Essential Role of C-Rel in Nitric-Oxide Synthase-2 Transcriptional Activation: Time-Dependent Control by Salicylate

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ABSTRACT

To determine the role of C-Rel in nitric-oxide synthase-2 (NOS-2) transcriptional activation, we evaluated the effect of lipopolysaccharide and interferon-γ (LPS/IFNγ) on C-Rel DNA binding in RAW 264.7. LPS/IFNγ-stimulated C-Rel binding peaked at 4 to 8 h and declined at 24 h. Transfection of cells with a C-Rel small interfering RNA abrogated C-Rel binding at all time points. LPS/IFNγ produced superoxide at 4 h, which subsided at 8 h. C-Rel binding and NOS-2 expression were abrogated by superoxide dismutase or apocynin at 4 h, suggesting a key role that superoxide plays in mediating C-Rel binding and NOS-2 transactivation only at 4 h. We have reported previously that salicylate at 10^{-5} M inhibited LPS/IFNγ-induced CCAAT/enhancer binding protein β (C/EBPβ) binding at 4 h but not at 8 or 24 h. A single dose of salicylate did not inhibit C-Rel binding at any time point. The addition of a second dose of salicylate 4 h before an indicated endpoint suppressed C-Rel but not C/EBPβ or interferon-γ-regulated factor-1 binding at 8 and 24 h. A single dose of salicylate added with LPS/IFNγ inhibited NOS-2 expression only at 4 h. However, salicylate supplement inhibited NOS-2 promoter activities and mRNA and protein levels throughout 24 h. Signal profiling with a panel of inhibitors revealed time-dependent switch of signaling pathways. These results demonstrate temporal regulation of transactivator binding by LPS/IFNγ via evolving signaling pathways. We propose that salicylate inhibits C/EBPβ binding at 4 h and C-Rel binding at 8 and 24 h by targeting related kinases.

Nitric-oxide synthase-2 (NOS-2, also known as inducible NOS) plays an important role in inflammation, septic shock, and tissue injury (Bredt and Snyder, 1994; Nathan, 1997). It is highly inducible by cytokines and lipopolysaccharide (LPS). Its transcriptional activation by LPS and interferon-γ (IFNγ) has been extensively investigated in inflammatory cells such as RAW 264.7, a murine macrophage cell line. LPS and IFNγ synergistically stimulate NOS-2 promoter function by inducing binding of several transcriptional activators to their respective enhancer elements located at two clusters of the promoter region within approximately 1 kilobase from the transcription start site of murine NOS-2 (Lowenstein et al., 1993; Xie et al., 1993). Among the transactivators, NF-κB has been shown to bind κB sites on the NOS-2 promoter and activate NOS-2 expression (Xie et al., 1994). NF-κB comprises five isoforms, three in the Rel family (C-Rel, RelA, and RelB) and two in the NF-κB family (NF-κB1 or p50, and NF-κB or p52). RelA was demonstrated to be essential for NOS-2 expression (Xie et al., 1994). Although C-Rel was reported to bind NOS-2 promoter, its role in NOS-2 transac-

ABBREVIATIONS: NOS-2, nitric-oxide synthase-2; COX, cyclooxygenase; C/EBPβ, CCAAT/enhancer binding protein β; LPS, lipopolysaccharide; IFNγ, interferon γ; IRF-1, interferon-γ regulated factor-1; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; RSK, p90 ribosomal S6 kinase; mTOR, mammalian target of rapamycin; Jak, Janus-activated kinase; PI-3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; GFP, green fluorescent protein; NF-κB, nuclear factor κB; BZT, 1,2,3-benzenetriol; SOD, superoxide dismutase; qPCR, quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation; PBSI, phosphate-buffered saline buffer containing Na$_3$VO$_4$, NaF, β-glycerophosphate, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and dithiothreitol; PD98059, 2′-amino-3′-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; AG490, α-cyano-(3,4-dihydroxy)-N-benzylcinnamide; GF109203X, 3-[1-[3-(dimethylaminopropyl)-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione monohydrochloride.

tivation is unclear (Xie et al., 1994). C-Rel is critical for lymphocyte development and function (Kontgen et al., 1995; Liou et al., 1999; Sanjabi et al., 2000), but its physiological function in nonlymphoid cells is less clear.

Salicylate at millimolar concentrations was reported to suppress NF-κB transactivation, but its isoform target was not characterized (Kopp and Ghosh, 1994). We have observed that salicylate at micromolar concentrations suppresses NOS-2 and cyclooxygenase-2 (COX-2) transcriptional activation in RAW 264.7 cells stimulated with LPS/IFNγ for 4 h by selectively inhibiting binding of CCAAT/enhancer binding protein-β (C/EBPβ) to C/EBP enhancer elements on both genes (Cieslik et al., 2002). Salicylate also abrogates COX-2 transcriptional activation in human fibroblasts and endothelial cells (Xu et al., 1999; Saunders et al., 2001) stimulated with phorbol 12-myristate 13-acetate and interleukin-1β for 4 h via suppression of C/EBPβ binding (Saunders et al., 2001). Thus, salicylate at micromolar concentrations targets C/EBPβ DNA binding induced by proinflammatory mediators. However, it is unclear whether salicylate exerts any action on C-Rel DNA binding or C-Rel-mediated NOS-2 transcriptional activation in RAW 264.7 cells stimulated with LPS/IFNγ. The purpose of this study was to determine the role of C-Rel in LPS/IFNγ-induced NOS-2 expression and the influence of salicylate on C-Rel DNA binding and NOS-2 promoter activation. The results show that LPS/IFNγ induced C-Rel binding to a κB probe in a time-dependent manner. LPS/IFNγ-induced NOS promoter activity was abrogated by a specific C-Rel small interfering RNA (siRNA). Salicylate added with LPS/IFNγ inhibited NOS-2 expression at 4 h but not at subsequent time points. However, supplemental salicylate added 4 h before each indicated endpoint suppressed COX-2 promoter activity by ~50%, and inhibited C-Rel DNA binding at 8 and 24 h but not at 4 h. In contrast, supplemental salicylate did not inhibit C/EBPβ binding at 8 or 24 h. Signal profiling using a panel of inhibitors revealed time-dependent switch of signaling pathways via which C-Rel binding and NOS-2 transcription were activated. Changes in signaling pathways over time may account for differential inhibition of C-Rel and C/EBPβ binding at different time points.

Materials and Methods

Materials. Lipofectamine 2000, Dulbecco's modified Eagle's medium high glucose medium, OPTI-MEM 1 medium, and antibiotics were obtained from Invitrogen (Carlsbad, CA); rabbit polyclonal anti-NOS-2 antibodies were from Upstate Biotechnology (Lake Placid, NY); mouse monoclonal antiactin and rabbit polyclonal anti-RelA antibodies were from Oncogene (Boston, MA); and rabbit polyclonal anti-RelB and anti-C-Rel were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence solution and bicinchoninic acid reagent for protein concentration assay were obtained from Pierce (Rockford, IL); IFNγ was from Roche (Indianapolis, IN); fetal bovine serum, LPS (*Escherichia coli* 0111:B4), 1,2,3-benzenetriol (BZT), and Zn/Cu superoxide dismutase (SOD) were from Sigma (St. Louis, MO). Apocynin, a selective inhibitor of NADPH oxidase, was obtained from Avocado Research Chemicals Ltd. (Heysham, Lancashire, UK). High-Capacity cDNA Archive Kit, TaqMan Universal PCR Master Mix, 5-carboxyfluorescein-labeled NOS-2 probe, and VIC-labeled 18S probe were obtained from Applied Biosystems (Foster City, CA). Signaling inhibitors including PD98059, LY294002, SB203580, AG490, GF109203X, and rapamycin were obtained from Calbiochem (San Diego, CA).

Cell Culture. RAW 264.7 cells obtained from American Type Culture Collection (Manassas, VA) were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin (Cieslik et al., 2002). Cells were washed and cultured in serum-free medium for 24 h before experiment. After washing, they were incubated in fresh serum-free medium in the presence or absence of LPS (2 μg/ml) and IFNγ (40 U/ml) for 4, 8, and/or 24 h.

