Salicylate Is a Transcriptional Inhibitor of the Inducible Nitric Oxide Synthase in Cultured Cardiac Fibroblasts*

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We have previously reported that salicylate inhibits the inducible NO synthase (NOS 2) in cytokine-induced cardiac fibroblasts (Farivar, R. S., Chobanian, A. V., and Brecher, P. (1996) Circ. Res. 78, 759–768). To define further the mechanism of inhibition of NOS 2 by salicylate, we investigated NOS 2 mRNA induction by cytokines and determined the kinetics of inhibition by salicylate as compared to dexamethasone. Interferon-γ plus tumor necrosis factor-α induced NOS 2 mRNA synergistically in a time- and dose-dependent manner. Both dexamethasone and salicylate equally inhibited the induction of NOS 2 mRNA in a time- and dose-dependent fashion, both before and after cytokine induction. Salicylate also inhibited interferon-γ plus interleukin-1β-induced NOS 2 mRNA. After 24 h of cytokine stimulation, salicylate stopped the induction of NOS 2 mRNA, whereas dexamethasone delayed the accumulation of transcript. In half-life experiments of NOS 2 mRNA, we found that dexamethasone reduced the half-life of NOS 2 mRNA from 7 to 4 h, whereas salicylate had no effect on mRNA stability. Tumor necrosis factor-α and interferon-γ induced NF-κB (p50/p65) and STAT-1, respectively, as assessed by gel shift assays. Salicylate did not inhibit the cytokine induction of NF-κB or STAT-1. This study suggests that the anti-inflammatory mechanism of salicylate involves inhibition of NOS 2 transcription and shows that the effect is independent of NF-κB activation.

The mammalian Ca2+-independent NOS,1 or NOS 2, has been implicated in a variety of inflammatory diseases. Several inflammatory diseases, such as rheumatoid arthritis (1), heterotopic allograft cardiac transplant rejection (2), and septic shock (3), have been associated with an activation of the NOS 2 gene in various diverse cell types, including cardiac myocytes (4), hepatocytes (5), and macrophages (6). In each disease, up-regulation of NOS 2 initially may serve a beneficial function for the host, such as control of parasites (7), vasodilation of blood vessels, or reduction of cardiac beat rate (8). Yet chronic activation of NOS 2 may lead to undesirable consequences, such as cellular destruction, formation of highly reactive oxygen intermediates (9), and reduction of cardiac contractility (10). NO produced by NOS 2 appears to be either deleterious or protective, depending on its local milieu (11).

Inhibitors of NOS 2 have been described at the transcriptional and posttranscriptional levels. Fe3+ has been reported to inhibit the transcription of NOS 2 in macrophages without affecting NOS 2 mRNA half-life (12). In activated murine peritoneal macrophages, transforming growth factor-β1 had no effect on transcription of the NOS 2 gene but had a destabilizing effect on NOS 2 mRNA (13). In contrast, in vascular smooth muscle cells treated with IL-1β, TGF-β1 inhibited NOS 2 mRNA accumulation by inhibiting transcription with no effect on mRNA half-life (14). These cell-specific effects point to complex regulation of the NOS 2 gene.

A transcriptional inhibitor of NOS 2 is desirable because of the need to diminish the large amounts of NO produced in inflammatory diseases without deleting the protective effects of endothelium-derived NO from NOS 3 (endothelial cell NOS). In a previous study (15), we showed that the chronic administration of an inhibitor of all forms of NOS (L-NAME) sensitized rats to the fibrotic effects of exogenously added angiotensin II.

