Salicylate Suppresses Macrophage Nitric-oxide Synthase-2 and Cyclo-oxygenase-2 Expression by Inhibiting CCAAT/Enhancer-binding Protein-β Binding via a Common Signaling Pathway*

Katarzyna Cieslik, Ying Zhu, and Kenneth K. Wu‡
From the Vascular Biology Research Center, Institute of Molecular Medicine and Division of Hematology, University of Texas-Houston Health Science Center, Houston, Texas 77030

We determined whether salicylate at pharmacological concentrations inhibits nitric-oxide synthase-2 (NOS-2) and cyclo-oxygenase-2 (COX-2) expressions in RAW 264.7 stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Cells were treated with sodium salicylate (10⁻⁷–10⁻⁴ M) or vehicle for 30 min followed by LPS+IFN-γ for up to 24 h. Salicylate suppressed NOS-2 and COX-2 protein levels and promoter activities stimulated by LPS+IFN-γ for 4 h in a concentration-dependent manner but had no effect on NOS-2 expression stimulated by the combined agonists for 24 h. Results from promoter analysis indicate that the binding of CCAAT/enhancer-binding protein β (C/EBPβ) to its cognate site at −150/−142 on the NOS-2 promoter region was essential for NOS-2 expression at 4 h but not at 24 h. Salicylate reduced C/EBPβ binding at 4 h and did not alter its binding at 24 h. NOS-2 and COX-2 protein levels and C/EBPβ binding stimulated by LPS+IFN-γ for 4 h were inhibited by a similar battery of signaling inhibitors, suggesting a common pathway for NOS-2 and COX-2 expression. Kinetic analysis indicates that NOS-2, similar to COX-2 expression, at 4 h was largely due to the action of LPS, which induced C/EBPβ binding, whereas its expression at a longer time point was contributed by IFN-γ. Our findings implicate two distinct pathways for NOS-2 expression induced by LPS+IFN-γ. Salicylate at pharmacological concentrations is capable of suppressing the early phase of NOS-2 and COX-2 expression by blocking C/EBPβ binding.

Nitric-oxide synthase-2 (NOS-2, also known as inducible nitric-oxide synthase), a member of the NOS protein family, defends against microbial infection but may contribute to inflammation and tissue injury when overexpressed (1). NOS-2 is inducible by lipopolysaccharide (LPS) and a myriad pro-inflammatory cytokines (2). Transcriptional activation of NOS-2 by these stimuli has been extensively characterized in murine RAW 264.7 macrophages. LPS combined with interferon-γ (IFN-γ) was reported to activate NOS-2 promoter synergistically. Promoter analysis reveals that two clusters of enhancers located at −54 to −236 and −731 to −962 of NOS-2 promoter region are essential for the sustained expression of NOS-2 in response to LPS+IFN-γ stimulation (3, 4). The binding of NFκB and interferon regulatory factor-1 to the enhancer elements of these two regions are indispensable for NOS-2 transcription (5, 6). It has been recently reported (7, 8) that a CAAT/enhancer-binding protein (C/EBP) element situated at −142 to −150 is also involved in NOS-2 expression, but the exact transactivators that bind to this site have not been characterized. COX-2 is also inducible in this murine macrophage by diverse pro-inflammatory mediators. Its induction by these stimuli is typical of an immediately early gene with a rapid and transient time course. Several regulatory elements on murine COX-2 promoter including a cyclic AMP response element, two C/EBP sites, and a single NFκB site have been shown to be involved in COX-2 promoter activation (9, 10). The C/EBP sites have been demonstrated to be required for COX-2 expression in murine cells induced by diverse stimuli including LPS (10–12). However, the binding of C/EBPβ to these two sites have not been fully characterized. Results from our laboratory demonstrate that C/EBPβ binding to the C/EBP site in human COX-2 promoter is required for COX-2 transcriptional activation by pro-inflammatory mediators such as phorbol 12-myristate 13-acetate and interleukin-1β (13). Aspirin and sodium salicylate at pharmacological concentrations suppress COX-2 transcription by blocking the binding of C/EBPβ (13, 14). The effect of salicylate on NOS-2 expression is controversial. Although several reports have shown that salicylate suppresses NOS-2 expression at suprapharmacological concentrations (>5 mM) by reducing NFκB binding (15, 16), to our knowledge, there is no report on NOS-2 expression influenced by pharmacological concentrations of salicylate. Because NOS-2 and COX-2 expressions are concurrently induced by LPS+IFN-γ in RAW 264.7 cells, we used this cell line as a model to test the hypothesis that salicylate at pharmacological concentrations (10⁻⁴ to 10⁻⁶ M) inhibits NOS-2 and COX-2 expressions stimulated by LPS+IFN-γ by blocking a common C/EBPβ-dependent transcriptional pathway. The results show that salicylate suppressed NOS-2 and COX-2 protein levels and promoter activities by blocking the binding of C/EBPβ to its respective C/EBP elements on NOS-2 and COX-2 promoters. Our results further show that NOS-2 promoter activity stimulated by LPS+IFN-γ for 4 h required C/EBPβ binding, and at this time period of stimulation, salicylate was effective in suppressing C/EBPβ binding, thereby reducing the extent of NOS-2 expression. In-

