Salicylate Inhibition of Extracellular Signal-Regulated Kinases and Inducible Nitric Oxide Synthase

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Abstract—The expression of inducible nitric oxide synthase (iNOS) is a characteristic response to inflammation and can be inhibited with sodium salicylate. We used the cytokine-induced iNOS induction in cardiac fibroblasts as a model system in which to test the hypothesis that effects on mitogen-activated protein kinases (MAPKs) may explain the mechanism by which salicylate exerts its anti-inflammatory effects. Tumor necrosis factor-α (TNF-α) alone can induce extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase activity in a rapid and transient manner, whereas interferon-γ (IFN-γ) can induce only ERK. The inhibition of either the ERK pathway or p38 MAPK activity with selective inhibitors blocked cytokine-induced iNOS protein and nitrite production. Salicylate treatment inhibited iNOS expression induced by TNF-α and IFN-γ and attenuated the phosphorylation of ERK by TNF-α and IFN-γ either alone or in combination. Salicylate had no obvious effect on the activation of p38 MAPK or c-Jun N-terminal kinase. The results showed that salicylate inhibited the phosphorylation of ERK and iNOS expression induced by cytokines in a dose-dependent manner and suggested that salicylate exerts its anti-inflammatory action in part through inhibition of the ERK pathway and iNOS induction. (Hypertension. 1999;34:1259-1264.)

Key Words: inflammation ■ nitric oxide synthase ■ protein kinases ■ fibroblasts

The expression of the inducible nitric oxide (NO) synthase (iNOS) gene is an important part of the immune response to infection.1-2 The NO generated by iNOS from its substrate l-arginine has beneficial effects (eg, antimicrobial, anti-atherogenic, antiapoptotic),3-6 whereas the overproduction of NO has detrimental consequences (eg, direct cellular injury, proinflammatory).7,8 Anti-inflammatory agents often are administered to prevent the action of components of the host response to inflammation or infectious stimuli, but little is known regarding the impact of such agents on induced NO production. Earlier reports9,10 from workers in this laboratory documented that sodium salicylate or aspirin inhibits iNOS expression induced by cytokines in rat cardiac fibroblasts and suggested that the possible therapeutic role for salicylate and aspirin might involve a reduction in iNOS expression and NO production.

The anti-inflammatory actions of aspirin and its metabolite sodium salicylate have been attributed in part to inhibition of prostaglandin synthesis via inhibition of cyclooxygenase activity.11 Several recent studies have reported additional effects of these agents that also may contribute to their pharmacological effect. Sodium salicylate and aspirin have been shown to inhibit activation of the transcription factor nuclear factor-κB (NF-κB) elicited in response to inflammatory agents such as lipopolysaccharide or interleukin-1,12,13 and this inhibition was ascribed to the ability of salicylate to prevent phosphorylation and subsequent degradation of the inhibitory protein IκB-α.12,14 More recently, Yin et al15 demonstrated that salicylate specifically inhibits IκB kinase-β activity in vitro and vivo. IκB kinase-β catalyzes the transfer of phosphate moieties from ATP to IκB, thereby allowing activation by NF-κB of the gene involved in the pathogenesis of the inflammatory response.

Studies have suggested that the iNOS gene is regulated at multiple levels: transcriptional, posttranscriptional, and posttranslational.16 However, the role of the mitogen-activated protein kinase (MAPK) cascades in the control of iNOS expression has not been completely defined. In this study, we investigated whether the MAPK signaling pathway was involved in the regulation of iNOS expression and whether sodium salicylate influenced MAPK activity. Our findings suggest that the activation of extracellular signal-regulated kinase (ERK)1/2 and p38 MAPK may be necessary for the induction of iNOS expression in response to interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in cardiac fibroblasts and that sodium salicylate blocks ERK1/2 activity in a dose-dependent manner. Thus, it is possible that sodium salicylate inhibits iNOS gene expression in part through the ERK signaling pathway.

Methods

Materials

Rat recombinant TNF-α and IFN-γ were purchased from Genzyme Corp and R&D Systems. DMEM/F-12, FCS, and tissue culture reagents were obtained from Life Technologies, Inc. [α-32P]dCTP (10 mCi/mL) and [γ-32P]ATP (10 mCi/mL) were purchased from...
DuPont NEN. The p38 MAPK kit, phospho-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) antibody, phospho-p42/44 MAPK (Thr202/Tyr204) E10 monoclonal antibody, and phospho-MAPK kinase (MEK)1/2 (Ser217/221) antibody were obtained from New England Biolabs. The iNOS antibody was purchased from Calbiochem-Novabiochem Corp. Sulfanilamide, N-1-naphthylamine dihydrachloride, and sodium nitrite were obtained from Aldrich Chemical Co. Sodium salicylate (Sigma Chemical Co) was dissolved and diluted with culture medium. The pyridyl imidazole SB203580, a specific inhibitor of the p38 MAPK cascade, was from Alexis Corp and was used from a stock solution (20 mmol/L) prepared in DMSO. The inhibitor PD98059 (Calbiochem-Novabiochem Corp), which specifically blocks the ERK pathway, was used from a stock solution (25 mmol/L) prepared in DMSO.