Dexamethasone, a known inhibitor of NOS 2, may have both transcriptional and posttranscriptional effects on NOS 2, depending on the species and cell type (14, 16, 17), thus acting at multiple levels to regulate NOS 2 expression. In rat glomerular mesangial cells exposed to IL-1β, dexamethasone was able to inhibit NOS 2 protein expression and nitrite accumulation without affecting NOS 2 mRNA levels (18). Surprisingly, nuclear run-on experiments demonstrated that dexamethasone markedly attenuated IL-1β-induced gene transcription. However, this was counteracted by a prolongation of the half-life of NOS 2 mRNA, accounting for the unchanged steady-state levels of NOS 2 mRNA. The decrease in nitrite was due to reduced translation and increased degradation of NOS 2 protein (18). Similarly, insulin-producing RIN m5F cells stimulated with IL-1β made less nitrite with dexamethasone addition, although NOS 2 mRNA levels were not changed (19). These observations are in contrast with the findings of Perella et al. (14), who reported that dexamethasone does not inhibit nitrite accumulation in IL-1β-induced smooth muscle cells, although it modestly decreased transcriptional rate and increased NOS 2 mRNA half-life.

We have previously demonstrated that the salicylate-like drugs have the ability to inhibit the accumulation of steady-state levels of NOS 2 mRNA and subsequent NOS 2 enzyme and nitrite production in cultured neonatal rat cardiac fibroblasts (20). The nonsteroidal anti-inflammatory drugs aspirin and salicylate, but not indomethacin or acetaminophen, could inhibit nitrite and NOS 2 mRNA accumulation in cell culture at concentrations that were equivalent to plasma levels in pa-
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Patients undergoing treatment for rheumatoid arthritis (21). Because salicylate could reduce NOS 2 mRNA levels and had been shown to have no direct effect on the enzyme, we further investigated the mechanism and are now providing evidence that this effect occurred at the transcriptional level. In addition, we tested whether this effect was mediated through the inhibition of the transcription factors NF-κB and STAT, proteins that are known to be involved in NOS 2 expression. Salicylate did not affect the induction of either NF-κB or STAT-1 by EMSA, and we suggest a novel, NF-κB-independent ability of salicylate to inhibit the transcription of NOS 2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse TNF-α and mouse IFN-γ were purchased from Genzyme Corp. (Cambridge, MA). Dulbecco’s Modified Eagle’s Medium/ Ham’s F12 medium, fetal calf serum, and penicillin/streptomycin/amphotericin B mixture were purchased from Life Technologies, Inc. α-[32P]dCTP (10 mCi/ml) was purchased from DuPont NEN. Poly(dI-dC) (polydeoxyinosinic-polydeoxyctydilic acid) was purchased from Pharmacia Biotech. Supershift antibodies against IRF-1, IRF-2, STAT-1, and STAT-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Supershift antisera against p50, p65, Rel B, and c-rel were kindly provided by Dr. Nancy R. Rice (National Cancer Institute-Frederick Cancer Research and Development Center). Sodium salicylate, fatty acid-free bovine serum albumin, insulin/transferrin/selenium mixture, protamine, dexamethasone, cycloheximide, actinomycin D, and all other reagents were purchased from Sigma. All reagents were of the highest purity available unless noted otherwise.

**Cell Culture**—Primary cultures of neonatal rat cardiac fibroblasts were prepared as described previously with only minor modifications (20). Cells in the fourth to sixth passages were used for all experiments and were serum-starved in 0.5% fatty acid-free serum for 24–48 h prior to use.

Stock solutions of the various cytokines were used as recommended by the manufacturer. All agents were diluted directly into the culture medium. Control cultures treated with the diluent (typically phosphate-buffered saline) gave results that were indistinguishable from untreated controls. All experiments were repeated at least twice for different batches of cells, and representative data are shown.

**RNA Isolation, Gel Electrophoresis, and Analysis**—Total cellular RNA was isolated from fibroblasts by the acid guanidinium thiocyanate-phenol-chloroform method (22). Northern analysis was carried out as described previously (20, 23). The cDNA probes for rat NOS 2, rat β-actin, and human GAPDH were labeled with α-[32P]dCTP by a random prime labeling method (Amersham Corp.). Densitometry was performed on a Molecular Dynamics model 300A laser densitometer.

