Superinduction of Cyclooxygenase-2 by NO• and Agonist Challenge Involves Transcriptional Regulation Mediated by AP-1 Activation†

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ABSTRACT: Superinduction of cyclooxygenase-2, in murine RAW 264.7 macrophages as well as human pulmonary type II A549 epithelial cells, is achieved by the simultaneous addition of agonists such as lipopolysaccharide or interleukin-1β and the NO• donor *S*-nitrosoglutathione. NO•-evoked superinduction of cyclooxygenase-2 in the presence of agonists was dose-dependent and required transcriptional as well as translational regulation. We sought to further analyze NO•-elicited superinduction at the level of the transcription factor NF-κB that is obligatory for cyclooxygenase-2 expression. NO•-mediated NF-κB activation was restricted to low concentrations of *S*-nitrosoglutathione (50−200 μM), while a higher dose of *S*-nitrosoglutathione (1 mM) was ineffective. Not observing a correlation between NF-κB activation and cyclooxygenase-2 expression under NO•-delivery stimulated our interest in analyzing AP-1. NO• efficiently activated AP-1 at all concentrations tested. The involvement of AP-1 in promoting cyclooxygenase-2 superinduction was established in cells transfected with the dominant-negative c-Jun mutant, TAM-67. Enhanced expression of cyclooxygenase-2 by lipopolysaccharide/*S*-nitrosoglutathione-treatment was attenuated in TAM-67 transfectants, while the response to lipopolysaccharide alone remained unaffected. We conclude that AP-1 activation exclusively conveys the NO• signal that is required for superinduction of cyclooxygenase-2. Superinduction of cyclooxygenase-2 is restricted to a situation where both, NF-κB and AP-1 are activated. Under inflammatory conditions this might be achieved by the costimulatory signals provided by agonist challenge and NO•.

Cyclooxygenase-2 (Cox-2),[1] also known as prostaglandin (PG) H synthase, catalyzes formation of prostaglandins that fulfill immune and inflammatory functions (*1*). Cox-2 is induced by lipopolysaccharide (LPS) and various cytokines under cellular conditions and is expressed at the site of inflammation in vivo (*2*, *3*). Inflammation generates not only prostaglandins but also high amounts of NO•, mainly as a result of macrophage activation (*4*). NO• is produced to achieve host defense. NO•-evoked toxicity seems to display a cell-selective response, because not all systems are equally affected. Recently, we reported Cox-2 induction by NO• as a protective principle against apoptosis in RAW 264.7 macrophages (*5*), with the notion that Cox-2 induction in activated macrophages is well-established (*6*). During inflammation, expression of Cox-2 may protect macrophages from self-destruction via initiation of apoptosis, thereby prolonging the survival of active immune competent cells.

Superinduction of many early response genes, including Cox-2, has been described for agonist stimulation in combination with translational blockers such as cycloheximide (CHX) (*7*). CHX has been reported to activate the transcription factor NF-κB in pulmonary type II A549 epithelial cells (*8*). Interestingly, NF-κB signaling is one determinant for immediate early gene expression of Cox-2. This has been established for cytokine- and NO•-induced Cox-2 expression (*9*−*11*). Besides NF-κB, another redox sensitive transcription factor, AP-1, is involved in Cox-2 transcriptional regulation. This was noticed when NO•-mediated Cox-2 induction was attenuated by the mitogen-activated protein kinase inhibitor PD98059 (*11*), taking into consideration that AP-1 activation is achieved via the MAP-kinase pathway (*12*, *13*). Our observations led to the hypothesis that activation of the transcription factor AP-1 in response to exogenously added NO• might occur and stimulated our interest to explore the effect of NO• in combination with LPS and IL-1β during Cox-2 induction.

AP-1 activation is achieved by many stimuli including growth factors, cytokines, T-cell activators, neurotransmitters, UV irradiation, and NO• as well as the phorbolester 12-*O*-tetradecanoylphorbol-13-actetate (*12*−*16*). Hence, the name TPA responsive element (TRE) is used for its recognition site (*17*). AP-1 is composed of c-Jun homodimers or heterodimers formed by members of the c-Jun- and the c-Fos-protooncogene family (*13*). Although Jun-Fos heterodimers show a higher transactivating potential than Jun homodimers, both complexes are involved in AP-1-dependent gene activation. AP-1-evoked gene expression is found in association with proliferation, differentiation, or stress responses (*18*).

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[1] Abbreviations: NO•, nitric oxide radical; Cox-2, cyclooxygenase-2; GSNO, S-nitrosoglutathione; LPS, lipopolysaccharide; CHX, cycloheximide; IL-1β, interleukin-1β; CRE, cAMP response element; NF-κB, nuclear factor kappa B.; AP-1, activator protein-1.

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A potential impact of AP-1 on Cox-2 expression has been described (*19*). Interestingly, the binding site for AP-1 in the Cox-2 promoter is a CRE (cAMP responsive element) rather than a TPA responsive element (TRE). Binding of AP-1 to CRE-sites located in various promoters has been identified (*20*) and transactivation is obtained directly by AP-1 or an enhancing effect of AP-1 in combination with the transcription factor CREB (CRE-binding protein) (*21*).

Here, we report that in murine RAW 264.7 macrophages and human pulmonary type II A549 epithelial cells, expression of Cox-2 is one of the molecular mechanisms achieved by NO•. To a great extent, this is evoked by a strong synergism between LPS/NO•- or IL-1β/NO•-treatment. Our results point to AP-1 activation by NO• as the underlying mechanism, as shown by transient transfection of the dominant negative c-Jun mutant TAM-67. We conclude that abrogation of AP-1 activity attenuates Cox-2 expression after NO• generation.

