes an alternative pathway for activation of p38 MAPK.

Prostaglandins are formed by the dual action of a phospholipase A2 which releases arachidonic acid from membrane phospholipids and cyclooxygenase which converts its substrate to prostaglandin H2 which is subsequently converted to prostaglandins by cell-specific synthases. Cyclooxygenases (Cox-1 and Cox-2) function as rate-limiting enzymes in the biosynthesis of prostaglandins. Cox-2 has been identified as an inducible enzyme stimulated by cytokines, tumor promoters, and hormones (39). Previous work from our laboratory (21) has suggested that Cox-2 can be activated by IL-1 stimulation of renal mesangial cells. Cytokine treatment induces concomitant activation of JNK/SAPK and p38 MAPK (20, 21). Further evidence has demonstrated that pharmacologic inhibition of p38 MAPK by SC68376 inhibited cytokine-induced stimulation of Cox-2 and PGE2 synthesis, suggesting that p38 MAPK pathway is one of the important signaling mechanisms modulating cytokine-induced Cox-2 gene expression and prostaglandin biosynthesis (20).

In this study we show that 3T3 cells activated to overexpress ∆MEKK1 exhibit up-regulated expression of Cox-2 and in-

FIG. 7. MEKK1 does not induce MKK3 and MKK6 phosphorylation and activation. A, Western blot assay for MKK3/MKK6 phosphorylation. MEKK1 inducible NIH 3T3 cells were stimulated with 1 mM IPTG for the indicated time periods and MKK3/MKK6 phosphorylation was determined by Western blot analysis using an anti-phospho-specific MKK3/ MKK6 antibody. Lanes 1–5, MEKK1 inducible NIH 3T3 cells treated with 1 mM IPTG for 0 (lane 1), 6 (lane 2), 12 (lane 3), 24 (lane 4), and 36 (lane 5) h; lanes 6–10, NIH 3T3 cells treated by 100 μg/ml anisomycin for 0 (lane 6), 5 (lane 7), 15 (lane 8), 30 (lane 9), and 60 (lane 10) minutes. B, immunocomplex MKK3 activity assay utilizing GST-p38 MAPK as the substrate. MEKK1 inducible NIH 3T3 cells were stimulated with 1 mM IPTG for the indicated time periods. MKK3 was immunopurified by an anti-MKK3 antibody and MKK3 activity was detected by immunocomplex kinase assay with GST-p38 MAPK as the substrate. Lanes 1, NIH 3T3 cells without IPTG stimulation; lanes 2–5, MEKK1 inducible NIH 3T3 cells treated with 1 mM IPTG for 0 (lane 2), 12 (lane 3), 24 (lane 4), and 36 (lane 5) h. C, immunocomplex MKK6 activity assay utilizing GST-p38 MAPK as the substrate. MEKK1 inducible NIH 3T3 cells were stimulated with 1 mM IPTG for the indicated time periods. MKK6 was immunopurified by an anti-MKK6 antibody and MKK6 activity was detected by immunocomplex kinase assay with GST-p38 MAPK as the substrate. Lanes 1, NIH 3T3 cells without IPTG stimulation; lanes 2–5, MEKK1 inducible NIH 3T3 cells treated with 1 mM IPTG for 0 (lane 2), 12 (lane 3), 24 (lane 4), and 36 (lane 5) h.

SEK-1AL inhibited Cox-2 expression (Fig. 8C) and PGE2 production (Fig. 8D). This data clearly suggests that SEK1/MKK4 is an important intermediate kinase which regulates MEKK1-induced p38 MAPK activation and Cox-2 expression. Together, we believe that MEKK1 → SEK1/MKK4 → p38 MAPK cascade is an important upstream signaling mechanism promoting Cox-2 expression and PGE2 production.
antibody. D, Western blot assay for p38 MAPK phosphorylation. popRSV1 (lane 1), popRSV1 SEK-WT (lane 2), popRSV1 SEK-AL (lane 3), or popRSV1 SEK ED (lane 4) were stably transfected in NIH 3T3 cells and p38 MAPK phosphorylation was detected by Western blot assay using an anti-phospho-specific p38 MAPK antibody.

Cox-2 plays a critical role in MEKK1-induced PGE2 biosynthesis. Thus, we believe that the activation of MEKK1 increases Cox-2 expression and PGE2 production whereas dominant negative SEK1 inhibits this response. SEK1-induced Cox-2 expression and PGE2 production are completely inhibited by p38 MAPK inhibitor. Together, these results illustrate the ability of SEK1/MKK4 to mediate MEKK1-induced Cox-2 expression and PGE2 production and serve as an upstream kinase modulating p38 MAPK activation.

The implications of the data presented link the MAPK pathway and the prostaglandin generative pathway. Previous work has identified ERK as an activator of cPLA2 in Chinese hamster ovary cells (18). Furthermore, recent data generated in thrombin-stimulated platelets indicate the possible role of p38 MAPK as an activator of cPLA2 (19, 35). In contrast, our data demonstrates that NIH 3T3 cells stimulated to overexpress ΔMEKK1 and activate p38 MAPK do not exhibit any perceptible changes for cPLA2 total protein. We cannot exclude the possibility that increased cPLA2 activity may be involved in the regulation of MEKK1-induced prostaglandin biosynthesis. However, our data demonstrate that the Cox-2 inhibitor can completely block MEKK1-induced PGE2 production. Therefore, we believe that even if cPLA2 is involved in this regulation, it would have a minimal role in PGE2 production when Cox-2 is inhibited. These results suggest that cPLA2 is not the only target for activation by the MAPK pathway, but Cox-2 is a key mediator.

Our experiments demonstrate that overexpression of ΔMEKK1 increases Cox-2 expression and PGE2 production which is completely blocked by SC68376, a pharmacologic inhibitor of p38 MAPK. ΔMEKK1 overexpression results in activation of both JNK/SAPK and p38 MAPK. Induction of ΔMEKK1 increases SEK1/MKK4 but not MKK3 or MKK6 activity. Overexpression of the constitutively active form of SEK1 increases both p38 MAPK and JNK/SAPK phosphorylation, and enhances PGE2 production and Cox-2 expression which...
can be completely inhibited by a p38 MAPK inhibitor. Overexpression of the dominant negative SEK1-AL decreases the phosphorylation of both p38 MAPK and JNK/SAPK and reduces Cox-2 expression. Collectively, this data suggests that MEKK1 → SEK1/MKK4 may function as an upstream kinase pathway which activates both p38 MAPK and JNK/SAPK and, in turn, induces Cox-2 expression and PGE₂ production. Together, our current findings suggest a novel signaling pathway by which MEKK1 → SEK1/MKK4 → p38 MAPK → Cox-2. This pathway provides further evidence for the role of the MAPK pathway in the mediation of prostaglandin biosynthesis and as a potential target for modulation of the inflammatory response.

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