**Preparation of cDNA Probes**—Rat NOS 2 cDNA plasmid for Northern analysis was prepared by polymerase chain reaction as reported previously (24). An insert of KpnI-BamHI restriction fragment of the rat NOS 2 plasmid (kindly provided by Dr. Robert A. Star, University of Texas, Southwestern Medical Center) was used as the cDNA probe for Northern analysis. The cDNA probe for human GAPDH was purchased from the American Type Culture Collection (Rockville, MD). The cDNA probe for rat β-actin was a gift from Dr. S. Farmer (Boston University School of Medicine) (25).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared by the method of Schreiber et al. (26) from monolayer cultures in 10-cm Petri dishes. 2 μl of extract containing 5 μg of protein was used for EMSA.

The NF-κB double-stranded oligonucleotide corresponding to the NF-κB consensus sequence in the κ light chain enhancer in B cells (27) (5′-AGG CTAG GCC GAC TTT GCC CCC AGG C-3′; Promega) was end-labeled with γ-[32P]ATP and T4 polynucleotide kinase (Promega) and purified by P-60 Biogel columns (Bio-Rad). Nuclear extracts were added to [32P]-labeled NF-κB oligonucleotide (≥10,000 counts per minute/lane) in a binding buffer containing 2 μg of poly(dI-dC) (Pharmacia), 20 mM HEPES, pH 7.9, 10% glycerol, 1 mM EDTA, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and salt (final concentration, 105 mM NaCl for NF-κB and 50 mM KCl for sir-inducible element (SIE)). Reaction mixtures were incubated for 20 min at room temperature. DNA-protein complexes were resolved on 4.0% nondenaturing polyacrylamide gel (30:1: acrylamide: bisacrylamide). Electrophoresis was for 3 h at 12 V/cm in 50 mM Tris, 0.38 mM glycine, and 2 mM EDTA for SIE, and 0.5 × TBE (1 × = 90 mM Tris-Borate, pH 8, 2 mM EDTA) for NF-κB. In competition experiments, unlabeled oligonucleotide was added to the nuclear extracts for 10 min prior to addition of radiolabeled probe. The

**RESULTS**

**Time- and Dose-dependent Induction of NOS 2 mRNA by IFN-γ plus TNF-α**—Northern analysis was performed on total RNA from cultured cardiac fibroblasts treated with cytokines. Three distinct bands were observed in the hybridization for NOS 2 as reported previously (20, 29), and only the most abundant 4.4-kb band is shown in Fig. 1. In Fig. 1A, NOS 2 mRNA was not present in control cells, nor was the transcript observable in cells treated with either TNF-α or IFN-γ as a single agent. In combination IFN-γ plus TNF-α treatment, there was a dramatic increase in NOS 2 mRNA that was apparent when TNF-α was present at 280 units/ml and reached a plateau at 1400 units/ml, with only a minimal increase at higher doses. In response to IFN-γ plus TNF-α treatment, NOS 2 mRNA was observable at 6 h (Fig. 1B) and reached a maxi-

![Fig. 1. Dose response and time course of cardiac fibroblast NOS 2 mRNA induction by IFN-γ plus TNF-α. A, fibroblasts were treated for 24 h with the indicated concentrations of IFN-γ plus TNF-α, and total RNA was obtained at the end of the incubation period. B, fibroblasts were exposed to IFN-γ (240 units/ml) plus TNF-α (850 units/ml), and total RNA was extracted from the cells at the indicated times.](Image)
Induction of the NOS 2 Gene by IFN-γ plus TNF-α Requires New Protein Synthesis—To determine whether new protein synthesis was required for the induction of NOS 2 mRNA by IFN-γ plus TNF-α, cycloheximide (1 or 10 μg/ml) was added to the fibroblasts 1 h before cytokine addition. Cycloheximide completely prevented the induction of NOS 2 mRNA by IFN-γ plus TNF-α at 6 h after cytokine addition (Fig. 2). Cycloheximide alone did not induce NOS 2 mRNA.