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‡ To whom correspondence should be addressed: Vascular Biology Research Center, Institute of Molecular Medicine and Division of Hematology, University of Texas-Houston Health Science Center, Houston, Texas 77030. Tel.: 713-500-6801; Fax: 713-500-6812; E-mail: Kenneth.K.Wu@uth.tmc.edu.

1 The abbreviations used are: NOS-2, nitric-oxide synthase-2; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; IFN-γ, interferon-γ; C/EBP, CCAAT/enhancer-binding protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/microsignal-regulated kinase kinase; PKC, phosphatidylinositol-3-kinase; PKA, protein kinases C and A, respectively; Jak, Janus activated kinase.

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EXPERIMENTAL PROCEDURES

Cell Culture—RAW 264.7 mouse macrophages (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). For all experiments, medium was removed, and cells were washed and incubated in serum-free medium for 24 h before stimulation with 2 μg/ml LPS (Escherichia coli serotype 0111:B4, Sigma), 40 units/ml IFN-γ (Roche Molecular Biochemicals), or LPS + IFN-γ for indicated time periods.

Transfection and Promoter Activity—A wild-type murine 1.63-kilobase NOS-2 promoter/enhancer fragment (~1486/~130) (Integrated DNA Technologies, Coralville, IA) and a 0.9-kilobase COX-2 promoter/enhancer fragment (~891/~75) were cloned into pGL3 basic luciferase expression vectors. RAW 264.7 cells at ~60% confluence were transfected with the luciferase expression construct according to a procedure described previously (14). A mixture of 15 μg of Lipofectin (Invitrogen) and 4 μg of DNA was added to cells and incubated in serum-free medium for 6 h. The medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 18 h. Cells were washed with serum-free medium, growth-arrested for 24 h, and stimulated with LPS and IFN-γ for an indicated period of time. Cells were harvested using Promega lysis buffer, and luciferase activity was measured on a Turner TD-20/20 luminometer. On the same cell lysate, total protein assay was performed.

Western Blot—Western blot analysis was performed as described previously (13). Cells were washed with cold phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride, harvested, and spun down at 3000 rpm for 5 min. Cell pellet was dissolved in solubilizing buffer (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1 mM Na3VO4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na3VO4, 0.06 mg/ml aprotinin). Cell pellet was then spun down at 10,000 rpm for 15 min. Supernatant was collected, and its protein concentration was determined. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, each lane was loaded with 20 μg of protein. The gel was electrophoretically transferred, and proteins were identified by specific antibodies: anti-NOS-2 antibody (Upstate Biotechnology, Lake Placid, NY), anti-COX-2 antibody (Cayman, Ann Arbor, MI), anti-actin antibody (Oncogene, Boston, MA), and anti-CEBPβ (Santa Cruz Biotechnology, Santa Cruz, CA). The protein bands were visualized by enhanced chemiluminescence system (Pierce, Rockford, IL).

Western blot analysis was performed as described previously (17). 400 μg of nuclear extract proteins in 400 μl of phosphate-buffered saline buffer containing multiple protease inhibitors (1 mM Na3VO4, 10 mM NaF, 25 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 0.06 mg/ml aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml dithiothreitol) were incubated with a mixture of 4 μg of double-stranded biotinylated oligonucleotides containing murine NOS-2 promoter C/EBP sequence at ~150 to ~142 (underlined in 5’-CACAGGTTGATGTAATCAAGCA-3’) or COX-2 C/EBP sequence at ~138 to ~130 (5’-GGTTCTGGCCGACTCACCCT-3’) (Integrated DNA Technologies, Coralville, IA) and 40 μl of 4% beaded-agarose conjugated with streptavidin (Sigma) for 2 h on a rocking platform at room temperature. The beads were collected by centrifugation at 3000 rpm for 1 min, washed three times with phosphate-buffered saline buffer containing multiple inhibitors, and resuspended in 50 μl of Laemmli sample buffer. Nuclear proteins bound to the beads were dissociated by incubation in water bath at 95 °C for 5 min and applied to Western blot analysis.