MATERIALS AND METHODS

Materials. LPS (*Escherichia coli* serotype 0127:B8) and cycloheximide were purchased from Sigma, Deisenhofen, Germany. The Cox-2 antibody was bought from Transduction Laboratories, Lexington, USA. Recombinant human IL-1β was provided by Boehringer Mannheim, Mannheim, Germany. RPMI, cell culture supplements, and fetal calf serum were ordered from Biochrom, Berlin, Germany. Oligonucleotides were provided by Eurogentec, Seraing, Belgium. The c-Jun and c-Fos-supershift antibodies were obtained from Santa Cruz Biotechnology, Heidelberg, Germany. All other materials were as specified (*5*, *11*).

Cell Culture. The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI). All experiments were performed using complete RPMI. A549 cells were grown to confluency as described (*8*). Human pulmonary type II A549 epithelial cells were incubated overnight in serum-free media before changing to fresh media containing cytokines, drugs or vehicle. GSNO, cycloheximide, IL-1β, and LPS were dissolved in water and added as indicated.

RNA Extraction and Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). RNA was extracted using RNAClean (AGS, Heidelberg, Germany) according to the distributors manual. Reverse transcription reactions and PCR for murine and human Cox-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using the SuperScript RNase H^- reverse transcriptase (GIBCO BRL, Karlsruhe, Germany) and recombinant Taq DNA Polymerase (GIBCO BRL, Karlsruhe, Germany). The sequence of the primers was as follows: Human Cox-2 (574-878) (*32*), $T_A = 52$ °C: 5′>3′ TTC AAA TGA GAT TGT GGG AAA AT; 3'>5' TTC TAT GAG TCC GTC TCT ACT AGA. Murine Cox-2 (1337-1915) (*33*), $T_A = 42$ °C: 5′>3′ CTC ACT TTG TTG AGT CAT TC; 3′>5′ GTA ATT GGG ATG TCA TGA TTA G. GAPDH (murine 135-717, human 155-759) (*34*, *35*), $T_A = 60$ °C: 5′>3′ GAA GGC CAT GCC AGT GAG CTT CC; 3′>5′ CCA TCA ACG ACC CCT TCA TTG ACC.

Annealing temperatures were calculated using the primer design program of Lasergene DNASTAR, Madison, Wisconsin, USA. The number of amplification cycles (25 for GAPDH; 30 for human and murine Cox-2) was necessary to achieve exponential amplification where product formation is proportional to starting cDNA. This was verified by densitometric quantification (data not shown). Products were run on 1% agarose gels and visualized by ethidiumbromide staining. Controls of isolated RNA omitting reverse transcription were used during PCR to guarantee genomic DNA-free RNA-preparations (data not shown).

Nuclear Protein Extraction. Preparation of crude nuclear extract was basically as described (*36*). Briefly, following cell activation for the times indicated, 4×10^6 RAW 264.7 macrophages were washed in 1 mL of ice-cold PBS, centrifuged at 1000*g* for 5 min, resuspended in 400 μL ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15000*g* for 30 s. Pelleted nuclei were gently resuspended in 50 μL ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15000*g* for 5 min at 4 °C. Aliquots of the supernatant, that contain nuclear proteins where frozen in liquid nitrogen and stored at −70 °C. Protein was determined using a Bio-Rad II Kit.

Electrophoretic Mobility Shift Assays. An established EMSA-method, with slight modifications, was used (*37*). Nuclear protein (5 μg) was incubated for 20 min at room temperature with 20 μg bovine serum albumin, 2 μg poly-(dI-dC) (Pharmacia, Freiburg, Germany), 2 μL buffer D (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.5 mM PMSF, pH 7.9), 4 μL buffer F (20% Ficoll-400, 100 mM HEPES/KOH, 300 mM KCl, 10 mM DTT, 0.5 mM PMSF, pH 7.9), and 20 000 cpm of a (^{32}P)-labeled oligonucleotide in a final volume of 20 μL. Supershift antibodies (2 μg) were added as indicated. DNA−protein complexes were resolved at 180 V for 4 h in a taurine-buffered, native 6% polyacrylamide-gel, dried, and visualized (autoradiography using a Fuji X-ray film). Oligonucleotide probes were labeled by a filling reaction using the Klenow fragment (Boehringer Mannheim, Mannheim, Germany). A 1 pmol sample of an oligonucleotide was labeled with 50 μCi of (α-^{32}P)-dCTP (3000 Ci/mmol, Amersham, Braunschweig, Germany), cold nucleotides (dATP, dTTP, dGTP from GIBCO, Eggenstein, Germany), purified on a CHROMA SPIN-10 column (Clontech, Heidelberg, Germany), and stored at −20 °C until use. The following oligonucleotide sequences were used. The NF-κB-site (-401/-393, italicized letters) from the murine Cox-2 promoter (*38*). 5′-GAG GTG AG*G GGA TTC CC*T TAG-3′ 3′-AC TC*C CCT AAG GG*A ATC AATC-5′. The AP-1 site from the human collagenase gene (17): 5′-AGC TAA AGC ATG AGT CAG ACA GCC T-3′ 3′-TT TCG TAC TCA GTC TGT CGG ATC GA-5′ (this oligonucleotide was kindly provided by Dr. Peter Angel, Deutsches Krebsforschungszentrum, Heidelberg, Germany).

The CRE-site from the from the murine Cox-2 promoter (*20*): 5′-AGC GGA CTC CAC GTG ACG TAG TGG T-3′ 3′-T GAG GTG CAC TGC ATC ACC AGG-5′.