Dexamethasone or Salicylate Inhibited Steady-state Levels of NOS 2 mRNA both before and after Induction by IFN-γ plus TNF-α—To compare the effect of a steroidal anti-inflammatory drug on the induction of NOS 2 mRNA by IFN-γ plus TNF-α, we exposed cells to various doses of dexamethasone (10 nM to 1 μM) 30 min before cytokine addition. 24 h later, total RNA was extracted from cells for Northern analysis. Dexamethasone inhibited NOS 2 mRNA induction in a dose-dependent manner (Fig. 3A). To determine whether salicylate, a nonsteroidal anti-inflammatory drug, inhibited NOS 2 mRNA induction by cytokines, we exposed cardiac fibroblasts to salicylate for 30 min before the addition of IFN-γ plus TNF-α. Salicylate inhibited NOS 2 mRNA accumulation in a dose-dependent manner (Fig. 3B). At 4 mM, which is the concentration found in the plasma of human patients administered salicylate for rheumatoid arthritis (21), salicylate reduced mRNA levels of NOS 2 to 21% of IFN-γ plus TNF-α levels. To demonstrate that the effect is not limited to stimulation with TNF-α, salicylate (4 mM) reduced mRNA levels of NOS 2 to 40% of IFN-γ plus IL-1β levels (Fig. 3C).

To determine whether dexamethasone or salicylate could inhibit NOS 2 mRNA levels after induction by IFN-γ plus TNF-α, we treated fibroblasts with the drugs either before (30 min) or after (2 or 6 h) the addition of cytokines. Dexamethasone (1 μM) or salicylate (4 mM) decreased the NOS 2 mRNA levels to approximately 30% of induced levels both before and after cytokine addition (data not shown).

Effect of Dexamethasone and Salicylate on NOS 2 mRNA after Prolonged Induction by IFN-γ plus TNF-α—To determine whether salicylate or dexamethasone could reduce levels of NOS 2 mRNA after a prolonged induction by cytokines, after 24 h of IFN-γ plus TNF-α stimulation, salicylate (4 mM) or dexamethasone (1 μM) was added. Total RNA was extracted at 4, 8, and 24 h after salicylate or dexamethasone addition (cytokines were included during this period), and Northern anal-
ysis was performed for NOS 2 mRNA (Fig. 4). Both dexamethasone and salicylate were able to reduce mRNA levels to 40% of induced levels when present with IFN-γ plus TNF-α for 4 h. Dexamethasone was not able to maintain the suppression of NOS 2 mRNA, yet in salicylate-treated cells NOS 2 mRNA levels remained attenuated throughout the 24-h drug treatment period.

**Salicylate Does Not Affect NOS 2 mRNA Half-life, whereas Dexamethasone Destabilizes NOS 2 mRNA**—Because steady-state mRNA levels as shown by Northern analysis reflect transcription of new mRNA as well as the breakdown of existing mRNA, NOS 2 mRNA half-life was determined. To assay mRNA half-life, cardiac fibroblasts were exposed to IFN-γ plus TNF-α for 24 h, then vehicle (0.1% ethanol), salicylate (4 mM), or dexamethasone (1 μM) was added for 3 h. After this incubation period, actinomycin D (7 μg/ml) was added to the fibroblasts. Total RNA was extracted from the fibroblasts at the indicated times after the administration of actinomycin D. Northern analysis was performed using 10 μg of total RNA/lane. Also shown is the ethidium bromide-stained gel. Beneath each lane is the densitometric analysis of NOS 2 mRNA, expressed as a percentage of IFN-γ plus TNF-α stimulation.