Cell Culture
Primary cultures of neonatal rat cardiac fibroblasts were obtained and maintained in culture as previously described. Cells in the fourth to sixth passages were used for all experiments and serum starved in 0.5% FCS for 24 hours before use.

RNA Isolation and Northern Blot Analysis
Each assay was performed with 80% to 90% confluent monolayers of cardiac fibroblasts cultured in 100-mm-diameter culture dishes for the preparation of total RNA. Total cellular RNA was extracted according to the acid guanidinium thiocyanate–phenol–chloroform method, and Northern blot analysis for iNOS and β-actin was performed as previously described.

Nitrite Determination
 Fibroblasts were grown in 24-well plates. The medium was changed to DMEM/F12 lacking phenol red for 24 hours before the addition of cytokines or other agents. Aliquots of the culture medium (50 μL) were collected at the indicated time points, and nitrite content in the medium was measured with the Griess reagent as previously described.

Immunooassay for MAPKs
Immunoprecipitation experiments and p38 MAPK and JNK immunooassays were performed with use of the MAPK assay kits (catalog no. 9820) provided by New England Biolabs and essentially according to the manufacturer’s instructions. Briefly, cells were rinsed with ice-cold PBS and solubilized in lysis buffer consisting of 50 mmol/L Tris, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin. In the p38 MAPK assay, the lysates containing 100 μg of protein were immunoprecipitated through incubation at 4°C overnight with 1 μg of p38 MAPK antibody and then with protein A–Sepharose beads (Amersham Pharmacia Biotech) for 1 hour at 4°C. The beads were washed twice with PBS and twice with kinase buffer (25 mmol/L Tris, pH 7.4, 5 mmol/L β-glycerophosphate, 10 mmol/L MgCl2, 1 mmol/L DTT, and 1 mmol/L sodium orthovanadate). MAPK activity was assayed through the addition of 25 μL of kinase buffer containing 100 μmol/L cold ATP and 1 μg of GST-ATF-2 fusion protein as substrate. After incubation for 30 minutes at 30°C, the reaction was terminated by the addition of 3× SDS sample buffer (187.5 mmol/L Tris, pH 6.8, 6% SDS, 30% glycerol, 150 mmol/L DTT, and 0.3% bromphenol blue). The immunoprecipitates were separated through 10% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories), and analyzed through immunoblotting with phospho-ATF-2 (Thr28) antibody as a primary antibody. In the JNK assay, the procedures were mainly the same as for the p38 MAPK assay except for the use of the phospho-SAPK/JNK (Thr183/Tyr185) antibody instead of p38 MAPK antibody during the immunoprecipitation. In the ERK and MEK assays, whole-cell lysates were used to perform the direct immunooassay through Western blot analysis as described later.

**Figure 1.** iNOS induction by IFN-γ and TNF-α. A, Nitrite was measured with Griess reagent for designated times. Data represent mean ± SEM from triplicate assays. B, Western blot analysis of iNOS protein was performed with 20 μg of total protein obtained from cardiac fibroblasts stimulated with IFN-γ (200 U/mL) plus TNF-α (1000 U/mL) for 1 to 72 hours. C, Northern blot analysis of iNOS mRNA was performed with 10 μg of total RNA extracted from cells stimulated by cytokines for ≥72 hours.

**Results**

**Time-Dependent Induction of iNOS With IFN-γ and TNF-α**
Rat cardiac fibroblasts were stimulated with IFN-γ and TNF-α for different times, and the activity of iNOS was measured through the production of nitrite, a soluble product of NO in the culture medium. Figure 1A shows that IFN-γ (200 U/mL) and TNF-α (1000 U/mL) induced nitrite production in a time-dependent manner. A progressive increase in nitrite accumulation in the medium was found after 24 hours and reached a maximum after 72 hours. When added alone, neither IFN-γ nor TNF-α was able to induce nitrite production during the experimental time period (data not shown).

Immunoblot analysis with antibody against mouse macrophage iNOS clearly showed that TNF-α and IFN-γ markedly increased the levels of iNOS protein, consistent with the production of nitrite (Figure 1B). Although nitrite released into the culture medium was not detected during the first 6 hours, steady-state mRNA levels of iNOS were detected within 6 hours, reached a maximal level by 24 hours, and remained high for ≥72 hours. No detectable iNOS mRNA
was visible in the untreated cells or the cells treated for 1 hour (Figure 1C).