Immunoblot Analyses. Cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0) and sonication (Branson

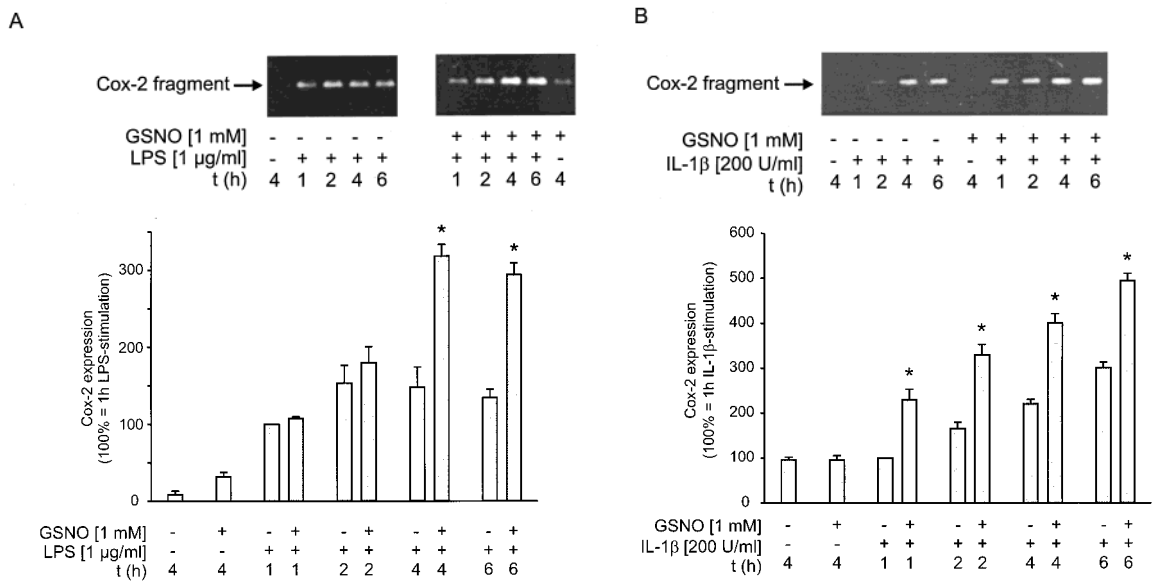

FIGURE 1: Superinduction of Cox-2 mRNA. LPS (1 μg/mL) and IL-1β (200 U/mL) alone or in combination with GSNO (1 mM) were assayed for Cox-2 RNA induction. Cells were harvested as indicated and RNA was prepared for semiquantitative RT-PCR. (A) Murine RAW 264.7 macrophages were exposed to LPS and/or GSNO as indicated. (B) Human A549 type II pulmonary epithelial cells were exposed to IL-1β and/or GSNO as indicated. Representative agarose-gels are shown and densitometric quantifications of three determinations are given (100% = 1 h LPS- or IL-1β-stimulation; *p $\leq$ 0.05, LPS-, or IL-1β-treatment vs LPS/GSNO− or IL-1β/GSNO-addition at individual time points). PCR-results are normalized for GAPDH-expression. For details see Materials and Methods.

sonifier, 20 s, duty cycle 100%, output control 60%). Following centrifugation (14000*g*, 5 min) protein was determined. Proteins (100 μg) were resolved on 10% polyacrylamide gels and blotted onto nitrocellulose. Equal loading was confirmed by Ponceau S staining. Filters were incubated overnight at 4 °C with the Cox-2 antibody (1:250, Dianova, Hamburg, Germany). Proteins were detected by a horseradish peroxidase (HRP)-conjugated polyclonal antibody (1:10 000), using the ECL method (Amersham, Braunschweig, Germany).

Transient Transfection of a Dominant Negative c-Jun Mutant into RAW 264.7 Macrophages. Targeting transcription factor activation by transient transfection of upstream signaling components requires high transfection efficiency and/or selection of cells expressing the mutant protein. One day before transfection, cells were seeded at a density of 1 $\times$ 10^6 cells/mL into 10 cm non cell culture plates. RAW 264.7 macrophages were transiently transfected with 15 μg of the pRc/CMV-TAM67 expression vector (abbreviated TAM-67), which contains the sequence of a dominant-negative c-Jun mutant (kindly provided by Dr. E. Gulbins, Tübingen, Germany) or 15 μg of a vector control (pRc/CMV, Invitrogen, Groningen, The Netherlands) (*39*, *40*). For positive selection, 5 μg of the vector pMACS4, designed to express a truncated human CD4 molecule, were cotransfected. Transfection was achieved using a Pro Gentor II electroporator (Hoefer Scientific Instruments, San Francisco, USA). 3 $\times$ 10^6 cells were resuspended in 400 μL complete medium, transferred to a cuvette, and pulsed (260 V, 1080 μF, 26 ms). Transfected cells were pooled and seeded in 10 mL complete medium into a 10 cm non cell-culture Petridish. Overnight (15 h) cultured cells were harvested and CD4-positive clones were enriched using a Mini-MACS system (Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany) according to the manufacturers instructions. Briefly, transfected cells were harvested in PBS supplemented with 5 mM EDTA; 10^7 cells were resuspended in 320 μL PBS/0.5% BSA/5 mM EDTA (PBE) and 80 μL MACSelect 4 Microbeads were needed to achieve magnetic labeling of transfected cells. After 15 min on ice, the volume was adjusted to 2 mL with PBE. Cells were applied to a positive selection column (MS^+), which was placed in the magnetic field of a Mini-MACS separator. Unbound cells were washed out (2 mL PBE), the column was removed from the separator, and positive cells were collected, pooled, and seeded. In control examinations, 15 h has been determined as the most effective period for allowing CD4 surface marker expression in RAW 264.7 macrophages. Control transfections with the empty vector construct were performed (data not shown).

RESULTS

Superinduction of Cyclooxygenase-2 mRNA. With the notion that cyclooxygenase-2 (Cox-2) is under the influence of the second messenger nitric oxide ($NO^\bullet$) and the current concept that $NO^\bullet$ may cause gene regulation, we analyzed transcription of Cox-2 in RAW 264.7 macrophages and human pulmonary type II epithelial A549 cells. Relative transcription of Cox-2 was determined by semiquantitative RT-PCR analyses using 30 PCR cycles in response to LPS, IL-1β, and/or the $NO^\bullet$-donor GSNO.

In a first set of experiments, we used RAW 264.7 macrophages and demonstrated upregulation of Cox-2 mRNA in response to LPS (Figure 1A). LPS is known as a specific stimulus for macrophages. LPS evoked Cox-2 transcription after 1 h and kept relative transcription at an elevated level for 6 h. GSNO by itself promoted Cox-2 transcription at a weak rate, only (Figure 1A). However, application of LPS together with GSNO amplified Cox-2 transcription two- to 3-fold. Statistical quantification confirmed these results (Figure 1 A, lower panel). Superinduction of Cox-2 mRNA formation became evident at 4 and 6 h, appeared weak at 2 h, and was absent at a 1 h coincubation period.