Transfection and Promoter Activity. A 1.6-kilobase NOS-2 promoter fragment ($-1486/+145$) was cloned into pGL3 luciferase vector as described previously (Deng et al., 2003a). RAW 264.7 cells at 60% confluence were transfected with 4 μg of the NOS-2 promoter construct using Lipofectamine according to a procedure described previously (Cieslik et al., 2002). Eighteen hours after transfection, cells were incubated in serum-free medium for 24 h followed by stimulation with LPS and IFNγ. Cells were harvested, and luciferase activity was measured in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). To monitor transfection efficiency, we cotransfected cells with a green fluorescent protein (GFP) expression vector and analyzed GFP expression by fluorescent microscopy. The transfection efficiency based on GFP expression is consistently ~50% with a low level of variability. In each experiment, cellular proteins were measured, and the result was expressed as relative light unit per microgram of protein. The assay was performed in triplicate, and each experiment was repeated at least three times.

Whole-Cell Lysate. Cells were washed with cold PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), harvested and spun down at 550g for 5 min. Cell pellet was dissolved in PBS buffer containing 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl$_2$, 1 mM EDTA, 0.1 mM PMSF, 50 mM NaF, 1 mM Na$_3$VO$_4$, and 0.06 mg/ml aprotinin. Cell pellet was then spun down at 6500g for 5 min. Supernatant was collected, and protein concentration was determined.

Nuclear Extract Preparation. Cells were harvested in 1 ml of PBSI buffer (phosphate-buffered saline buffer containing multiple protease inhibitors: 1 mM Na$_3$VO$_4$, 10 mM NaF, 25 mM β-glycerophosphate, 0.1 mM PMSF, 0.06 mg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM dithiothreitol) and spun down at 550g for 5 min. Pellet was resuspended in 10 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 300 mM sucrose, 0.5% Nonidet P-40, 0.1 mM PMSF, 0.06 mg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM dithiothreitol and placed on ice for 10 min. Cells were centrifuged at 2600g for 20 s. Supernatant containing cytosolic proteins was kept for further analysis. Pellet was dissolved in PBSI and sonicated for 30 s. Nuclear homogenate was spun down at 10,400g for 5 min and the supernatant was aliquoted and kept at $-80°$C.

Western Blot Analysis. Western blotting was performed by a procedure described previously (Cieslik et al., 2002). A 20-μg sample of whole-cell lysate or nuclear extract proteins was loaded to each lane, separated by 4 to 15% gradient SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Proteins were detected by enhanced chemiluminescence method using specific antibodies.

Protein-DNA Binding Assay. The binding assay was performed as described previously (Zhu et al., 2002; Deng et al., 2003a). Nuclear extract proteins (400 μg) in 600 μl of PBSI buffer were incubated with a mixture of 4 μg of double-stranded biotinylated oligonucleotides containing a murine NOS-2 promoter NF-κB sequence (underlined) at -974 to -964 (5'-CTAGG<u>GGGGATTTTCCC</u>TCTC-3') (synthesized by Integrated DNA Technologies, Coralville, IA) and 40 μl of 4% beaded-agarose conjugated with streptavidin for 2 h (Sigma) on a rocking platform at room temperature. The beads were collected by centrifugation at 550g for 1 min, washed three times with PBSI, and resuspended in 40 μl of Laemmli sample buffer. Nuclear proteins bound to the beads were dissociated by incubating the mixture at 95°C for 5 min and then applied to Western blot analysis.

Luminol-Dependent Chemiluminescent Assay. We measured reactive oxygen species (ROS) by luminol-dependent chemilumines-

cent assay (Vilim and Wilhelm, 1989) using an assay kit from Calbiochem. Cell pellets were resuspended in superoxide anion medium containing 125 μM chemiluminescence enhancer and 100 μM luminol. Chemiluminescence was measured in a Turner TD-20/20 luminometer. The protein concentration in the sample was determined, and the result was expressed as relative light unit per microgram of protein.

Quantitative PCR. Quantitative PCR was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using a TaqMan two-step reverse transcription-PCR method. Total cellular RNA was isolated by a kit from RiboPure, Ambion (Austin, TX) according to manufacturer's protocol. Total RNA (1 μg) was used for reverse transcription (High-Capacity cDNA Archive Kit), on GeneAmp PCR 9700 system (Applied Biosystems). Transcribed DNA (10 ng) was applied to qPCR reaction. As a target probe, we used TaqMan MGB murine NOS-2 labeled with 5-carboxyfluorescein dye (Applied Biosystems). As an endogenous control, we used TaqMan MGB18S probe labeled with VIC dye (Applied Biosystems). We used relative quantitation standard curve method to calculate the amount of target mRNA normalized to an endogenous reference (18S). Each sample was tested in triplicates to ensure reproducibility. Evaluation of threshold cycle, amplification plot, and spectra was done using an ABI PRISM 7000 Sequence Detection System Version 1.0 (Applied Biosystems).
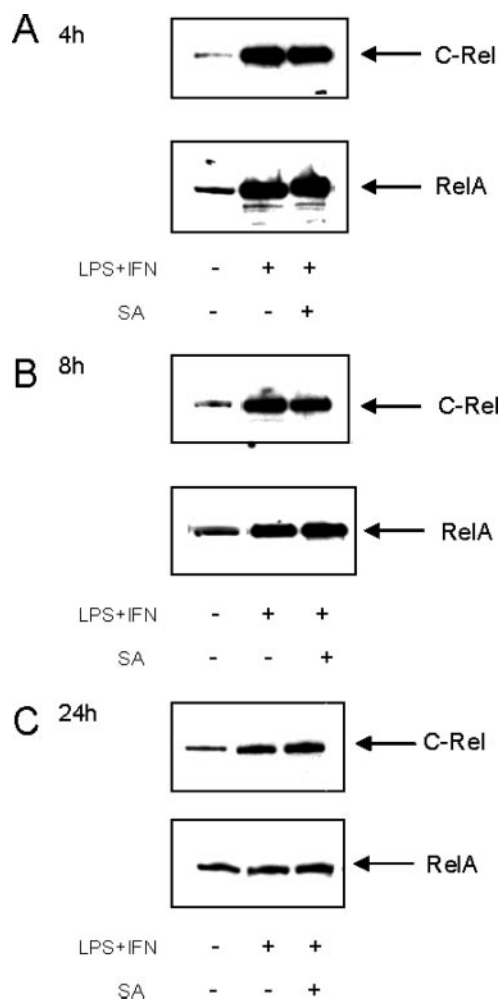