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Inhibitors of Signaling Pathways—PD98059, an inhibitor of MEK1/2; LY294002, an inhibitor of phosphatidylinositol-3 kinase (PI3K); SB203580, an inhibitor of p38 MAPK; AG490, an inhibitor of Janus activated kinase 2/3 (JAK2/3); GF109203X, an inhibitor of protein kinase C (PKC); and rapamycin, an inhibitor of mammalian target of rapamycin, were obtained from Calbiochem. RAW 264.7 cells were pretreated with these agents for 30 min prior to the addition of LPS, IFN-γ, or LPS + IFN-γ.

RESULTS

Concurrent Suppression of NOS-2 and COX-2 Expressions in RAW 264.7 Cells by Sodium Salicylate—It is well established that RAW 264.7 cells expressed both COX-2 and NOS-2 proteins in response to LPS + IFN-γ stimulation. To determine whether sodium salicylate at pharmacological concentrations suppresses COX-2 and NOS-2 expressions concurrently, we evaluated the effects of sodium salicylate on COX-2 and NOS-2 protein levels and promoter activities. Sodium salicylate suppressed COX-2 protein levels in serum-starved RAW 264.7 cells stimulated with LPS + IFN-γ for 4 h in a concentration-dependent manner with a maximal inhibition of ~50% at 10−5 to 10−4 M (Fig. 1a). NOS-2 protein was detected after stimulation of RAW 264.7 cells with LPS + IFN-γ for 4 h and was suppressed by sodium salicylate (Fig. 1b). Sodium salicylate at 10−5 M suppressed COX-2 and NOS-2 promoter activities stimulated...
with LPS + INF-γ for 4 h to a similar extent (Fig. 2). We have observed in RAW 264.7 as well as in human fibroblasts that salicylate inhibited NOS-2 and COX-2 expressions only in serum-starved cells. When cells were cultured in the presence of 10% fetal bovine serum, salicylate did not inhibit NOS-2 or COX-2 protein levels stimulated by LPS + INF-γ for 4 h (data not shown).

Inhibition by Salicylate of NOS-2 Promoter Activity at 4 h but Not at 24 h—In response to LPS + INF-γ stimulation, COX-2 promoter activity peaked at 4 h and declined to near basal level at 8 h and thereafter (Fig. 3). In contrast, NOS-2 promoter activity peaked at 4 h and declined to near basal level at 24 h (Fig. 4). Salicylate (10−5 M) reduced NOS-2 promoter activity at 4 h by 50% but had no effect on NOS-2 promoter activity stimulated with LPS + INF-γ for 24 h (Fig. 5).

Suppression of C/EBPβ Binding to NOS-2 and COX-2 Promoters by Salicylate—Of several C/EBPβ isoforms, only the 38-kDa C/EBPβ was detectable in quiescent cells and its level was not altered by LPS + INF-γ stimulation for 4 h (Fig. 6a). C/EBPβ binding to the NOS-2 promoter sequence was detected at basal state, which was enhanced by LPS + INF-γ stimulation for 4 h (Fig. 6b). Basal binding to COX-2 C/EBP β sequence was not detected, but the binding was detectable at 4 h of stimulation (Fig. 6c). Sodium salicylate at 10−5 M did not alter C/EBPβ protein levels (Fig. 6a) but suppressed C/EBPβ binding to NOS-2 and COX-2 promoter C/EBP sequences (Fig. 6b and c). By contrast, C/EBPβ binding to NOS-2 sequence became undetectable after a 24-h treatment with LPS + INF-γ, which is correlated with a marked reduction of C/EBPβ protein levels (Fig. 7). Sodium salicylate had no effect on C/EBPβ binding or protein level at 24 h of stimulation (Fig. 7).