A rather similar appearance of Cox-2 mRNA induction was noticed in human epithelial A549 cells (Figure 1B),

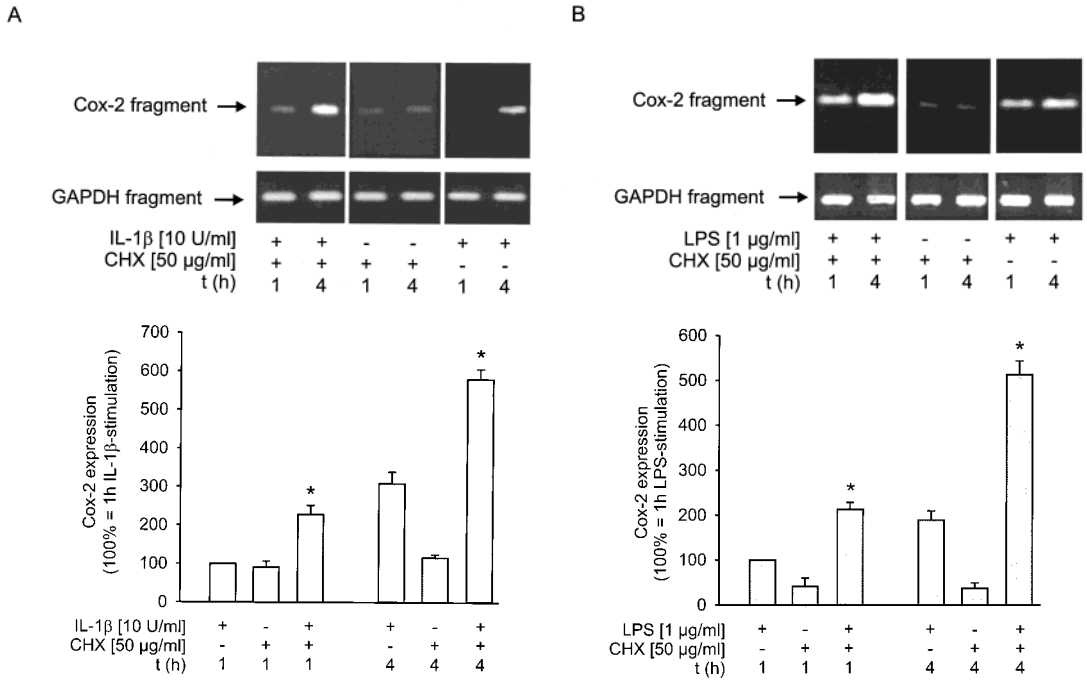

FIGURE 2: Superinduction of Cox-2 by cycloheximide. The transcriptional blocker cycloheximide (CHX, 50 μg/mL), agonists, or a combination of CHX with agonists (LPS, 1 μg/mL or IL-1β, 10 U/mL) are used for Cox-2 RNA induction. Cells were harvested after 1 and 4 h as indicated and RNA was prepared for semiquantitative RT-PCR. (A) Human A549 type II pulmonary epithelial cells were treated as indicated. (B) Murine RAW 264.7 macrophages were exposed as indicated. Representative agarose-gels are shown and densitometric quantifications of three determinations are given (100% = 1 h LPS- or IL-1β-stimulation; *p $\leq$ 0.05, LPS- or IL-1β-treatment vs LPS/CHX- or IL-1β/CHX- addition). PCR-results are normalized for GAPDH-expression. For details see Materials and Methods.

following IL-1β addition, which is an established cellular agonist for these cells in evoking Cox-2 expression. IL-1β elicited Cox-2 transcription at 4 and 6 h with a minor response at lower time points. GSNO alone was without any effect in A549 cells but significantly enhanced the IL-1β signal. The combination of GSNO and IL-1β actively transcribed Cox-2 at 1 and 2 h compared to either IL-1β or GSNO. Superinduction of Cox-2 following the addition of IL-1β/GSNO was also evident at 4 and 6 h when compared to the IL-1β response. These results were verified by statistical quantification (Figure 1B, lower panel). We conclude that the NO•-releasing compound GSNO enhanced Cox-2 transcription in LPS-stimulated murine macrophages or IL-1β-treated human epithelial A549 cells.

Superinduction of Cox-2 mRNA is known and has been established in A549 cells for the combination of IL-1β and cycloheximide (CHX), with the interpretation that superinduction involves increased transcription and not mRNA stabilization (*8*). Our analysis of Cox-2 PCR-fragments in A549 cells corroborated these studies (Figure 2A). Superinduction of Cox-2 by IL-1β and CHX was noted after 1 h and appeared approximately 2-fold after 4 h. Relative appearance of Cox-2 fragments were verified in proportion to the occurrence of GAPDH PCR-fragments.

We then asked whether CHX would promote superinduction of Cox-2 in macrophages as well (Figure 2B). The combinatory efficacy of LPS and CHX was analyzed at 1 and 4 h. Apparently, the addition of CHX-enhanced LPS-mediated Cox-2 transcription, especially after a 4 h incubation period. CHX by itself led to a minor Cox-2 induction, only (Figure 2A and B). Densitometric analyses of at least three independent experiments demonstrated statistical significance (Figures 2A and B, lower panels). Enhanced transcription of Cox-2 in response to LPS or IL-1β under the influence of GSNO or CHX raised the question whether molecular mechanisms of mRNA superinduction are similar for both, NO•-donating agents (GSNO) and compounds known to interfere with protein translation (CHX). Therefore, we studied protein expression of Cox-2 in macrophages and epithelial cells in response to LPS or IL-1β with or without the further addition of GSNO.