**Activation of MAPK in Response to TNF-α and IFN-γ**

To evaluate whether the MAPK pathways are involved in the induction of iNOS, we examined the ability of cytokines to activate each of the 3 MAPKs in cardiac fibroblasts. The confluent cells were treated with TNF-α (1000 U/mL) or IFN-γ (200 U/mL) alone or in combination for various times. As shown in Figure 2, TNF-α alone stimulated the phosphorylation of ERK1/2 and JNK but with different kinetics. ERK1/2 in TNF-α-stimulated cells was detected by 5 minutes, and the activity was sustained for ≈30 minutes. TNF-α induced a rapid and transient p38 MAPK activation, which increased to 5- to 7-fold more than control (untreated cells) within 10 to 15 minutes and then decreased to baseline after 15 minutes. Because the transcription factor ATF-2 is phosphorylated by both activated p38 MAPK and JNK, we performed the assay for JNK by modifying the instructions given for the p38 MAPK kit (New England Biolabs). Instead of p38 MAPK antibody, phospho-SAPK/JNK antibody was used to selectively immunoprecipitate JNK from cell lysates. The resulting immunoprecipitate was then incubated with ATF-2 fusion protein in the presence of ATP and kinase buffer. Using phospho-specific ATF-2 antibody, the phosphorylation of ATF-2 was detected with the use of Western blotting. We demonstrated that TNF-α induced the phosphorylation of ATF-2 from the JNK immunoprecipitates and that the maximal effect was reached at ≈15 minutes. When TNF-α treatment alone was compared with combined IFN-γ and TNF-α treatment, p38 MAPK and JNK were similar, whereas the peak activity for ERK was delayed, reaching a maximal value at 15 to 30 minutes after coincubation with IFN-γ and TNF-α. IFN-γ alone transiently increased the phosphorylation of ERK and slightly induced JNK activity but was unable to activate p38 MAPK significantly over control.

**Inhibition of p38 MAPK and ERK Prevents iNOS Induction With Cytokines**

To further evaluate the potential contribution of the MAPK signaling pathways in cytokine-mediated iNOS induction, we used the selective inhibitor SB203580 to block p38 MAPK activity. Cells were untreated or pretreated for 1 hour with increasing concentrations of SB203580 followed by IFN-γ and TNF-α exposure for 6 hours (with the inhibitor remaining present in the pretreated cells). Northern blot analysis was performed (Figure 3A), and significant inhibition with 1 μmol/L SB203580 and a maximal effect at ≈20 μmol/L were found. The cells were pretreated for 1 hour with SB203580 or left untreated and further treated with both IFN-γ and TNF-α for 12 hours. Cells were then immunoblotted for iNOS protein (Figure 3B). Cell medium that received IFN-γ and TNF-α treatment for 24 hours was used for nitrite determination (Figure 3C). SB203580 reduced both protein and NO production in a similar dose-dependent fashion.

The role of ERK in the regulation of cytokine-stimulated iNOS induction was studied with PD98059, a specific inhibitor of the MEK1-dependent pathway. As shown in Figure 4, cells were pretreated with PD98059 for 1 hour and then treated with IFN-γ and TNF-α for 6 hours; iNOS mRNA expression obviously did not decrease compared with IFN-γ and TNF-α treatment without the drug (Figure 4A). After IFN-γ and TNF-α treatment for 12 hours, iNOS protein was attenuated by 50% in the presence of 50 μmol/L PD98059 (Figure 4B), and NO release was similarly affected by PD98059 after cytokine treatment for 24 hours (Figure 4C). When the two inhibitors (SB203580 and PD98059) were added together, they caused a strong inhibition of iNOS mRNA, protein, and nitrite production, respectively (Figure 5, A to C). It is plausible that p38 MAPK regulates iNOS at a transcriptional and posttranscriptional level, whereas ERK possibly influenced only posttranscriptional events.
Effect of Sodium Salicylate on Induction of iNOS With Cytokines

Cardiac fibroblasts were pretreated for 1 hour with different concentrations of sodium salicylate or left untreated and then treated for an additional 24 hours with IFN-γ plus TNF-α. Figure 6 shows that iNOS mRNA, protein, and nitrite production was inhibited in a dose-dependent fashion with sodium salicylate. Measurement of iNOS protein indicated that concentrations of sodium salicylate required for the maximal and half-maximal inhibitions were 7.5 and 2.5 mmol/L, respectively (Figure 6B). iNOS mRNA (Figure 6A) and nitrite (Figure 6C) production had similar sensitivities to sodium salicylate, with an IC50 value of ~5 mmol/L.