Fig. 1. LPS/IFNγ-induced C-Rel binding to κB probe was not inhibited by salicylate. A, RAW 264.7 cells were treated with LPS/IFNγ in the presence or absence of sodium salicylate (10^{-5} M) for 4 h (A), 8 h (B), or 24 h (C). Nuclear extracts were prepared, and C-Rel and RelA binding was analyzed by streptavidin-agarose pulldown assay. The figures are representative of three experiments with similar results.
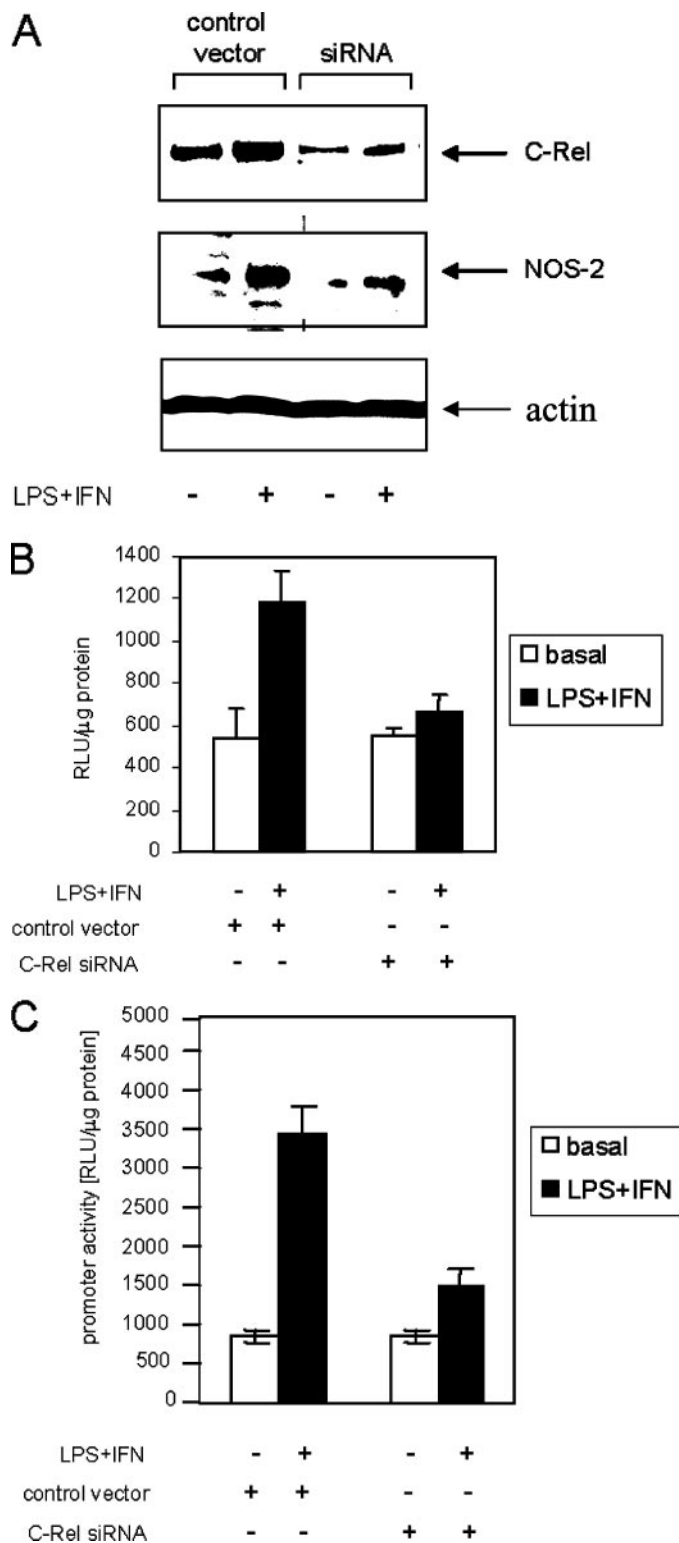

Fig. 2. Suppression of NOS-2 protein expression and promoter activity by C-Rel-specific siRNA. RAW 264.7 cells were transfected with siC-Rel or control vector, and the transfected cells were treated with or without LPS/IFNγ for 4 h. A, C-Rel, NOS-2, and actin proteins were analyzed by Western blots. A representative blot from three experiments is shown. B and C, siC-Rel transfected cells were transfected with a NOS-2 promoter luciferase expression vector and were treated with or without LPS/IFNγ for 4 (B) or 24 h (C). Luciferase activity was determined. Each bar denotes mean $\pm$ S.E.M. of three experiments.

Chromatin Immunoprecipitation Assay. The chromatin immunoprecipitation (ChIP) assay was used to determine binding of C-Rel to NOS-2 promoters according to procedures described previously (Deng and Wu, 2003b). In brief, RAW 264.7 at 80 to 90% confluence were serum-starved for 24 h followed by treatment with LPS/IFNγ in the presence or absence of SOD for 4 h and then incubated with formaldehyde (1%) at 37°C for 20 min. Cells were washed and lysed in a lysis buffer containing multiple protease inhibitors. One third of the lysates was used as DNA input control. The remaining two thirds were used in immunoprecipitation with a C-Rel antibody or control IgG. After extensive washing of the precipitates, cross-linked chromatin-protein complex was reversed at 65°C for 5 h followed by proteinase K for 3 h at 50°C. DNA was isolated and used as a template for PCR amplification with specific NOS-2 promoter primers (5′ primer: − 498 CTGCCCAAGCTGACT-TACTAC −478 and 3′-primer: −1 GACCCTGGCAGCAGCCATCAG −21). The resulting product of 498-base pair NOS-2 promoter fragment was separated and detected by agarose gel electrophoresis.

Statistical Analysis. Statistical significance was analyzed by Student's t test.

Results

C-Rel Played an Essential Role in LPS/IFNγ-Induced NOS-2 Expression. LPS/IFNγ increased C-Rel binding to a κB probe in a time-dependent manner (Fig. 1). The C-Rel DNA binding activity reached plateau at 4 h, persisted at 8 h, and decreased 24 h after LPS/IFNγ treatment. For comparison, we determined the time-dependent DNA binding of RelA and RelB. RelA binding was increased by LPS in a time-dependent manner closely resembling C-Rel binding (Fig. 1), whereas RelB binding was not regulated by LPS/IFNγ (data not shown). To determine whether LPS/IFNγ-induced transcriptional activation requires C-Rel, we transfected RAW 264.7 cells with C-Rel siRNA or control vector and treated the transfected cells with LPS/IFNγ for 4 and 24 h. C-Rel protein levels were reduced by siRNA by ∼75% (Fig. 2A), confirming a significant knockdown of C-Rel. LPS/IFNγ-induced NOS-2 proteins (Fig. 2A) and promoter activities (Fig. 2B) at 4 h were inhibited by siRNA to the basal level. NOS-2 promoter activities remained suppressed in siRNA-transfected cells treated with LPS/IFNγ for 24 h (Fig. 2C). These results indicate C-Rel plays a key role in LPS/IFNγ-stimulated NOS-2 expression.
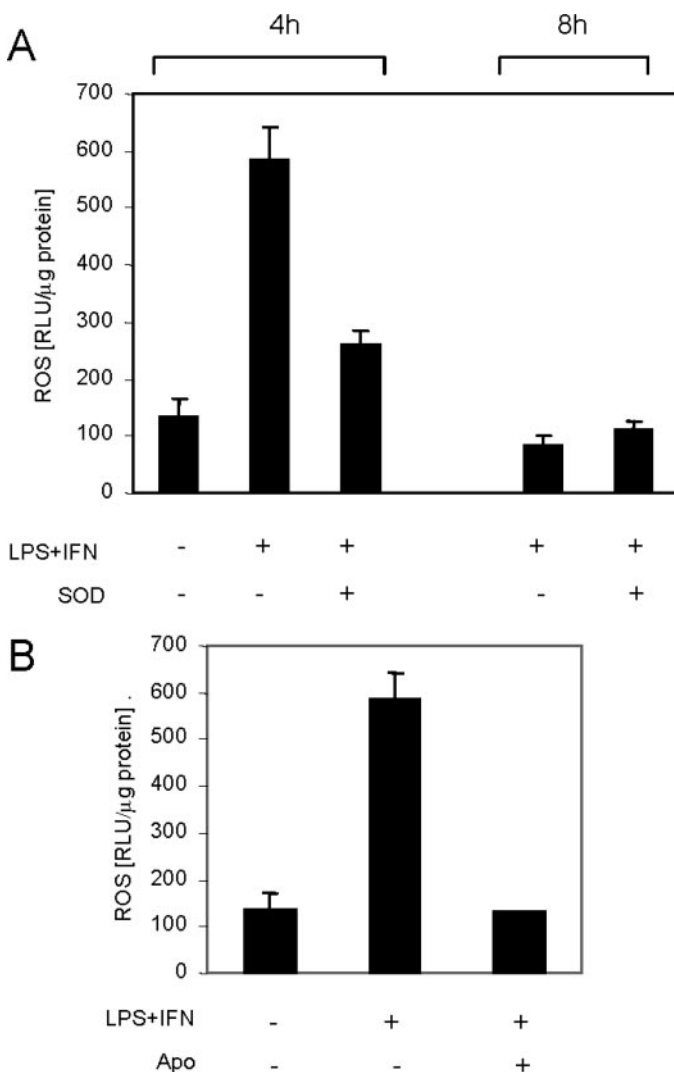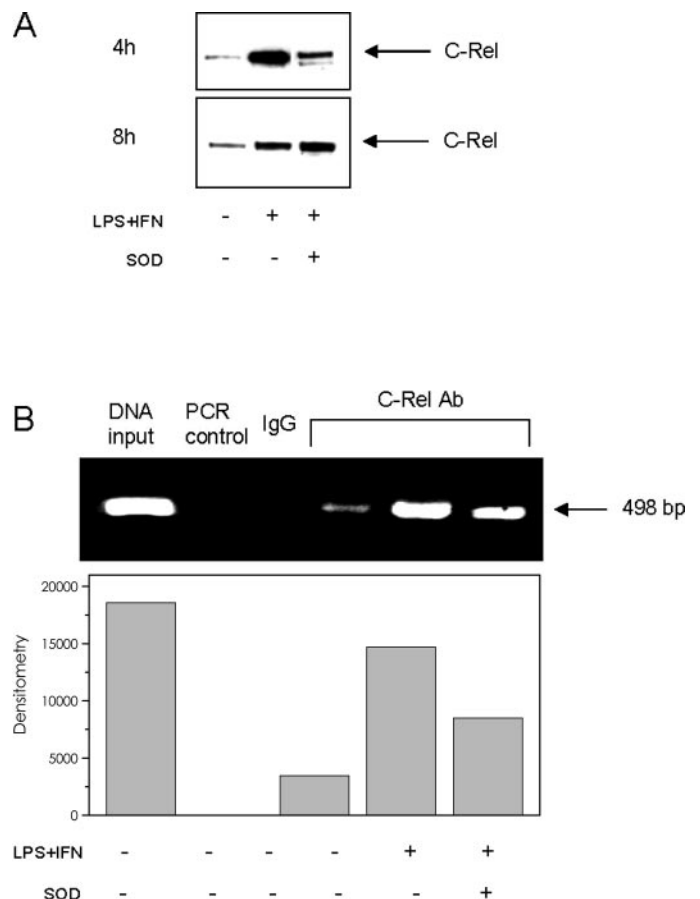

