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ORIGINAL ARTICLE

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Nitric oxide prevents inducible cyclooxygenase expression by inhibiting nuclear factor-κB and nuclear factor-interleukin-6 activation

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Abstract Stimulation of J774 macrophages with lipopolysaccharide (LPS) leads to the release of large amounts of prostaglandins (PGs) generated by the inducible isoform of cyclooxygenase (COX-2). Nitric oxide (NO), a pleiotropic free radical, has been demonstrated to modulate the release of a broad range of inflammatory mediators, amongst these PGs. In the present study we investigated the molecular mechanism by which NO affects cyclooxygenase pathway. Incubation of J774 cells with LPS caused an increase of prostaglandin E_2 production and COX-2 protein expression which was prevented in a concentration-dependent fashion by pre-incubating cells with sodium nitroprusside (SNP) and *S*-nitroso-gluthatione (GSNO), two NO-generating agents. Electrophoretic mobility shift assay indicated that both NO-generating agents blocked LPS-induced activation of nuclear factor-κB (NF-κB) by increasing IκB-α protein expression and blocking nuclear translocation of NF-κB subunits p50 and p65. SNP and GSNO also inhibited nuclear factor-interleukin-6 (NF-IL6) activation. These results show for the first time that SNP and GSNO down-regulate LPS-induced COX-2 expression by inhibiting NF-κB and NF-IL6 activation and suggest a negative feed-back mechanism that may be important for limiting excessive or prolonged PGs production in pathological events.

Keywords Macrophages · Nitric oxide · Prostaglandins · Nuclear factor-κB · Nuclear factor-interleukin-6

Introduction

Cyclooxygenase (COX) is the key enzyme for prostaglandins (PGs) and other eicosanoid generation from arachidonic acid (Xie et al. 1992). Nitric oxide synthase (NOS) is the enzyme that catalyses the formation of nitric oxide (NO) from the guanidino nitrogen atom of L-arginine (Nathan 1992). Two isoforms of both enzymes occur: a constitutive one, COX-1, and the inducible counterpart COX-2 (Lee et al. 1992); also NOS has a constitutive counterpart (endothelial NOS or eNOS and neuronal NOS or nNOS) and an inducible isoform, called iNOS (Nathan 1992). After stimulation with LPS or interleukin-1, many cell types, including endothelial cells and macrophages, express the inducible isoforms of either COX or NOS which are responsible for the production of large amounts of PGs and NO, respectively (Lee et al. 1992; Maier et al. 1990; Schmidt and Ulrich 1994). These inducible enzymes are essential components of the inflammatory response and implicated in the pathogenesis of several inflammatory diseases (Ialenti et al. 1992, 1993; Iuvone et al. 1994; Mitchell et al. 1993). Several studies have suggested an important link between the NOS and COX pathways, although the precise mechanism underlying this interaction is far from being identified (Di Rosa et al. 1996). Conflicting data present in literature have described both an inhibitory and stimulatory action of NO on COX pathway either in vitro or in vivo (Clancy et al. 2000; Di Rosa et al. 1996; Salvemini 1997). Recent findings have suggested transcription factors, such as nuclear factor-κB (NF-κB), as potential molecular targets for the biological actions of NO. Nevertheless, the effects of NO on NF-κB are quite controversial. In fact, NO can activate the NF-κB DNA binding activity in some cell types (Diaz-Cazorla et al. 1999; Simpson and Morris 1999) while having an inhibitory effect in others (delaTorre et al. 1997, 1999; Matthews et al. 1996; Park et al. 1997; Peng et al. 1995, 1998; Spiecker et al. 1997; Umansky et al. 1998). It has been demonstrated that treatment of cells with NO-generating compounds, such as sodium nitroprusside (SNP) and *S*-nitrosoglutathione (GSNO), inhibits activation of the NF-κB (Peng et al. 1995). The promoter region of COX-2 genes in mouse (Fletcher et al. 1992), rat (Sirois et al. 1993) and human (Kosaka et al. 1994) has been cloned and sequenced. This promoter region contains a canonical TATA box and various putative transcriptional regulatory