Superinduction of Cyclooxygenase-2 Protein Expression. Expression of Cox-2 was examined by Western blot analyses in RAW 264.7 macrophages. Exposure of macrophages for 15 h to 1 μg/mL LPS showed protein expression of Cox-2 relative to an unstimulated control (Figure 3A). To assess Cox-2 expression in response to NO•, we employed GSNO at concentrations ranging from 50 μM to 1 mM. GSNO promoted significant Cox-2 expression at 0.2 mM, only. Interestingly, GSNO enhanced LPS-evoked Cox-2 expression significantly (Figure 3A, lower part). Superinduction of Cox-2 was noticed at 0.2−1 mM GSNO. Under these conditions, the coadministration of LPS and GSNO promoted at least a 2-fold higher Cox-2 expression compared to the single application of either LPS or GSNO as shown by densitometric analyses (Figure 3B).

Extending investigations on Cox-2 expression in human A549 cells revealed an analogous situation. GSNO by itself turned out to be a weak Cox-2 inducing agent (Figure 3C). A dose−response relationship performed in A549 cells showed no Cox-2 expression at concentrations up to 0.5 mM GSNO. The NO• donor at concentrations of 1−2 mM elicited minor induction of Cox-2. However, the signal appeared weak compared to the IL-1β-response. The potency of GSNO to induce Cox-2 expression became more dramatic when GSNO was coadministered with IL-1β (Figure 3C, lower

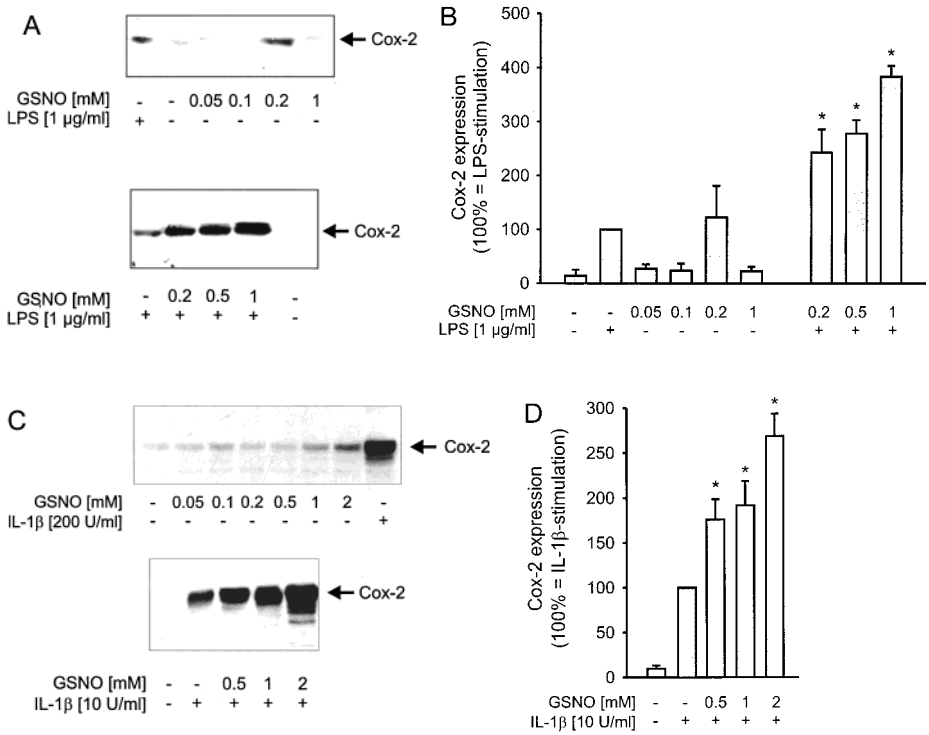

FIGURE 3: Protein expression of Cox-2. (A) Increasing concentrations of GSNO alone or in combination with LPS were assessed for Cox-2 protein expression in murine RAW 264.7 macrophages. Cells were stimulated with 50, 100, 200, and 1000 μM GSNO for 15 h (upper panel) or with 200, 500, and 1000 μM GSNO in combination with 1 μg/mL LPS (lower panel). (B) Densitometric quantifictaion of at least three experiments (100% = 15 h LPS stimulation. *p $\leq$ 0.05, LPS-treated sample vs LPS/GSNO-addition). (C) Increasing concentrations of GSNO alone or in combination with IL-1β were assessed for Cox-2 protein expression in human A549 type II pulmonary epithelial cells. Cells were stimulated with the indicated concentrations of GSNO for 15 h (upper panel) or with 500, 1000, and 2000 μM GSNO in combination with 10 U/mL IL-1β (lower panel). Cox-2 expression was analyzed by Western blotting as described under Materials and Methods. (D) Densitometric quantification of at least three experiments (100% = 15 h IL-1β-stimulation. *p $\leq$ 0.05, IL-1β-treated sample vs IL-1β/GSNO-addition).

part). GSNO at concentrations of 0.5−2 mM significantly enhanced IL-1β-evoked protein expression of Cox-2, at least 2-fold as verified by densitometric quantification (Figure 3D). As a result of these studies, we conclude that GSNO not only enhanced transcription of Cox-2 but also led to increased protein expression of Cox-2. Similar results were obtained for the NO donor spermine-NO (data not shown). Conclusively, NO$^\bullet$ donors promoted superinduction of Cox-2 at the mRNA and protein level.

Transcriptional Regulation of Cyclooxygenase-2 Induction. To obtain further insights into the molecular mechanism of NO$^\bullet$ action during Cox-2 induction, we determined transcription factors such as AP-1 and NF-κB, since they are known to affect Cox-2 promoter activity. AP-1 and NF-κB activation were analyzed by electrophoretic mobility shift analyses. In the absence of agonists, AP-1 was not activated (Figure 4A). Assessment of AP-1 oligonucleotide binding under conditions that have been specified for Cox-2 expression revealed a strong gel-shift signal. LPS in combination with 0.2, 0.5, and 1 mM GSNO promoted strong AP-1 activation. Significant activation of AP-1 was shared by GSNO itself (Figure 4B). GSNO at concentrations between 0.2 and 1 mM evoked significant AP-1 activation. The response was as strong as the effect elicited by the combination of LPS and GSNO. In contrast, LPS caused no or a very minor AP-1 response, after a 2, 4, and 6 h lasting exposure period (Figure 4C). We conclude that AP-1 activation is achieved as a result of NO$^\bullet$ delivery rather than LPS addition, which was confirmed by the densitometric quantification of at least three independent experiments (Figure 4D).