Fig. 3. Generation of ROS in LPS/IFNγ-treated cells. A, RAW 264.7 were pretreated with SOD (30 U/ml) for 30 min followed by LPS/IFNγ for 4 and 8 h. ROS was measured as described under *Materials and Methods*. B, cells were pretreated with apocynin (Apo) (1 mM) followed by LPS/IFNγ for 4 h. Each bar represents the mean ± S.E.M. of three experiments.

Fig. 4. Role of ROS in C-Rel binding. A, RAW 264.7 cells were treated with SOD followed by LPS/IFNγ for 4 or 8 h, and C-Rel binding was analyzed by the streptavidin-agarose pulldown assay using a biotinylated κB probe. B, ChIP analysis of C-Rel binding to NOS-2 promoter region in immunoprecipitated chromatin. IgG denotes precipitation with a nonimmune IgG. Densitometric analysis of the 498-base pair band is shown at the bottom.

Superoxide-Mediated C-Rel Binding Was Not Inhibited by Salicylate. We have shown that a single dose of salicylate (10^{-5} M) added to cultured cells 30 min before, at the same time with, or 30 min after LPS/IFNγ inhibited NOS-2 expression via blocking C/EBPβ DNA binding at 4 h but not at 8 or 24 h (Cieslik et al., 2002). To determine whether salicylate influences C-Rel DNA binding, we incubated RAW 264.7 cells with sodium salicylate (10^{-5} M) and LPS/IFNγ for 4, 8, or 24 h, and at each time point, binding of nuclear extract C-Rel to a κB probe was assayed. Salicylate did not inhibit C-Rel binding to the κB probe at any of the three time points (Fig. 1, A–C). Likewise, salicylate did not inhibit RelA binding (Fig. 1, A–C). Lack of an effect on C-Rel and RelA binding activity might be due to the generation of ROS in RAW 264.7 cells stimulated with LPS/IFNγ (Allen and Tresini, 2000), which may oxidize and inactivate salicylate or mediate NOS-2 transcriptional activation via a sig-

naling pathway not inhibited by salicylate. To confirm that LPS/IFNγ-treated RAW 264.7 cells produce ROS, we measured ROS generation at 4 and 8 h after LPS/IFNγ stimulation. A significant amount of ROS was detected at 4 h, which was blocked by SOD or apocynin (Fig. 3, A and B) consistent with generation of superoxide anions via NADPH oxidase. ROS generation returned to the basal level at 8 h. To evaluate the effect of ROS on C-Rel binding, we pretreated RAW 264.7 cells with SOD for 30 min followed by LPS/IFNγ for 4 or 8 h. Binding of nuclear extract C-Rel and RelA to the κB probe was determined by the streptavidin-agarose pull-down assay. LPS/IFNγ-induced C-Rel binding was reduced by SOD treatment at 4 but not at 8 h (Fig. 4A). Analysis of C-Rel binding to chromatin NOS-2 promoter by ChIP confirmed that SOD abrogated LPS/IFNγ-induced C-Rel binding at 4 h (Fig. 4B).
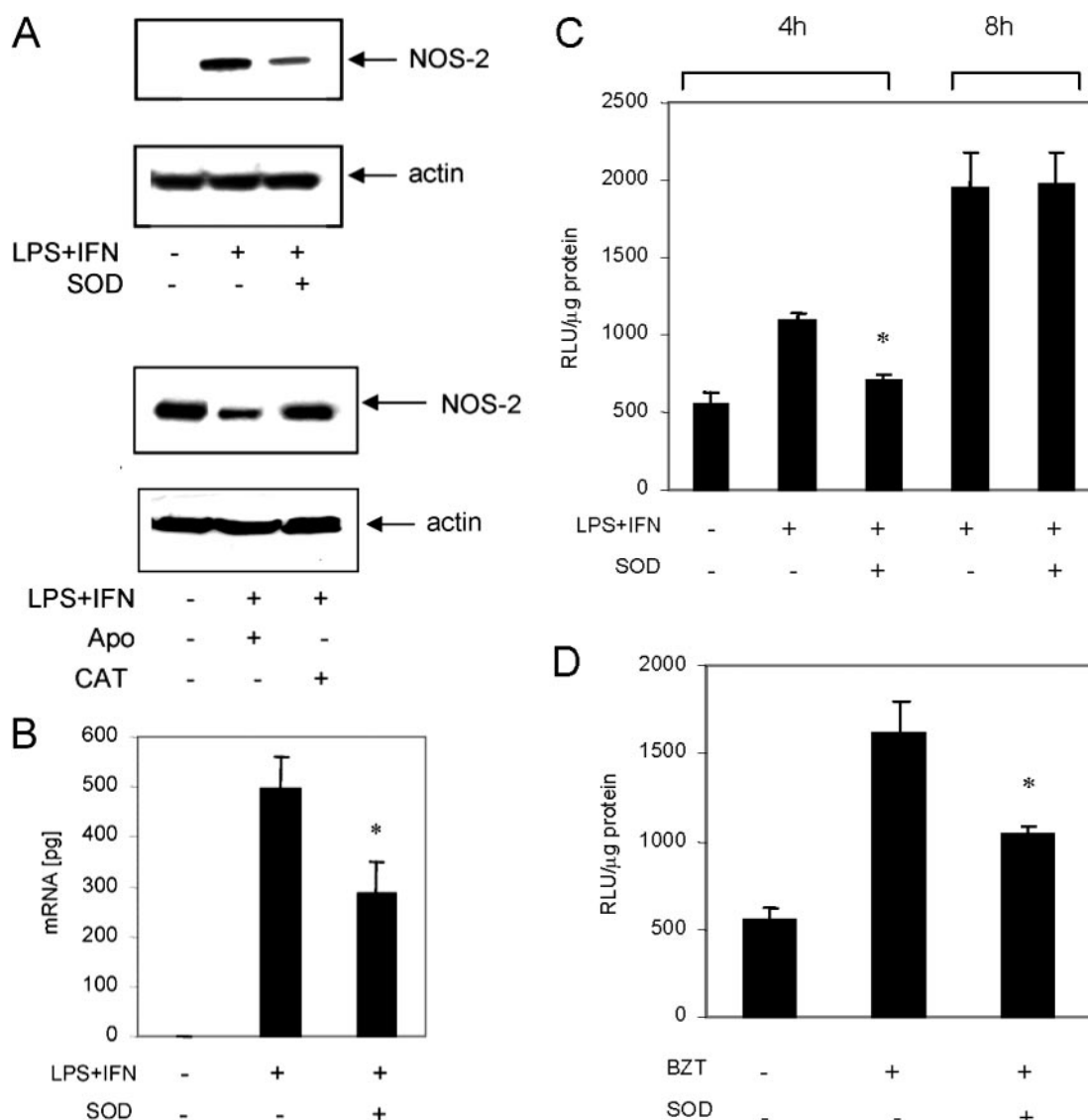
Superoxide anions have been reported to serve as an in-