Salicylate Inhibited LPS- but Not INF-γ-induced NOS-2 Protein Expression—LPS has been reported to induce COX-2 expression in RAW 264.7 cells at early time points (18), but the kinetics of NOS-2 expression had not been analyzed. We determined the kinetics of NOS-2 expression induced by LPS and INF-γ individually. Under LPS stimulation, NOS-2 proteins were detected at 4 h and peaked at 12 h (Fig. 8). In contrast, NOS-2 proteins were undetectable at 4 h after INF-γ stimulation became detectable at 8 h and peaked at 24 h (Fig. 8). LPS + INF-γ treatment resulted in synergistic stimulation of NOS-2 expression. Robust expression was detected at 4 h, peaked at 12 h, and declined significantly at 24 h. We next determined whether sodium salicylate inhibited NOS-2 protein expression stimulated by LPS, INF-γ, or LPS + INF-γ for 4 and 24 h. Salicylate (10−5 M) inhibited LPS and LPS + INF-γ-induced NOS-2 protein levels at 4 h to a similar extent (Fig. 9). It had no effect on INF-γ-induced NOS-2 protein levels (Fig. 9). Taken together, these results suggest that salicylate inhibited LPS-induced C/EBPβ binding, thereby suppressing C/EBPβ-mediated COX-2 and NOS-2 expression.

NOS-2 and COX-2 Expression at 4 h Was Mediated by a Similar Signaling Pathway—To gain insight into the signaling pathway via which salicylate blocks C/EBPβ binding, we compared the signaling pathways through which NOS-2 and COX-2 expressions are stimulated by LPS + INF-γ for 4 h.

NOS-2 protein levels were significantly reduced by LY294002,
SB203580, and AG490 (Fig. 10a), consistent with the involvement of PI3K, p38 MAPK, and Jak2/3 in NOS-2 expression. COX-2 protein levels at 4 h were also inhibited by LY2942002, SB203580, and AG490 with an almost identical profile (Fig. 10b). LY2942002 and SB203580 abrogated LPS + IFN-γ stimulation for 4 h. These results suggest a common signaling pathway through which NOS-2 and COX-2 promoter activities are stimulated by LPS versus IFN-γ at 4 h.

In view of different kinetics of NOS-2 expression in response to LPS versus IFN-γ stimulation, we were interested in knowing whether the signaling pathway for NOS-2 expression stimulated by LPS + IFN-γ for 4 h is attributable to LPS. We pre-treated cells with LY294002 or PD98059 for 30 min followed by

FIG. 5. Sodium salicylate (10⁻⁵ m) suppressed NOS-2 promoter activity stimulated by LPS + IFN-γ for 4 h but not 24 h. Each bar denotes the mean ± S.D. of three experiments.

FIG. 6. Effect of sodium salicylate (10⁻⁵ m) on C/EBPβ protein levels determined by Western blots (a), C/EBPβ binding to NOS-2 promoter sequence (b), and C/EBPβ binding to COX-2 promoter sequence by the streptavidin-agarose pulldown assay (c). Please refer to “Experimental Procedures” for a detailed description of this assay. Lane 1, basal level; lane 2, the level stimulated with LPS + IFN-γ for 4 h; and lane 3, pretreatment with sodium salicylate (10⁻⁵ m) for 30 min before stimulation with LPS + IFN-γ for 4 h. Each bar denotes the mean ± S.D. of three experiments.

FIG. 7. Disappearance of C/EBPβ proteins and C/EBPβ binding to NOS-2 promoter sequence after stimulation with LPS + IFN-γ for 24 h. Lane 1, basal level; lane 2, LPS + IFN-γ stimulation for 24 h; and lane 3, pretreatment with sodium salicylate (10⁻⁵ m) followed by LPS + IFN-γ stimulation for 24 h.

FIG. 8. Time course of NOS-2 protein expression stimulated by LPS (2 μg/ml), IFN-γ (40 units/ml), or LPS + IFN-γ. This figure is representative of two experiments with similar results. The numbers shown with each panel denote hours.

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LPS, IFN-γ, or LPS + IFN-γ for 4 h. Cells were harvested, and NOS-2 proteins were determined. NOS-2 protein expression induced by LPS alone or LPS + IFN-γ was similarly inhibited by LY294002 but not by PD98059 (Fig. 12). IFN-γ did not induce detectable NOS-2 at 4 h, and pretreatment with these inhibitors did not alter its effect on NOS-2 protein expression (Fig. 12). These results suggest that NOS-2 and COX-2 expressions after a 4 h stimulation by LPS + IFN-γ were contributed primarily by LPS via a signaling pathway involving several signaling molecules including PI3K.