Effect of Sodium Salicylate on Cytokine-Induced Activation of MAPKs

As shown in Figure 7A, the pretreatment of cardiac fibroblasts with increasing concentrations of salicylate inhibited the tyrosine phosphorylation of ERK induced by TNF-α or IFN-γ alone or in combination, with an IC50 value of ~5 mmol/L. Figure 7B shows that 5 mmol/L salicylate suppressed the cytokine-mediated ERK1/2 activity, whereas salicylate had no obvious effect on p38 MAPK and JNK activation.

To further explore the inhibitory effect of salicylate on ERK activity, we tested whether salicylate affected MEK1/2 activity, the kinase immediately upstream of ERK. As shown in Figure 8, either TNF-α or IFN-γ alone, or in combination, increased the phosphorylation of MEK1/2, and 5 mmol/L salicylate dramatically inhibited MEK activity induced by the cytokines in all combinations.

Discussion

The data reported here suggest that salicylate inhibited iNOS expression induced by cytokines in part through the suppression of the ERK1/2 MAPK signaling pathway. The suggestion is based on the following observations. First, TNF-α and IFN-γ individually increased the phosphorylation of ERK and in combination induced iNOS expression. Second, the inhibitor of MEK (PD98059) suppressed iNOS protein and nitrite production induced by cytokines. Third, salicylate dose-dependently attenuated iNOS expression and ERK activity. Fourth, salicylate inhibited the phosphorylation of
forms the transcription factor AP-1, and AP-1–binding sites have been identified in the murine iNOS gene promotor. iNOS induction by p38 MAPK may be explained through the use of several transcription factors: through AP-1 because p38 MAPK–mediated expression of c-jun and c-fos has been found and through NF-κB because p38 MAPK seems to be required for NF-κB–mediated transcriptional activation but it affects neither NF-κB DNA binding nor phosphorylation of its subunits and the murine iNOS gene promoter region contains 2 NF-κB–binding sites.

The regulation of iNOS expression may involve both transcriptional and posttranscriptional events. The inhibitor of p38 MAPK suppressed iNOS mRNA, protein, and NO production induced by cytokines. In contrast, the MEK inhibitor reduced only iNOS protein and nitrite release; it did not influence the level of iNOS mRNA. Discordance between the effects on iNOS protein and iNOS mRNA suggests that a posttranscriptional regulation may be involved.

The present evaluation of the roles of p38 and ERK in the regulation of iNOS expression in cardiac fibroblasts suggests that MAPKs may be involved in iNOS expression. Activation of MAPKs need not be an absolute requirement for iNOS induction, because stimulation by TNF-α alone fully activated p38 and ERK but did not induce iNOS expression. Furthermore, iNOS protein and nitrite release in the presence of 50 mmol/L PD98059 produced ~50% inhibition, whereas the same concentration of drug almost completely inhibited ERK activity when TNF-α and IFN-γ were used (data not shown). Cytokine-mediated iNOS expression may depend on pathways other than those activating either p38 or ERK, and it is conceivable that under certain conditions in specific cell types, iNOS could be induced independent of MAPK activation.

The action of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, sodium salicylate, and indomethacin, is the subject of debate. NSAIDs mediate their effects either through inhibition of prostaglandin biosynthesis or via mechanisms that are independent of effects on prostaglandin synthesis. For several reasons, salicylate probably falls within the former category. Unlike aspirin and other NSAIDs, salicylate does not inhibit cyclooxygenase activity in every experimental system. In particular, salicylate is a very weak inhibitor of the cyclooxygenase isomers in vitro. However, salicylate produces all of the classic effects of other NSAIDs in humans. Thus, various groups have sought other mechanisms through which salicylate can medulate the inflammatory response. Previous work from our laboratory and the present study demonstrated that sodium salicylate inhibited iNOS expression induced by cytokines in cardiac fibroblasts. We further showed that the pretreatment of cells with salicylate blocked the phosphorylation of ERK and MEK induced by TNF-α and IFN-γ either alone or together, whereas salicylate did not interfere with p38 MAPK and JNK activation. Taken together, these data suggest that at least some of the anti-inflammatory effects of salicylate may be due to the inhibition of ERK. The inhibitory site of salicylate on the ERK cascade may be between receptor engagement and Raf-1 activation. It has been proposed that salicylate intercalates into the plasma membrane, alters membrane viscosity, and disrupts receptor/target interactions.
wenger et al.\textsuperscript{26-27} proposed that salicylate exerts a global inhibitory effect on TNF-\(\alpha\) signaling by acting at a TNF-\(\alpha\) receptor proximal site.

In summary, we demonstrated that a significant inhibition of ERK and MEK phosphorylation can be detected with 5 mmol/L sodium salicylate, which is in a dose range probably achieved locally on the administration of salicylate during anti-inflammatory therapy.\textsuperscript{25} The results provide a possible mechanism to explain the potential anti-inflammatory effects of salicylate and suggest that ERK activation by cytokines may be involved in the subsequent induction of iNOS expression.

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References
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