**The Activation of NF-κB (p50/p65) and STAT-1 Is Associated with the Presence of TNF-α and IFN-γ, Respectively**—To characterize the transcription factors that were activated by cytokine addition, electrophoretic mobility shift assay was performed on nuclear extracts from fibroblasts treated with cytokines. In the presence of TNF-α (850 units/ml) for 1 h, a shift was induced that was specific for the NF-κB oligonucleotide (Fig. 6A). There was no observable band in either control cells or IFN-γ-treated cells. In IFN-γ plus TNF-α-treated cells, the shift for NF-κB was identical to that of TNF-α-treated cells. Competition experiments with nonradiolabeled NF-κB containing consensus oligonucleotides effectively eliminated the band shift in a dose-dependent manner, whereas an unrelated nonradioactive oligonucleotide for Sp1 had no effect. Supershift experiments with antisera directed against different NF-κB/Rel family members demonstrated that TNF-α activated p50 and p65 binding to the oligonucleotide but not RelB or c-rel binding (Fig. 6B).

To determine which transcription factors were activated by IFN-γ addition, EMSA was performed using oligonucleotides with consensus sequences specific for IFN-γ-activated proteins. When EMSA was performed using an oligonucleotide containing the consensus sequence for the SIE, a shift was observed only when IFN-γ was added (Fig. 7A). Nuclear extracts from control or TNF-α-treated cells did not bind to the SIE, whereas treatment with IFN-γ plus TNF-α produced a shift identical to that seen with IFN-γ-treated cells. To confirm the specificity of the induced band, unlabeled SIE oligonucleotide was added and effectively competed with the labeled oligonucleotide, whereas an oligonucleotide containing the consensus sequence for Sp1 was ineffective (Fig. 7B). To determine the protein in the SIE-shifted complex, monoclonal antibody to STAT-1 (p84/p91) was added to the binding mixture (Fig. 7C). This resulted in a supershift of the SIE complex, confirming that STAT-1 is a protein that is part of the complex. In contrast, anti-IRF-1, IRF-2, and STAT-3 antibodies did not induce a supershift. In contrast to the data obtained using the SIE oligonucleotide, when an oligonucleotide containing the consensus sequence for GAS/ISRE was used, no detectable gel shift was found.

**Salicylate Does Not Inhibit the Induction of NF-κB or STAT-1 by EMSA**—Because it has been reported that salicylate could inhibit NF-κB activation in cell culture (30–32), we performed EMSA on nuclear extracts from cells treated with TNF-α in the presence of salicylate. Salicylate, at concentrations of 1–20 mM in combination with TNF-α clearly did not prevent the gel shift of NF-κB (Fig. 8).
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These studies extend our previous findings, which indicated that the inhibitory effect of salicylate was independent of changes in cyclooxygenase activity and did not occur by direct effects of the drug on enzymatic activity (20). Several previous studies (34–36), including our own (20), have described the inhibition of the NOS 2 gene by various aspirin-like drugs, and a reduction in steady-state mRNA levels for NOS 2 occurred when salicylate was added either to rat alveolar macrophages (34) or rat cardiac fibroblasts. In one case, salicylate was reported to inhibit nitrite production, but changes in NOS 2 mRNA were not measured (36). In another study, salicylate reduced nitrite accumulation without reductions in NOS 2 mRNA (35). Indirect evidence was provided that salicylate may be inhibiting the transcription factor NF-κB as part of the final common pathway responsible for the inhibition of NOS 2 mRNA (20, 34); furthermore, it was not clear in any study whether salicylate affected transcription or mRNA stability.