Fig. 5. Role of superoxide generation in NOS-2 expression. A, RAW 264.7 cells were treated with SOD, apocynin (Apo), or catalase (CAT) (120 U/ml) for 30 min followed by LPS/IFNγ for 4 h. NOS-2 proteins in cell lysates were analyzed by Western blotting. The figures are representative of three experiments. B, cells were treated with SOD followed by LPS/IFNγ for 4 h. mRNA levels were determined by qPCR. C, cells transfected with NOS-2 promoter construct were treated with SOD followed by LPS/IFNγ for 4 or 8 h. Promoter activity was measured as luciferase expression. D, cells transfected with NOS-2 promoter construct were treated with SOD followed by BZT for 4 h, and luciferase expression was measured in a luminometer. Each bar shown in 4, B through D, represents mean ± S.E.M. of three experiments. $*$, $p < 0.05$.

tracellular signaling molecule to regulate NOS-2 expression (Beck et al., 1998; Kuo et al., 2000). We determined whether LPS/IFNγ-induced NOS-2 expression at 4 h was inhibited by SOD or apocynin. NOS-2 protein levels were reduced by SOD or apocynin by 50 to 60% but not by catalase (Fig. 5A). NOS-2 mRNA levels were reduced by SOD to an extent comparable with reduction of NOS-2 proteins (Fig. 5B). Furthermore, LPS/IFNγ-induced NOS-2 promoter activities were inhibited by SOD at 4 but not at 8 h (Fig. 5C). To further confirm the role of superoxide on NOS-2 promoter activation, we treated cells with BZT in the presence or absence of SOD. BZT stimulated NOS-2 promoter activity by more than 3-fold, which was suppressed by SOD (Fig. 5D). Taken together, these results show for the first time that superoxide plays a critical role in mediating C-Rel binding and C-Rel-dependent NOS-2 transcriptional activation induced by LPS/IFNγ at 4 but not at 8 h. Despite its suppression of LPS/IFNγ-induced NOS-2 expression at 4 h by blocking C/EBPβ DNA binding, salicylate does not inhibit superoxide-mediated C-Rel binding at 4 h.

Salicylate Supplement Selectively Inhibited C-Rel DNA Binding. We suspected that the inability of salicylate to inhibit LPS/IFNγ-induced C-Rel binding and NOS-2 expression at 8 and 24 h may be attributed to salicylate inactivation by ROS rendering it inactive at 8 and 24 h. We therefore determined whether the addition of supplemental salicylate (10^{-5} M) 4 h before the indicated endpoint might exert an effect. A second dose of salicylate added at 4 h reduced C-Rel binding to the κB probe at 8 h (Fig. 6A). The inhibitory effect of supplemental salicylate on C-Rel binding and NOS-2 protein expression was abrogated by the addition of BZT to LPS/IFNγ (Fig. 6B). These results support the notion that salicylate activity is limited by the presence of superoxide. A second dose of salicylate added at 20 h suppressed C-Rel binding activity at 24 h (Fig. 6A). Binding of RelA to the κB probe was suppressed at 8 and 24 h to an extent parallel to C-Rel (Fig. 6A). In contrast, C/EBPβ binding to a C/EBP probe was not inhibited by the second dose of salicylate at 8 h (Fig. 6A). C/EBPβ binding at 24 h after LPS/IFNγ treatment was undetectable, and salicylate did not have an effect (Fig. 6A). IRF-1 was reported to play an essential role in NOS-2 expression (Kamijo et al., 1994). To determine whether salicylate suppresses IRF-1 DNA binding, we treated cells with LPS/IFNγ in the presence or absence of salicylate for 4, 8, and 24 h. Nuclear extracts were prepared, and IRF-1 protein levels and IRF-1 binding were analyzed. Neither IRF-1 protein nor IRF-1 binding was detected at baseline. LPS/IFNγ increased nuclear IRF-1 proteins accompanied by increased IRF-1 binding to an IRF-1 probe at 4 h which persisted at 8 h. Its protein level declined, and binding completely dissipated at 24 h (Fig. 6C). A single dose of salicylate added at the beginning of LPS/IFNγ stimulation did not inhibit IRF-1 binding or protein level at 4 or 8 h. Salicylate supplement also had no effect on IRF-1 binding or protein level (Fig. 6C).

Sustained Inhibition of NOS-2 Transactivation by Salicylate Supplement. In view of inhibition of C-Rel binding by salicylate supplement at 8 and 24 h, it is likely that salicylate supplement could exert a sustained inhibition of NOS-2 expression at 8 and 24 h. To confirm this, we transfected RAW 264.7 cells with NOS-2 promoter vector and treated the transfected cells with LPS/IFNγ with or

without sodium salicylate (10^{-5} M) at time 0. Sodium salicylate was then added every 4 h up to 20 h after LPS/IFNγ treatment. At the indicated time points shown in Fig. 7, NOS-2 promoter activity was measured. LPS/IFNγ induced NOS-2 promoter activity in a time-dependent manner (Fig. 7). Salicylate added together with LPS/IFNγ inhibited NOS-2 promoter activity significantly at 4 but not at 8 h and subsequent time points (Fig. 7). Salic-
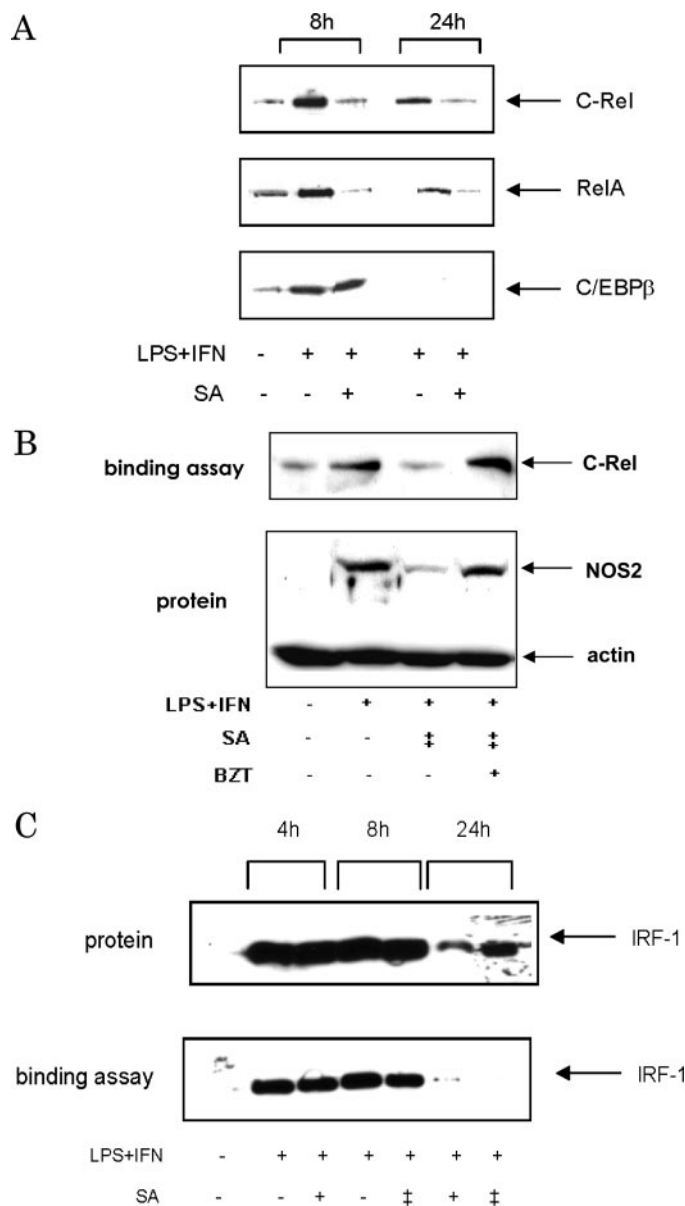