To verify binding of members of the AP-1 family of transcription factors to the CRE-site of the Cox-2 promoter, we used the specified oligonucleotides for EMSA and supershift analysis. As shown in Figure 4E, 1mM GSNO, supplied for 6 h to RAW 264.7 macrophages, provoked AP-1 binding to the CRE-oligonucleotide (Figure 4E, lane 2). Obviously, the complex consists of c-Jun/c-Fos-heterodimers as demonstrated by supershift analysis. Addition of c-Jun- (Figure 4E, lane 3) or c-Fos-antibodies (Figure 4E, lane 4) to the binding reaction clearly decreased complex binding, indicating the involvement of c-Jun and c-Fos. Addition of an unrelated antibody (PPAR-γ) had no influence on complex formation and, therefore, further guaranteed specificity (data not shown).

Next, we assayed NF-κB activation during a 6 h incubation period. NF-κB activation was nearly absent in unstimulated controls (Figure 5A). GSNO at concentrations of 50, 100, and 200 μM, dose-dependently achieved NF-κB activation. However, raising the dose of the NO$^\bullet$ donor to 1 mM revealed less prominent NF-κB activation, which again is mirrored by an abrogated Cox-2 expression at these concentrations. As expected, LPS caused NF-κB activation. The efficacy was comparable to the response achieved by 0.2 mM GSNO.

With the notion that Cox-2 expression was enhanced by coincubating LPS with GSNO, we looked for NF-κB

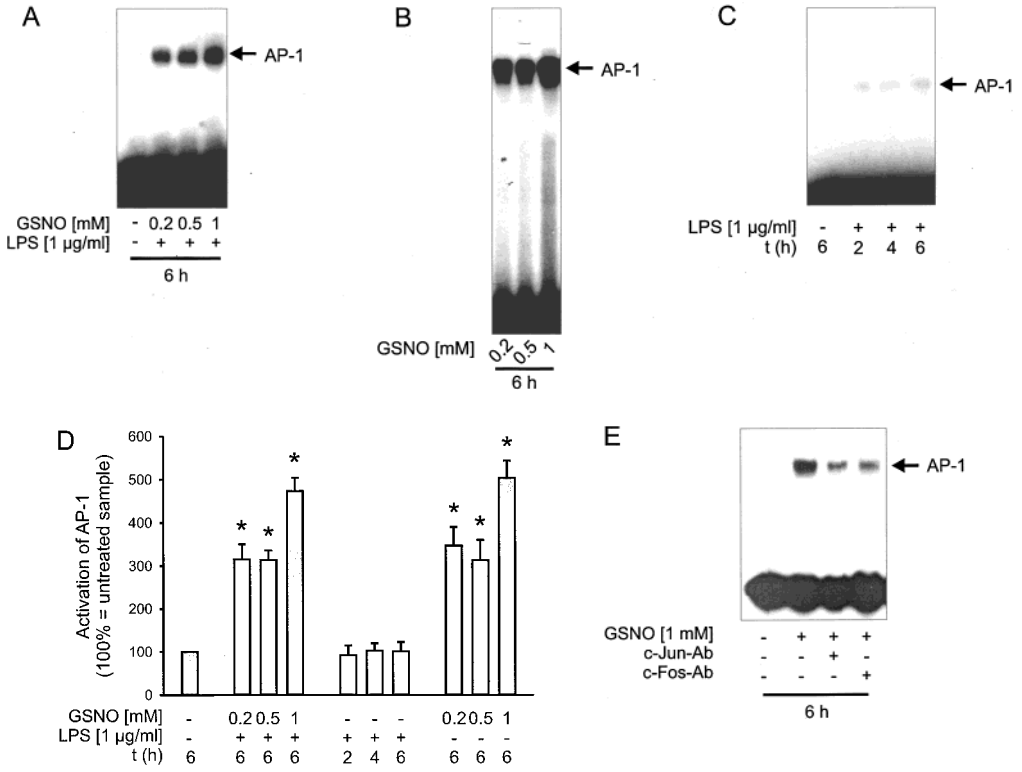

FIGURE 4: Activation of AP-1. (A) Activation of AP-1 by LPS (1 μg/mL) in combination with increasing concentrations of the GSNO (200, 500, and 1000 μM) was analyzed by an electrophoretic mobility shift assay (6 h). For controls, cell stimulation was omitted. (B) Cells were incubated for 6 h with 200, 500, and 1000 μM GSNO to achieve AP-1 activation. (C) Activation of AP-1 after LPS (1 μg/mL) challenge for the indicated times. For controls, cell stimulation was omitted. (D) Densitometric analysis of at least three independent experiments as shown in A−C (100% = untreated sample. *$p \leq 0.05$, 6 h LPS-treated sample vs GSNO or LPS/GSNO-addition). (E) AP-1 binding to the CRE site of the murine Cox-2 promoter. Supershift analysis of activated AP-1 was performed with the addition of a c-Jun antibody (lane 3) or a c-Fos antibody (lane 4). Macrophages were stimulated with 1 mM GSNO for 6 h. Data are representative of three independent experiments. For details see Materials and Methods.