Cycloheximide completely abolished the increase in steady-state mRNA for NOS 2 that was induced by the required combination of both TNF-α and IFN-γ, implicating new protein synthesis as a necessary requirement for the induction of NOS 2 mRNA in cardiac fibroblasts. Previous studies have reported that NOS 2 mRNA induction may be either dependent on or independent of new protein synthesis (5, 29, 37). In rat hepatocytes treated with a combination of four cytokines (IFN-γ + TNF-α + IL-1β + LPS), cycloheximide blocked the induction of NOS 2 mRNA (5). This differed from cultured vascular smooth muscle cells stimulated by IFN-γ plus LPS, in which cycloheximide had no effect on the induction of NOS 2 mRNA (37). The transcription factors implicated in cytokine-induced NOS 2 expression to date are NF-κB (38), IRF-1 (39), and STAT-1 (40), each of which is known to be present constitutively in the cytosol of target cells and therefore not susceptible to cycloheximide treatment. The transcription factor CIITA (major histocompatibility complex II transactivator) has been shown to be a cycloheximide-sensitive IFN-γ-inducible factor in human monocytes (41). CIITA is aberrantly processed in Bae lymphocyte syndrome, a rare disorder in which all lymphocytes are missing the class II major histocompatibility complex (42), but there is no information suggesting a role for this factor in NOS 2 gene regulation.

The effects of salicylate and dexamethasone were compared with respect to the kinetics of NOS 2 mRNA induction, and both agents effectively suppressed cytokine-induced NOS 2 mRNA when added either before or after the cytokines. Dexamethasone was the more potent inhibitor when compared on a molar basis, but both drugs were effective in a dose-dependent
manner over a concentration range considered reasonable for treatment of human disease. Our findings contrast with those of Perella et al. (14), in which dexamethasone was unable to inhibit NOS2 when given to vascular smooth muscle cells after induction by IL-1β. Dexamethasone is a steroidal anti-inflammatory drug that acts transcriptionally by binding to a cytosolic glucocorticoid receptor and then translocating to the nucleus and interacting directly in the promoter region of target genes. For the murine NOS2 gene, in the 1749 base pairs upstream of the transcriptional start site, there are no consensus sequences homologous to defined steroid-responsive elements (43). Thus, the effects of dexamethasone could be posttranscriptional, or they may occur by mechanisms analogous to those reported to occur by protein-protein interactions with the transcription factors AP-1 (fos/jun) (44) or the p65 subunit of NF-κB (45). It has been proposed that the inefficacy of steroid treatment of septic shock may be due to the timing of glucocorticoid administration, i.e., after NOS2 induction (14). Because dexamethasone was able to inhibit NOS2 mRNA after cytokine induction in cardiac fibroblasts, this may provide insight into why dexamethasone is used for the treatment of diseases such as myocarditis, in which cardiac fibroblast NOS2 mRNA may be induced.

Salicylate, a nonsteroidal anti-inflammatory drug, was not as potent as dexamethasone but was equally effective in its ability to inhibit NOS2 mRNA. The doses used were in the pharmacological range (1–4 mM), i.e. the plasma range for human beings undergoing salicylate treatment for inflammatory diseases, such as rheumatoid arthritis (21). Rheumatoid arthritis is one of a host of diseases, including septic shock (3), cardiac transplant rejection (2), and iatrogenic hypotension due to cytokine chemotherapy, in which NOS2 expression may be a final common pathway for hypotension and/or tissue destruc-

Fig. 7. STAT-1 induction by IFN-γ in cardiac fibroblasts. A, cardiac fibroblasts were treated with vehicle (control), IFN-γ (480 units/ml), TNF-α (850 units/ml), or IFN-γ plus TNF-α for 1 h before preparing nuclear extracts for analysis by EMSA with a probe for SIE. Probe indicates a reaction with no protein extracts, and NS refers to a nonspecific band. B, specificity of induced bands. Binding was carried out in the presence or absence of a molar excess of nonradioactive SIE (50 × and 500 ×) or Sp1 (500 ×). C, supershift analysis using antibodies directed against STAT-1, STAT-3, IRF-1, and IRF-2.
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and in IL-1β (20). A single agent, such as IL-1β via general inhibition of kinases, as has been recently suggested, is possible that the inhibitory effects of salicylate are mediated via STAT activation. It is anticipated, salicylate did not inhibit STAT activation. It is possible that the inhibitory effects of salicylate are mediated via general inhibition of kinases, as has been recently suggested, or by a more selective inhibition of a specific kinase or class of kinases.

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