Fig. 6. Time-dependent effect of salicylate on key transactivators binding. Cells were treated with LPS/IFNγ and salicylate (10^{-5} M) was added 4 h before each indicated time point. A, binding of C-Rel, RelA, and C/EBPβ to their respective probes. B, BZT was added at time 0, supplemental salicylate was added at 4 h, and C-Rel binding and NOS-2 protein levels were measured at 8 h after the addition of LPS/IFNγ and salicylate. C, IRF-1 proteins were analyzed by Western blots and DNA binding by streptavidin pulldown assay. ‡, salicylate supplement 4 h before the indicated endpoint.

ylate supplement reduced NOS-2 promoter activity by ~50% at each time point after 8 h (Fig. 7). A single dose of salicylate added to LPS/IFNγ at time 0 inhibited LPS/IFNγ-induced NOS-2 mRNA levels by ~50% at 4 but not at 8 or 24 h (Fig. 8). However, the addition of a supplemental dose of sodium salicylate 4 h before each indicated endpoint resulted in a ~50% reduction of NOS-2 mRNA levels at 8 and 24 h (Fig. 8). Corresponding to the mRNA data, a single dose of salicylate added at time 0 inhibited LPS/IFNγ-induced NOS-2 protein levels at 4 h (Cieslik et al., 2002) but not at 24 h (Fig. 9A). However, the addition of a single dose of salicylate at 20 h after LPS/IFNγ treatment suppressed NOS-2 protein levels at 24 h by ~50% (Fig. 9A). Because the salicylate supplement effect may simply reflect a concentration effect, we treated cells with higher concentrations of sodium salicylate together with LPS/IFNγ at time 0 and determined NOS-2 proteins at 24 h. Sodium salicylate at concentrations up to 10^{-3} M had no effect on NOS-2 proteins at 24 h (Fig. 9B). These data suggest that it is unlikely that NOS-2 inhibition by supplemental salicylate is simply due to a concentration effect. Supplemental dose provides active salicylate to inhibit LPS/IFNγ-induced C-Rel binding and NOS-2 expression at 8 and 24 h.

Temporal Response of NOS-2 Protein Expression to Signaling Inhibitors. The results shown above reveal time-dependent regulation of C-Rel and C/EBPβ DNA binding activities and NOS transcriptional activation by LPS/IFNγ and the consequent differential inhibition by salicylate. To determine whether the dynamic temporal regulation of NOS-2 expression might be related to temporal switch of signaling pathways, we evaluated the effect of inhibitors of several candidate signaling molecules on LPS/IFNγ-induced NOS-2 protein levels at 4, 8, and 24 h. RAW 264.7 cells were treated with each inhibitor 30 min before LPS/IFNγ and at 4, 8, and 24 h after LPS/IFNγ treatment, and NOS-2 proteins were analyzed by Western blots. The results confirm the involvement of different signaling kinases in NOS-2 protein expression at different time points. At 4 h after LPS/IFNγ treatment, NOS-2 protein elevation was blocked by AG490, LY294002, and SB203580 consistent with the involvement of Janus-activated kinase 2 (Jak2), phosphoinositide 3-kinase (PI-3K), and p38 mitogen-activated protein ki-

nase (MAPK) at this time point (Fig. 10A). At 8 h, NOS-2 proteins remained to be blocked by AG490 and LY294002 but was no longer inhibited by SB203580, whereas they were inhibited by rapamycin (Fig. 10B), consistent with involvement besides Jak2 and PI-3K of mammalian target of rapamycin (mTOR). p38 MAPK was no longer required. The pharmacological inhibitors exert a similar effect on LPS/IFNγ-induced C-Rel binding at 8 h (Fig. 10C). The signaling profile changed drastically at 24 h. Although NOS-2 protein expression was still inhibited by rapamycin, it was not blocked by AG490 or LY294002 and became inhibited by GF109203X, consistent with involvement of protein kinase C in transcriptional activation of NOS-2 at 24 h (Fig. 10D). Paradoxically, NOS-2 protein levels were enhanced by AG490, LY294002, SB203580, and PD98059 (Fig. 10D).

Discussion

Our results indicate that C-Rel plays an essential role in sustained expression of NOS-2 induced by LPS/IFNγ as evidenced by suppressing LPS/IFNγ-induced NOS-2 protein expression and promoter activity by a specific C-Rel siRNA. Our results show for the first time temporal regulation of C-Rel DNA binding by superoxide anions and salicylate. C-Rel binding at 4 h after LPS/IFNγ treatment depends on superoxide generation. Superoxide anions were reported previously to activate NOS-2 via NF-κB (Bonizzi et al., 1999; Eberhardt et al., 2000; Fries et al., 2003), but to our knowledge, this is the first report of its activation of C-Rel. However, C-Rel binding persists at 8 h despite a complete dissipation of superoxide production, suggesting the involvement of a superoxide-independent signaling mechanism. C-Rel is sequestered in cytosol at resting state by binding to IκBs. Stimulation of cells with LPS and/or cytokines results in phosphorylation and degradation of IκBs, and the dissociated C-Rel enters the nucleus, where it forms heterodimers with other NF-κB isoforms, which, in turn, bind to specific enhancer elements and activate NOS-2 transcription. Several kinases, including the canonical IκB kinases, have been shown to induce phosphorylation and degradation of IκBs (Karin, 1999). It is possible
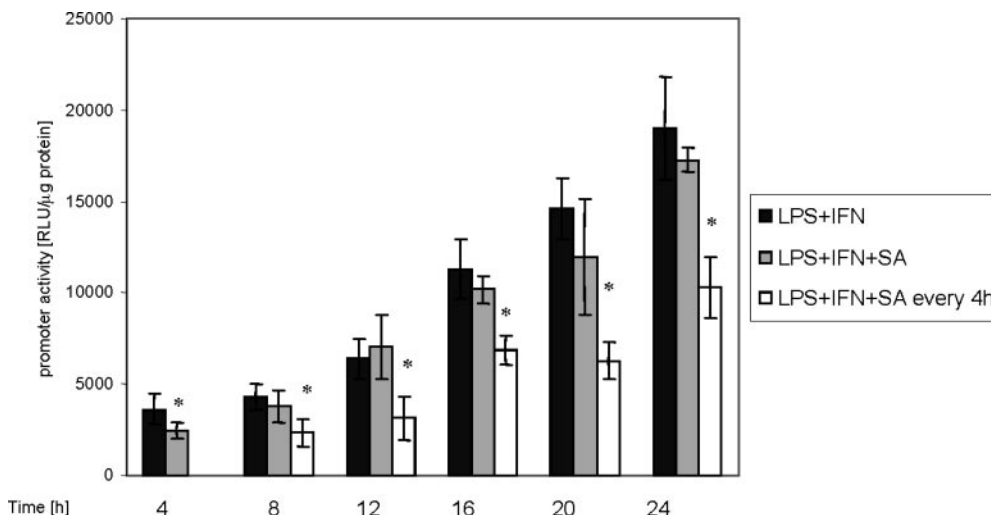
Fig. 7. Suppression of NOS-2 promoter activity by salicylate supplement. Cells transfected with NOS-2 promoter-luciferase constructs were treated with LPS/IFNγ and salicylate, and promoter activity was determined at indicated time points. In separate experiments, cells were similarly treated as above, and salicylate (10^{-5} M) was added every 4 h. Each bar shows mean ± S.E.M. of three experiments. *, $p < 0.05$ compared with LPS/IFNγ without salicylate.

that distinct classes of kinases are involved in IκB phosphorylation at 4 versus 8 and 24 h.