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elements such as CRE, AP2, Sp-1, GATA box, NF-IL6 and NF-κB (Inoue et al. 1994; Kim and Fischer 1998; Xie et al. 1994; Yamamoto et al. 1995). Mutation analysis of COX-2 murine promoter region (–512 bp to +123 bp) revealed that NF-κB and NF-IL6, but not AP2, acted as positive regulatory elements for the induction of COX-2 in murine osteogenic cell lines MC3T3-E1 (Yamamoto et al. 1995). Moreover, we have previously shown that incubation of J774 cells with ammonium pyrrolidinedithiocarbamate and *N*-α-*p*-tosyl-L-lysine chloromethylketone, two inhibitors of NF-κB activation, suppressed in a concentration-dependent manner both LPS-induced COX-2 protein expression and prostanoid generation (D'Acquisto et al. 1997). NF-κB is a member of the *Rel* family proteins and is typically a heterodimer of the p50 and p65 subunits. In quiescent cells, NF-κB resides in the cytosol in latent form bound to inhibitory proteins, IκBs. Stimulation of different types of cells with lipopolysaccharide, cytokines or oxidants triggers a series of signalling events that ultimately converge to the activation of one or more redox-sensitive kinases which specifically phosphorylate IκB, resulting in IκB polyubiquinitation and subsequent degradation. Once activated, the liberated NF-κB translocates into the nucleus and stimulates transcription by binding to cognate κB sites in the promoter regions of various target genes (for extensive review, see May and Ghosh 1998; Thanos and Maniatis 1995; Verma et al. 1995). The transcription factors CCAAT/enhancer-binding protein (C/EBP) comprise a family of isoforms encoded by separate genes. Seven members of C/EBP family identified so far share three structural components: C-terminal leucine zipper, a basic DNA-binding region and an N-terminal transactivating region. Multiple target genes in a variety of tissues have been reported for C/EBP family (Callejas et al. 2000; Wedel and Ziegler-Heitbrock 1995). C/EBPβ and especially C/EBPδ are highly inducible or activated via several signalling pathways in different biological systems, including monocyte/macrophages during the acute phase response elicited by LPS (Alam et al. 1992; Burgess-Beusse and Darlington 1998; Natsuka et al. 1992). These C/EBP isoforms participate in the expression of IL-1β (Pope et al. 1994), TNF-α (Matsusaka et al. 1993), IL-6, IL-8 (Margulies and Sehgal 1993) and other cytokines (Wedel and Ziegler-Heitbrock 1995). Most interestingly, a synergistic interaction between NF-κB and C/EBP transcription factors has been shown to mediate a tissue-specific response to different stimuli (Cha-Molstad et al. 2000; Maehara et al. 1999; Montaner et al. 1999; Ray and Ray 1995; Wadleigh et al. 2000). In this study, we demonstrate the involvement of NF-κB and NF-IL6 transcription factors in modulating COX-2 expression by NO in LPS-stimulated murine macrophage cell line J774.

Materials and methods

Cell culture. The murine monocyte/macrophage cell line J774 (American Tissue Culture Catalogue T1B pag. 231) was cultured at 37°C in humidified 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin. The cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well or 10-cm-diameter culture dishes at a density of 1×10^7 cells/ml per dish and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated with LPS (1 μg/ml). SNP (0.25, 0.5 and 1 mM), GSH (0.1 mM) or GSNO (0.1 mM) were added to the cells 5 min before LPS challenge. In parallel experiments spent SNP (0.25, 0.5 and 1 mM) was added to the cells. In some experiments SNP (1 mM) or GSNO (0.1 mM) was added 12 h after LPS challenge. The cell viability was determined by using MTT conversion assay (Mosmann 1983). Briefly, 100 μl MTT (5 mg/ml in complete DMEM) was added and the cells were incubated for an additional 3 h. After this time point the cells were lysed and the dark blue crystals solubilized with 500 μl of a solution containing 50% (v:v) *N*,*N*-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620-nm filter. The cell viability in response to treatment with test compounds was calculated as % dead cells =100– (OD treated/OD control) ×100.

PGE_2 determination. The accumulation of PGE_2 in the culture medium 24 h after LPS challenge was measured by ELISA kit according to the manufacturer's instructions (TEMA Ricerche, Milan, Italy).