**DISCUSSION**

Our findings indicate that salicylate at pharmacological concentrations is capable of suppressing NOS-2 and COX-2 expressions in RAW 264.7 macrophages by blocking C/EBPβ binding to specific binding sites at the NOS-2 and COX-2 promoter regions. Our results further demonstrate that the concurrent NOS-2 and COX-2 expressions in response to LPS + IFN-γ stimulation for 4 h is attributable to the action of LPS, because IFN-γ did not induce NOS-2 or COX-2 expression at this early time point. C/EBPβ binding has been shown to be required for LPS-induced COX-2 transcriptional activation (18). In this study, our results show that C/EBPβ binding is also essential for NOS-2 transcriptional activation by LPS at 4 h. Salicylate was capable of suppressing C/EBPβ binding to the promoters of both genes. These results suggest that salicylate suppresses NOS-2 and COX-2 transcription by a common C/EBPβ-dependent mechanism. We have previously demonstrated that COX-2 stimulation by phorbol 12-myristate 13-acetate and interleukin-1β (13) in human fibroblasts also requires C/EBPβ binding, and salicylate exerts a similar effect on blocking C/EBPβ binding, thereby suppressing COX-2 transactivation. In contrast, salicylate does not suppress COX-2 stimulation by tumor necrosis factor α whose stimulation does not require C/EBPβ binding. Taken together, these results indicate that C/EBPβ binding is a key target of salicylate action. It may be predicted that salicylate is capable of suppressing an array of C/EBPβ-dependent genes. This action of salicylate could explain the therapeutic effects of aspirin on diverse human diseases.

The mechanism by which salicylate blocks C/EBPβ binding has not been characterized. As C/EBPβ binding depends on phosphorylation, it is probable that salicylate disrupts C/EBPβ phosphorylation by inhibiting the kinase activity. The exact kinase that phosphorylates C/EBPβ and enhances its binding and transactivation properties remains to be identified. Several kinases including p90 ribosomal S6 kinase, calmodulin-dependent kinase, MAPK, PKC, and PKA have been shown to phosphorylate C/EBPβ at distinct tyrosine or serine/threonine
Our results demonstrate different kinetics of NOS-2 and COX-2 promoter activation by LPS+IFN-γ. COX-2 expression by the combined stimuli follows a time course that is characteristic of COX-2 induction by other agonists including LPS. Its promoter activity stimulated by LPS or LPS+IFN-γ peaked at 4 h and declined rapidly thereafter. The addition of IFN-γ does not potentiate the stimulatory effect of LPS nor does it prolong the time course of COX-2 stimulation. In contrast, NOS-2 expression by the combined stimuli has a prolonged time course, which is contributed primarily by IFN-γ. Our time course of NOS-2 expression by the combined stimuli is consistent with the reported data (23, 24). However, the time course of NOS-2 expression under the stimulation of LPS or IFN-γ had not been extensively investigated. Our results show a different time course of NOS-2 expression stimulated by these two agonists.

LPS induced an early and short-term NOS-2 expression resembling the time course of COX-2 expression, whereas IFN-γ stimulated NOS-2 expression with a late onset and a prolonged time course. This kinetic difference is attributable to distinct receptor-mediated signaling pathways used by these two agonists to induce the binding of different transactivators to their respective regulatory elements on the NOS-2 promoter. The synergistic action of LPS+IFN-γ could be explained by a complementary time course of stimulation, recruitment of multiple transactivators and coactivators to the promoter, and dynamic switch of transactivator binding over time caused by these two agonists. These processes are complex and possibly coordinately regulated. The results from this study provide direct evidence for a time-dependent change in C/EBPβ binding that correlates with the early short-term NOS-2 expression. C/EBPβ binding is absolutely required for NOS-2 expression at 4 h but not at 24 h. The binding of other transactivators, notably NFκB and interferon regulatory factor-1, is probably responsible for the late onset and sustained NOS-2 expression stimulated by LPS+IFN-γ. It is interesting to note that C/EBPβ proteins are degraded by prolonged LPS+IFN-γ stimulation, which results in the switch-off of the C/EBPβ binding. It will be important to determine the time course of binding of other functionally important transactivators in response to stimulation by LPS, IFN-γ, or LPS+IFN-γ and evaluate the effect of salicylate on the binding of these transactivators at different time points.