It is reasonable to assume that salicylate inhibits kinase activity, thereby suppressing C-Rel binding at 8 and 24 h, because salicylate has been shown to inhibit p90 ribosomal S6 kinase (RSK) via which it blocks C/EBPβ DNA binding (Cieslik et al., 2005). C/EBPβ at resting state possesses weak

DNA binding activity and is activated by phosphorylation (Wegner et al., 1992; Nakajima et al., 1993; Trautwein et al., 1993; Buck et al., 1999). Our previous work has shown that phorbol 12-myristate 13-acetate-induced C/EBPβ binding activity depends on phosphorylation at Thr266 by RSK (Cieslik et al., 2005). Because C/EBPβ is phosphorylated by several kinases besides RSK, LPS/IFNγ-induced C/EBPβ activation may not be necessarily mediated via RSK. However, given the fact that salicylate inhibits LPS/IFNγ-induced COX-2 and NOS-2 transactivation in RAW 264.7 cells at 4 h, it is likely that salicylate inhibits C/EBPβ binding at 4 h via the RSK pathway. The reason for its failure to block C/EBPβ binding at 8 h is unclear. One explanation is that once C/EBPβ binding is induced by RSK, it no longer requires RSK and therefore becomes resistant to salicylate. An alternative explanation is that C/EBPβ binding is triggered by different kinases at subsequent time points after LPS/IFNγ
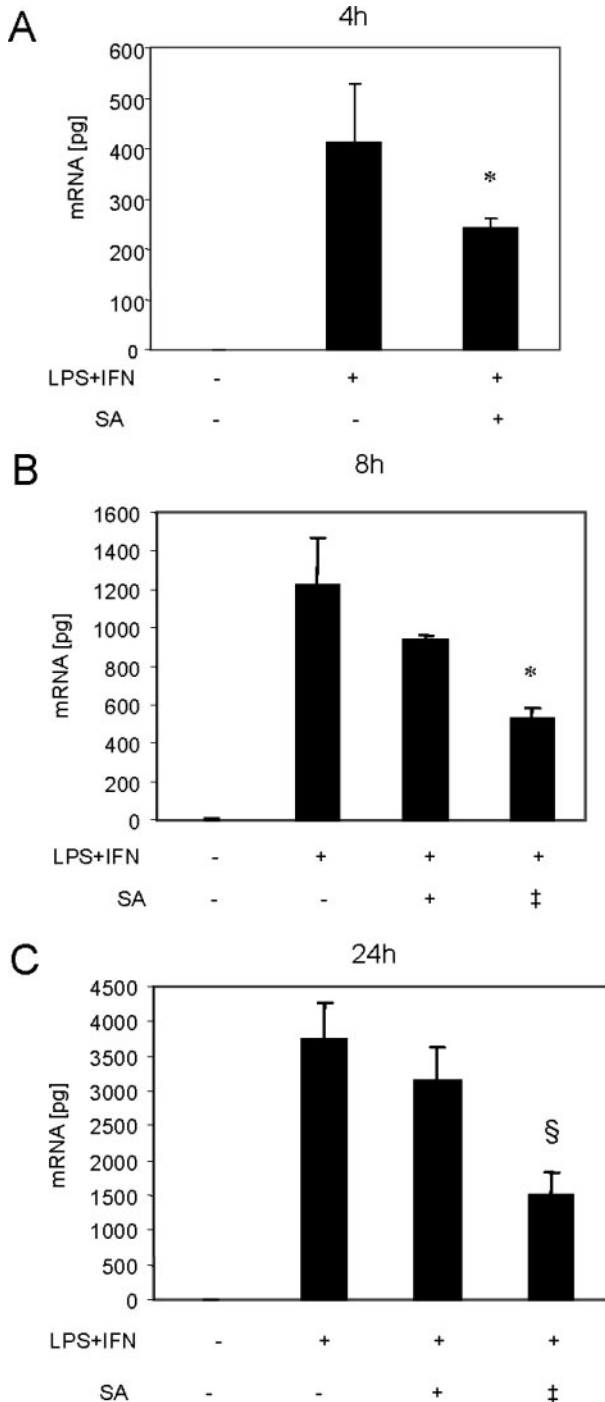

Fig. 8. Suppression of NOS-2 mRNA by salicylate supplement. RAW 264.7 cells were treated with LPS/IFNγ and salicylate for 4 (A), 8 (B), or 24 h (C). mRNA levels were measured by qPCR. Each bar shows mean ± S.E.M. of three experiments. ∗, $p < 0.05$, and §, $p < 0.01$ compared with LPS/IFNγ in the absence of salicylate. +, the addition of a single dose of salicylate (10^{-5} M) to LPS/IFNγ; ‡, the addition of a second dose of salicylate 4 h before the indicated time.
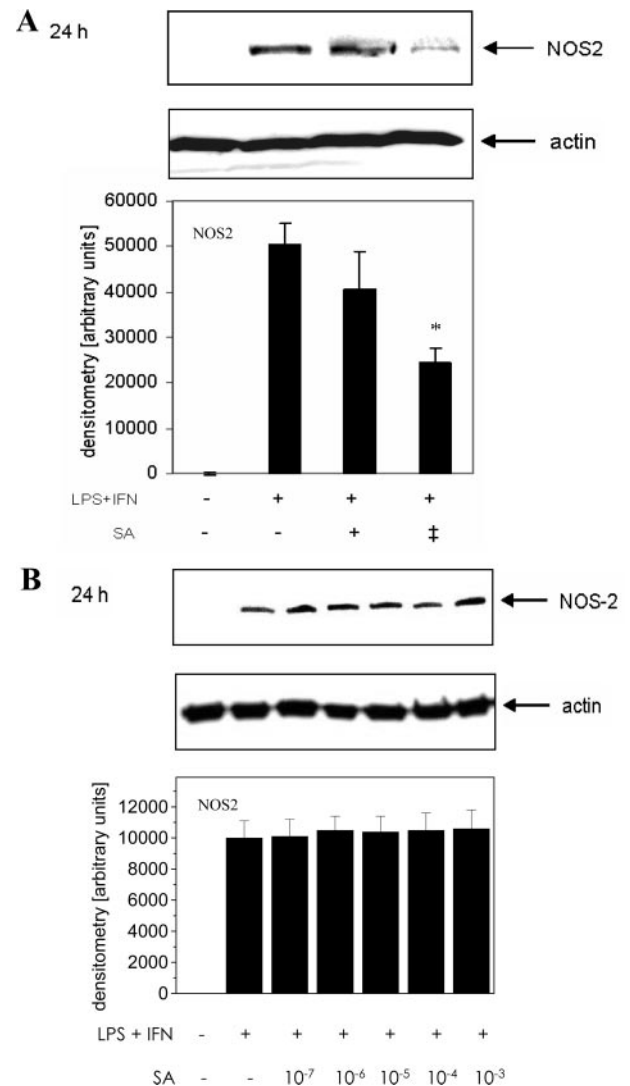

Fig. 9. Inhibition of NOS-2 protein by salicylate supplement but not high concentrations of salicylate. A, cells were treated with LPS/IFNγ and salicylate at 10^{-5} M for 24 h. In separate experiments, cells were treated with LPS/IFNγ and salicylate (10^{-5} M) for 20 h, at which time an additional dose of salicylate (10^{-5} M) was added. B. Cells were treated with LPS/IFNγ and salicylate at concentrations up to 10^{-3} M for 24 h. NOS-2 and actin proteins in cell lysates were measured by Western blotting. ‡, salicylate supplement at 20 h.

treatment as a result of signaling switch. Further studies are needed to elucidate the underlying mechanisms.

The temporal shift of inhibition of transactivator binding by salicylate is likely to be determined by time-dependent changes in LPS/IFNγ-induced signaling pathways. Analysis of the signaling inhibitor profile clearly shows changes in signaling kinases required for LPS/IFNγ-induced NOS-2 protein expressions at 4 versus 8 and 24 h. Although PI-3K and Jak2 are required for NOS-2 expression at 4 and 8 h, p38 MAPK was required at 4 but not at 8 h, and mTOR was required at 8 and 24 but not at 4 h. It has been reported that superoxide-induced gene expression is mediated via p38 MAPK (Eberhardt et al., 2000). It is highly likely that LPS/IFNγ-induced C-Rel binding and NOS-2 expression at 4 h are mediated in part via superoxide-activated p38 MAPK pathway, which is not inhibited by salicylate. LPS-IFNγ-induced C-Rel binding and NOS-2 protein expression at 8 h are suppressed by inhibitors of PI-3K, Jak2, and mTOR. These results suggest that mTOR plays a crucial role in C-Rel binding

and NOS-2 expression at 8 and 24 h, and its signaling pathway may be the target of salicylate action. We recognize that the data based on pharmacological inhibitors are confronted with flaws such as a lack of specificity and therefore should be interpreted with caution. Furthermore, because only a limited number of signaling kinase inhibitors are used in this study, it is difficult to pinpoint the specific signaling pathways that temporarily mediate C/EBPβ and NF-κB binding or to identify the exact kinases targeted by salicylate. Nevertheless, the inhibitor profile provides a useful guide for designing more thorough experimental approaches to identify kinases that are directly involved in C-Rel binding at different time points and to identify the target of salicylate inhibitory action.