Preparation of cytosolic and nuclear extracts. Cytosolic and nuclear extracts of macrophages stimulated for 2 h with LPS (1 μg/ml) in the presence or absence of SNP (0.25, 0.5 and 1 mM), GSH (0.1 mM) or GSNO (0.1 mM) were prepared as previously described (D'Acquisto et al. 1998). Briefly, harvested cells (2×10^7) were washed two times with ice-cold PBS and centrifuged at 180 *g* for 10 min at 4°C. The cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five or six times and the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13,000 *g*. The nuclear pellet was resuspended in 60 μl of high salt extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 *g* and supernatant was aliquoted and stored at –80°C. Protein concentration was determined by the Bio-Rad protein assay kit.

Electrophoretic mobility shift assay. Nuclear extracts containing 5 μg protein were incubated for 30 min with radiolabeled oligonucleotides ($2.5–5.0\times10^4$ cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg/ml bovine serum albumin, 10% (v/v) glycerol.

Five double-stranded oligonucleotides previously described by Yamamoto et al. (1995) were used as probes:

1. g1 (–409 bp to –385 bp) containing NF-κB and its vicinity
   - 5'-GAG GTG AGG GGA TTC CCT TAG TTA G-3'
   - 3'-CTC CAC TCC CCT AAG GGA ATC AAT C-5'
2. g2, a mutated probe for NF-κB in which GA of the NF-κB was changed to CC
   - 5'-GAG GTG AGG CCT TCC CTT AGT TAG-3'
   - 3'-CTC CAC TCC GGA AGG GAA TCA ATC-5'
3. gA (–155 bp to –121 bp) including AP2 and NF-IL6 binding site
   - 5'-GCC TGC CGC TGC GGT TCT TGC GCA ACT CAC TGA AG-3'
   - 3'-CGG ACG GCG ACG CCA AGA ACG CGT TGA GTG ACT TC-5'

4. gB, a mutant gA probe for AP2 binding site (GC was changed to TT)
   - 5'-GCC TGC CGC TTT GGT TCT TGC GCC AAC TCA CTG AAG-3'
   - 3'-CGG ACG GCG AAA CCA AGA ACG CGG TTG AGT GAC TTC-5'
5. gC, a mutant gA probe for NF-IL6 binding site (TTGCG was changed to CCGCT)
   - 5'-GCC TGC CGC TGC GGT TCC CGC TCA ACT CAC TGA AG-3'
   - 3'-CGG ACG GCG ACG CCA AGG GCG AGT TGA GTG ACT TC-5'

Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× TBE buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at –80°C for 20 h. Subsequently, the relative bands in nuclear fractions were quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, IBM).

Western blot analysis. Immunoblotting analysis of anti-COX-2, anti-p50, anti-p65 and anti-IκB-α was performed on J774 cells incubated with LPS (1 μg/ml) in the presence or absence of SNP, GSH or GSNO at different time points. Cytosolic and nuclear fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml of bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10,000 *g* for 10 min. Protein concentration was determined and equivalent amounts (75 μg) of each sample were electrophoresed in a 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-COX-2, anti-p50, anti-p65 and anti-IκB-α for 1 h at room temperature. The membranes were washed three times with 1% Triton 100-X in PBS and then incubated with anti-rabbit or anti-goat immunoglobulins coupled to peroxidase (1:1000). The immunocomplexes were visualised by the ECL chemiluminescence method (Amersham). Subsequently, the relative expression of COX-2, IκB-α, p50 and p65 proteins in cytosolic and nuclear fraction was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, IBM).