activation when both agonists were combined (Figure 5B). NF-κB gel-shift analyses revealed activation of the transcription factor by LPS during a 2−6 h incubation period. The further addition of 0.2 mM GSNO slightly enhanced NF-κB activation. The combinatory effect of GSNO and LPS was more pronounced but not significant at 5 and 6 h. A higher concentration of GSNO (1 mM), which revealed neither NF-κB activation (Figure 5C) nor Cox-2 induction (Figure 2A), left LPS-induced NF-κB activation unaltered when added simultaneously with the agonist (Figure 5C). To verify these data, densitometric quantification of at least three independent experiments was performed (Figure 5D). As a result from these experiments, we assume that the NO•-enhancing potency on Cox-2 expression might stem from AP-1 activation rather than enhanced NF-κB binding. Therefore, we designed experiments to suppress the AP-1 transactivating activity by transient transfection of TAM-67, which is a dominant-negative c-Jun mutant. Expression of Cox-2 was followed by Western blot analyses after 15 h in response to LPS and the combination of LPS/GSNO (Figure 6). Densitometric quantification of at least three independent experiments revealed that inhibition of AP-1-attenuated Cox-2 expression in response to NO•. LPS-evoked Cox-2 expression remained unaffected in TAM-67 transfected cells. In accordance with results presented in Figure 3, the combination of LPS and GSNO elicited superinduction of Cox-2. Of note, we used GSNO at a concentration (1 mM) that was unable to induce NF-κB and thereby to contribute to superinduction. Control transfections using the empty vector (pRc/CMV) were without any effect on Cox-2 expression (Figure 6).

Enhanced expression of Cox-2 resulting from LPS/GSNO apparently was attenuated in TAM-67 transfected macrophages. Under these conditions, expression of Cox-2 was lowered and appeared comparable to the efficacy achieved by LPS alone. Obviously, superinduction of Cox-2, which results from NO• delivery, is abrogated by blocking the AP-1 transactivating activity.

DISCUSSION

Increasing evidence implies that NO• itself can induce Cox-2 expression in different cell systems, albeit to a low extent (*5*, *22*−*24*). Here, we show that NO• efficiently promotes superinduction of Cox-2 mRNA and protein expression in the presence of agonists. The effect is not only restricted to NO• but can also be achieved by the simultaneous formation of superoxide as described by Guastadisegni et al. (*25*). The potency of NO• is in contrast to the effect of the translational blocker CHX, which is known to increase Cox-2 transcription after agonist challenge, only (*8*). For NO• it is mandatory to activate or enhance transcription and translation, presumably by activation of transcription factors. NO•-mediated activation of NF-κB and AP-1 has recently been demonstrated (*15*, *26*). This becomes important, especially for upregulation of Cox-2, since activation of both NF-κB and AP-1 is necessary for NO•-induced Cox-2 expression, whereas LPS-evoked Cox-2 expression requires activation of NF-κB, only (*11*).

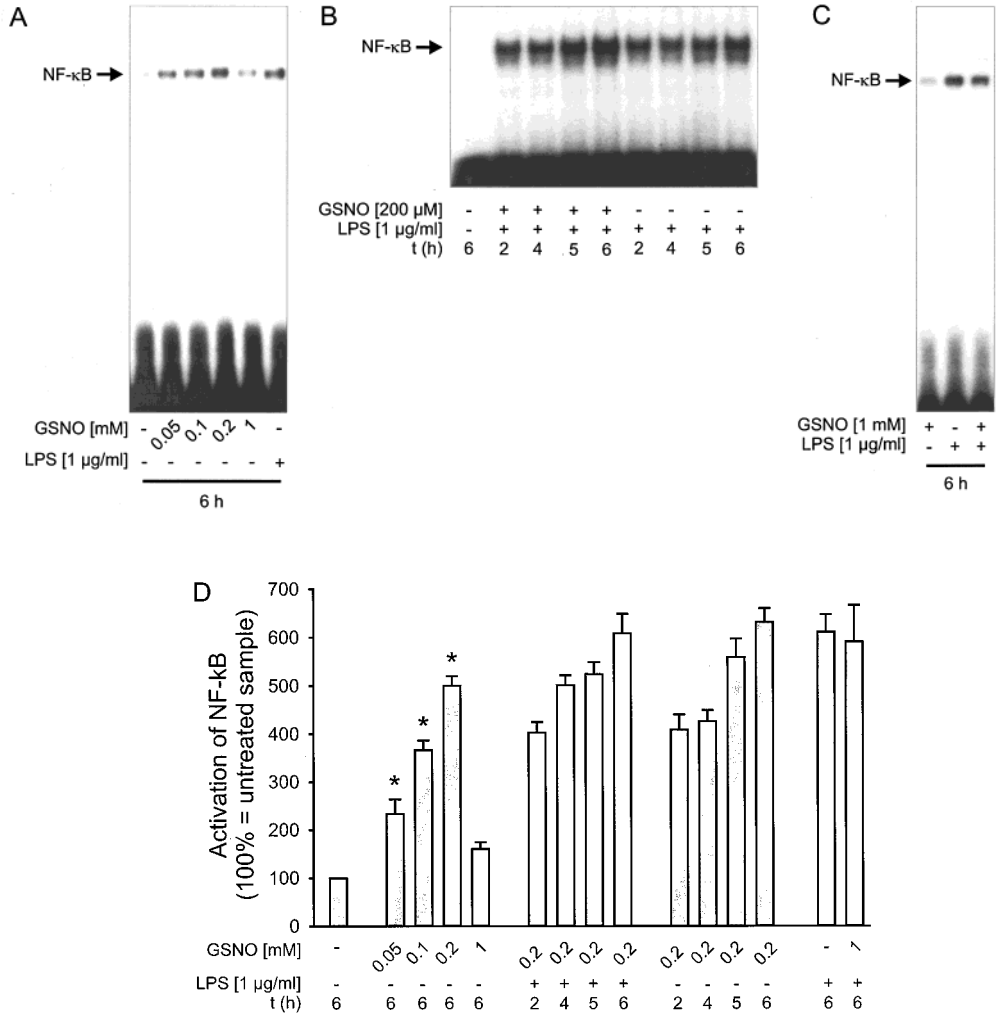

FIGURE 5: Activation of NF-κB. (A) Activation of NF-κB was analyzed after 6 h in response to GSNO (50, 100, 200, and 1000 μM) or LPS (1 μg/mL). For controls, cell stimulation was omitted. (B) Activation of NF-κB by LPS (1 μg/mL) alone or in combination with 200 μM of GSNO. Cells were incubated for the times indicated. For controls, cell stimulation was omitted. (C) Activation of NF-κB (6 h) by LPS (1 μg/mL), GSNO (1 mM), or a combination of LPS/GSNO. For details see Materials and Methods. (D) Densitometric analysis of at least three independent experiments as shown in A−C (100% = untreated sample. *$p \leq 0.05$, GSNO-treated samples versus unstimulated control).