The signaling profile at 24 h indicates a complex mechanism by which LPS/IFNγ regulates NOS-2 protein expression at this time point. NOS-2 protein levels were suppressed by inhibitors of mTOR or protein kinase C and, surprisingly, were enhanced by inhibitors of PI-3K, Jak2, mitogen-acti-
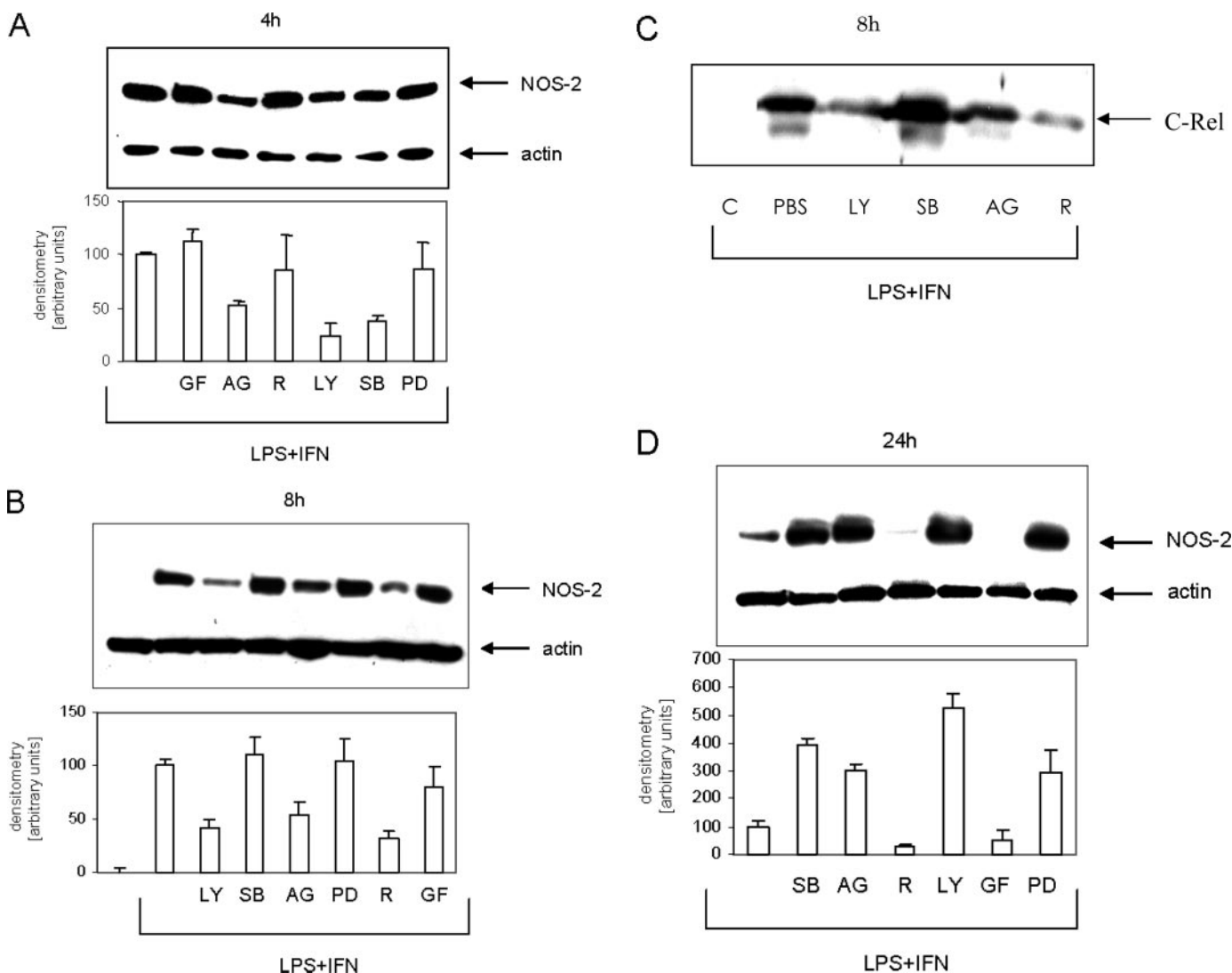

Fig. 10. Time-dependent differential inhibition of NOS-2 protein levels by inhibitors of signaling kinases. RAW 264.7 cells were pretreated with each inhibitor for 30 min, followed by LPS/IFNγ for 4 (A), 8 (B and C), and 24 h (D). A, B, and D, NOS-2 and actin proteins for each time point were analyzed by Western blots. Top, a representative Western blot; bottom densitometry of Western blots from three experiments. Each bar indicates mean ± S.E.M. C, a representative figure showing C-Rel binding determined by streptavidin pulldown assay. The concentrations for each inhibitor are the following: 50 μM LY294002 (LY), 50 μM AG490 (AG), 200 nM rapamycin (R), 500 nM GF109203X (GF), 2.6 μM SB203580 (SB), and 100 μM PD98059 (PD).

vated protein kinase kinase, or p38 MAPK. Kinetic analysis of NOS-2 expression has shown that NOS-2 proteins stimulated by LPS/IFNγ maximize at 12 h, decline at 16 h, and diminish at 24 h (Cieslik et al., 2002), whereas NOS-2 mRNAs and promoter activities do not decline at 24 h (Figs. 6A and 7). Taken together, these results suggest that NOS-2 transcriptional up-regulation at 24 h is offset by NOS-2 protein degradation via distinct signaling pathways. Salicylate probably inhibits transcriptional activation at 24 h by blocking mTOR-mediated C-Rel binding and probably has no effect on NOS-2 protein degradation. The mechanism by which LPS/IFNγ induces NOS-2 protein degradation is unclear. Previous reports have suggested that NOS-2 may be degraded via the ubiquitin-proteasome pathway (Rockwell et al., 2000; Shao et al., 2000). Because C/EBPβ and IRF-1 proteins are also degraded at 24 h, there may be a common mechanism by which LPS/IFNγ induces protein ubiquitination and degradation via the proteasome pathway. Our results suggest that protein degradation may be mediated by signaling pathways involving PI-3K, Jak2, p38 MAPK, and mitogen-activated protein kinase kinase. These findings are contrary to the reported data, which have shown that PI-3K and ERK signaling pathways protect proteins from degradation via ubiquitin-proteasome (Dan et al., 2004; Gardai et al., 2004). Further studies are needed to determine whether LPS/IFNγ induces NOS-2 degradation by the ubiquitin-proteasome pathway and how several kinases are involved in mediating the degradation.

In conclusion, results from this study provide novel insights into NOS-2 transcription induced by LPS/IFNγ: 1) C-Rel is essential for NOS-2 transcriptional activation; 2) C-Rel DNA binding activities are regulated temporally via different signaling pathways; 3) LPS/IFNγ-generated superoxide plays a crucial role in C-Rel binding and NOS-2 transactivation only at 4 h after LPS/IFNγ treatment; 4) supplement of salicylate at micromolar concentrations at 4-h intervals or at 4 h before an indicated endpoint achieves a sustained effect on controlling NOS-2 expression; and 5) the sustained effect of salicylate is mediated by inhibiting LPS/IFNγ-induced C/EBPβ binding at 4 h and C-Rel/RelA binding at 8 and 24 h. The results have important therapeutic implications. We propose that salicylate exerts its effect by inhibiting RSK activity at 4 h, thereby suppressing RSK-induced C/EBPβ activation and DNA binding activity. As the signaling pathways via which LPS/IFNγ exerts its NOS-2 transcriptional activation change over time, we speculate that salicylate may inhibit a different RSK family kinase such as mitogen and stress-activated kinase-1 at 8 and 24 h, thereby suppressing C-Rel activation and DNA binding activity (Vermeulen et al., 2003; Smith et al., 2004).

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