Reverse transcription-polymerase chain reaction. J774 macrophages (10×10^6) were resuspended in 1.0 ml TRIzol reagent (Life Technologies, Grand Island, N.Y., USA) and homogenized with a polytron homogenizer. After homogenization, samples were left at room temperature for 5 min and then chloroform was added (0.2 ml/1 ml TRIzol). Samples were shaken by hand for 15 s, left at room temperature for 2–3 min, and then centrifuged at 12,000 *g* for 30 min at 4°C. A 500-μl aliquot of the aqueous phase was placed in a microfuge tube and isopropyl alcohol (0.5 ml/1 ml TRIzol) was added to precipitate the RNA. The tubes were then centrifuged at 12,000 *g* for 20 min at 4°C to pellet the RNA. The supernatant was removed and the RNA was washed with 75% ethanol, vortexed and centrifuged again. The ethanol was removed and the RNA pellet was air-dried before dissolving in RNase-free water. The ratio of RNA/DNA and the tRNA concentration of the sample were determined with a GeneQuant II nucleic acid analyser. The extracted RNA was then reverse transcribed by adding 2.0 μg RNA per sample to a reaction mixture that contained 2 μl of 10× PCR buffer, 2 μl of mixed nucleotide triphosphates (10 mM), 2 μl of random nucleotide hexamers (N6; 900 pmol/μl), 0.5 μl of RNA guard (27 U), 11 μl of double-distilled water, and 1.5 μl of Superscript reverse transcriptase (300 U). Samples were placed in a thermocycler and reverse transcribed at 42°C for 50 min.

For PCR, 2 μl of the reverse-transcribed cDNA was placed in a tube that contained 2 μl of 2 mM mixed nucleotide triphosphates, 5 μl of 10× PCR buffer, and 35 μl of double-distilled water. Two microliters of each of the 5' and 3' primers was added for COX-2 (5'-CAC AGT ACA CTA CAT CCT GAC C-3'; 3'-GTT CTG TCT TAG TCT TCG CTC CT-5') and GAPDH (5'-CGG AGT CAA CGG ATT TGG TCG TAT-3'; 3'-AGC CTT CTC CAT GGT GGT GAA GAC-5'). GAPDH was used as an internal control. *Taq* polymerase was added with a hot start to reduce nonspecific binding. The samples were cycled as follows: 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min. Optimal amplification was achieved at 32 cycles for COX-2 and 22 cycles for GAPDH. The PCR products were run on a 1% agarose gel containing ethidium bromide. After separation the bands were visualised under UV light with a Gel Doc 2000, and analysed with Quantity One software (Bio-Rad).

Statistics. Results were expressed as the means ± SEM of *n* experiments. Statistical analysis was determined by Student's unpaired *t*-test with $P<0.05$ considered significant.

Reagents. LPS (from *S. thyphosa*) was from DIFCO (Detroit, Mich., USA). Phosphate buffer saline was from Celbio (Milan, Italy). DL-Dithiothreitol, phenylmethylsulfonylfluoride, soybean trypsin inhibitor, pepstatin A, leupeptin and benzamidine were from Calbiochem (Milan, Italy). [^{32}P]γATP was from Amersham (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-COX-2, anti-IκB-α, anti-p50, anti-p65, anti c-Rel, anti-C/EBPβ, anti-C/EBPδ and anti-AP2 antibodies were from Santa Cruz (Milan, Italy). Non-fat dry milk was from Bio-Rad (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). All the other reagents were from Sigma (Milan, Italy).

Results

Effect of SNP and GSNO on LPS-induced PGE_2 production and COX-2 expression

The production of PGE_2 by unstimulated J774 macrophages was undetectable (<3 nmol/10^6 cells, n=6). The stimulation of cells with LPS (1 μg/ml) for 24 h resulted in an accumulation of PGE_2 in the medium (35.5±1.3 nmol/10^6 cells, n=9). Moreover, upon stimulation with LPS (1 μg/ml) for 24 h, cells expressed a significantly high level of COX-2 protein when compared to control, untreated cells. Treatment of cells with SNP (0.25, 0.5 and 1 mM) or GSNO (0.1 mM) reduced in a concentration-dependent manner PGE_2 production (by 11.8±0.45%, 50.0±0.4%, 78.0±0.38% and 76.6±0.32%, respectively, n=6) at 24 h, while the treatment with GSH (0.1 mM) had no effect (Fig. 1). Spent SNP (0.25, 0.5 and 1 mM) also did