NOS-2 transcriptional activation in response to stimulation by LPS+IFN-γ at 4 and 24 h may be mediated through distinct signaling pathways. Our results indicate that at 4 h of stimulation, promoter activation is mediated via a pathway involving P38K, p38 MAPK, and Jak2/3. On the other hand, our data also reveal that NOS-2 transcriptional activation by LPS+IFN-γ for 24 h was inhibited by GF109203X and rapamycin and augmented by LY294002, SB203580, AG490, and PD98059, suggesting that NOS-2 expression at 24 h is mediated via a signaling pathway involving PKC and mammalian target of rapamycin and negatively regulated by P38K, p38 MAPK. Our results suggest that neither PKC nor p42/p44 MAPK is a target of salicylate, because the inhibition of PKC or MAPK did not alter C/EBPβ binding. Work is in progress to determine whether ribosomal S6 kinase, calmodulin-dependent kinase, or PKA is the target of salicylate inhibition.

residues (19–22). It is unclear whether all of the phosphorylation sites are required for C/EBPβ binding, nor is it clear whether all the kinases are activated by each stimulus. It is possible that each type of agonists may activate several kinases that act in concert to promoter C/EBPβ binding. Salicylate may block one of the kinase activity, thereby weakening the C/EBPβ binding. Our results suggest that neither PKC nor p42/p44 MAPK is a target of salicylate, because the inhibition of PKC or MAPK did not alter C/EBPβ binding. Work is in progress to determine whether ribosomal S6 kinase, calmodulin-dependent kinase, or PKA is the target of salicylate inhibition.

A

B

Fig. 11. Inhibition of 38-kDa C/EBPβ binding to NOS-2 sequence (A) and COX-2 sequence (B) by LY294002 (50 μM) and SB203580 (2.6 μM). A, uppermost panel, C/EBPβ protein levels in RAW 264.7 lysate analyzed by Western blots. Middle panel (A) and upper panel (B) show C/EBPβ binding. The lowest panel of A or B shows densitometric analysis of binding from three experiments. Each bar denotes the mean ± S.D. of three experiments. Lane 1, basal binding; lane 2, stimulation with LPS+IFN-γ for 4 h; lane 3, pretreatment with LY294002 prior to LPS+IFN-γ stimulation; and lane 4, pretreatment with SB 203580 before stimulation.

Fig. 12. Effect of LY294002 and PD98059 on NOS-2 protein levels stimulated by LPS, IFN-γ, or LPS+IFN-γ for 4 h. C denotes control in the absence of inhibitors. This figure is representative of two experiments with similar results.

MAPK, Jak2/3, and MEK1/2. Only a small number of reports address signaling pathways for LPS+IFN-γ-induced NOS-2 expression in RAW 264.7 cells. These reports provide information on signaling pathways for NOS-2 expression at 4–6 or 20–24 h but did not provide kinetic data. For example, Salh et al. (25) report the inhibition of NOS-2 expression at 6 h by LY294002, whereas Diaz-Guerra et al. (26) and Chen et al. (27) report the involvement of PKC in NOS-2 expression at 24 h. Diaz-Guerra et al. (28) report that NO production stimulated by LPS+IFN-γ for 24 h was augmented by LY294002. These results are consistent with our findings that PI3K mediates NOS-2 expression at 4–6 h and negatively regulates its expression at 24 h. Taken together, these results support the notion that NOS-2 expression stimulated by LPS+IFN-γ comprises two distinct phases that are signaled by different pathways: 1) an early phase of expression, which is signaled through PI3K, p38 MAPK, and Jak 2/3, and 2) a late phase of expression, which is signaled via PKC and mammalian target of rapamycin.

In this study, we have characterized considerably the early phase of expression. The early phase NOS-2 expression resembles the expression of an immediate early gene such as COX-2. Its signaling pathway leads to C/EBPβ binding and C/EBPβ-mediated transcriptional activation. We have demonstrated that salicylate is capable of suppressing the early phase NOS-2 expression by blocking C/EBPβ binding, and this inhibitory process also resembles its suppression of COX-2 transcriptional activation. In contrast, the late phase NOS-2 expression has not been characterized. Its signaling pathways appear to be complex, comprising positive and negative signaling pathways. The role of the negative signaling pathway in controlling the late phase NOS-2 expression is unclear. It is also unclear which transcriptional activators are activated through the positive signaling pathway and which transcription factors are controlled by the negative pathway. Our results have excluded the involvement of C/EBPβ in the late phase NOS-2 transcriptional activation. It is possible that NFκB and interferon regulatory factor-1 are the key transactivators for the late phase NOS-2 expression. Work is now in progress to characterize the signaling and transcriptional pathways for the late phase NOS-2 expression and the pharmacological control of its expression.

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