In the absence of a receptor agonist, NO$^\bullet$ alone dose-dependently activated NF-κB at concentrations from 50 to 200 μM. At higher GSNO-concentrations (1 mM), NF-κB activation was less obvious. A decreased gel-shift response at higher NO$^\bullet$ doses indicates less prominent or no NF-κB DNA-binding rather than conformational changes of the transcription factor, thus attenuating DNA-binding. This assumption is substantiated by the fact that 1 mM GSNO left LPS-evoked NF-κB activation unaltered (Figure 5C). Our results imply a concentration-dependent effect of NO$^\bullet$, with low or moderate concentrations leading to NF-κB activation (*11*, *26*) while high concentrations do not promote NF-κB activation (*27*).

Gel-shift analyses of NF-κB activation in response to the receptor agonist LPS in combination with 200 μM GSNO revealed a minor increase compared to LPS alone. This is in line with the report of Umansky et al. (*28*), who described enhanced NF-κB activation and an increase in IκB kinase-activity in TNF-α-stimulated endothelial cells after the coaddition of low NO$^\bullet$ concentrations. In another study, the NOS-inhibitor L-NAME blocked IFN-γ-induced PGE_2 production in osteoblasts (*29*). The authors reported that L-NAME-attenuated Cox-2 expression was partially restored after the addition of the NO$^\bullet$ donor sodium nitroprusside (SNP), with the implication that SNP itself upregulated Cox-2. As a result of these studies, it appears that NO$^\bullet$ at low concentrations promotes Cox-2 expression, whereas high doses of NO$^\bullet$-releasing agents do not activate NF-κB and do not evoke Cox-2 expression. However, in combination with an agonist such as LPS or IL-1β, superinduction of Cox-2 expression is also achieved by elevated NO$^\bullet$-concentrations.

A possible candidate for transmitting the NO$^\bullet$-superinducing effect is AP-1. In NO$^\bullet$-exposed macrophages or mesangial cells, activation of the stress-activated protein kinase pathway has been identified as one possible signaling system that potentially leads to AP-1 activation (*30*, *31*). Our gel-shifts revealed that AP-1 is concentration-dependently activated by NO$^\bullet$ donors, such as GSNO (Figure 4A) or spermine-NO (unpublished results). The essential role of AP-1 for NO$^\bullet$-enforced Cox-2 expression in the presence of agonists was verified in cells transfected with a dominant negative c-Jun mutant (TAM-67). Activation of AP-1 by NO$^\bullet$ was strong compared to LPS or IL-1β and binding of AP-1

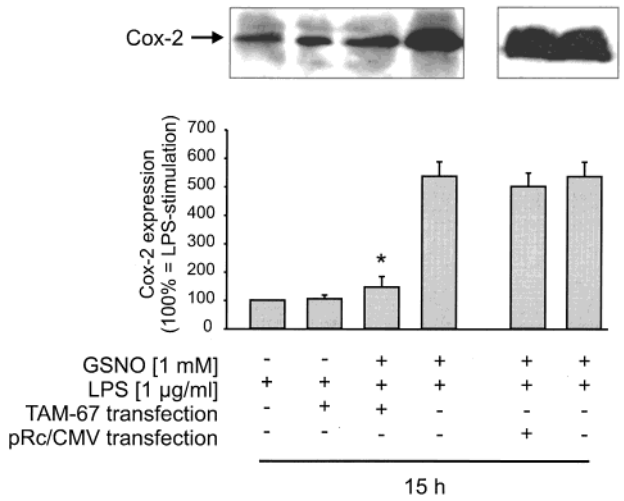

FIGURE 6: Cox-2 expression in TAM-67 transfected macrophages. Control cells, TAM-67 transfectants, or vector controls (pRc/CMV) were incubated for 15 h with 1 mM GSNO in the presence of LPS (1 μg/mL) as indicated. Cox-2 expression was analyzed by Western blotting as described under Materials and Methods. Blots of two separate representative experiments are shown (lanes 1−4, and lanes 5, 6). Densitometric quantification of three experiments is shown in the lower panel (100% = 15 h LPS-treated sample. *$p \leq 0.05$, LPS/GSNO-treatment in TAM-67 transfectants vs vector controls).

to the Cox-2 promoter is achieved via a homologous CRE-site (*20*). The binding complex consists of a c-Jun/c-Fos-heterodimer as shown by superhift analysis. The antibodies used were generated against the DNA-binding domain of the proteins and, therefore, decreased DNA-binding rather than a supershift was expected. Experimentially, we proved decreased Cox-2 expression in TAM-67 transfected cells after the coaddition of LPS and 1 mM GSNO, thus pointing to efficient expression of the dominant negative form of c-Jun.

Conclusively, AP-1 is the important NO•-induced transcription factor responsible for Cox-2 superinduction. In contrast, TAM-67 transfection left LPS-induced Cox-2 expression unaltered, further implicating that AP-1 exclusively conveys the NO•-stimulatory response. Therefore, enforced Cox-2 expression by NO• in the presence of agonists is due to activation of AP-1.

In conclusion, Cox-2 expression emerges as one of the molecular mechanisms evoked by NO• under inflammatory conditions. In the presence of agonists, NO• is able to superinduce gene and protein expression of Cox-2 by promoting activation of AP-1, whereas agonists achieve NF-κB activation that additionally is needed for Cox-2 expression. This is in close analogy to a recent report by Diaz-Cazorla et al. (*41*) who reported on the ability of endogenously produced NO to enhance Cox-2 expression in human mesangial cells. Our study further adds to the role of NO• in eliciting gene regulation that may be one important factor in inflammatory and immunological processes.

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