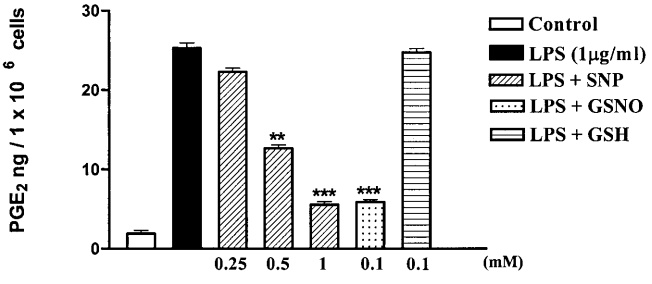

Fig. 1 Effect of SNP (0.25, 0.5 and 1 mM), GSNO (0.1 mM) or GSH (0.1 mM) on PGE_2 production by J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. The values are expressed as means ± SEM (n=4). $^{**}P<0.001$, $^{***}P<0.0001$ vs. control (LPS alone)

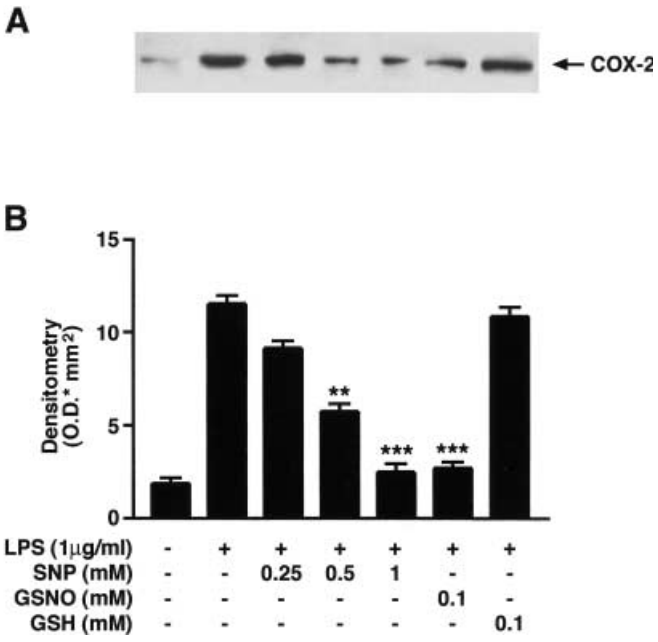

Fig. 2 Representative Western blot (**A**) as well as the densitometric analysis (**B**) show the effect of SNP (0.25, 0.5 and 1 mM), GSNO (0.1 mM) or GSH (0.1 mM) on COX-2 protein expression in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. Data in **A** are from a single experiment and representative of three separate experiments, while data in **B** are expressed as means ± SEM of three separate experiments. ***P*<0.001, ****P*<0.0001 vs. control (LPS alone)

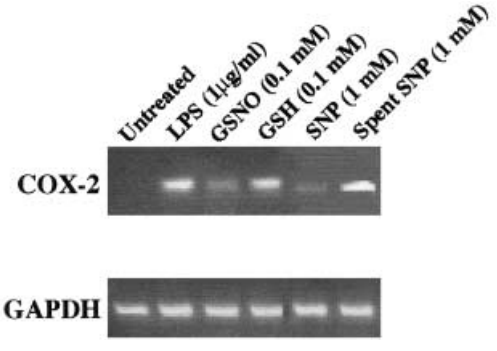

Fig. 3 Effect of GSNO, GSH, SNP and spent SNP on COX-2 expression. J774 macrophages were stimulated with LPS (1 μg/ml) in the presence or absence of GSNO (0.1 mM), GSH (0.1 mM), SNP (1 mM) and spent SNP (1 mM). mRNA levels of COX-2 were quantified by RT-PCR analysis. For details, see Materials and methods. Autoradiographs for COX-2 and GAPDH are representative of three determinations

not affect PGE_2 production (32.1±1.2, 29.3±2.3, 26.8±2.5 nmol/10^6 cells, *n*=9). Western blot analysis of LPS-stimulated J774 macrophages showed marked COX-2 protein induction which was inhibited in a concentration-dependent fashion by SNP (0.25, 0.5 and 1 mM) and GSNO (0.1 mM; 20.9±0.38%, 50.2±0.4%, 78.5±0.45% and 76.7±0.32%, respectively, *n*=6; Fig. 2). The addition of SNP (1 mM) or GSNO (0.1 mM) to the cells 12 h after LPS challenge did not affect COX-2 protein expression or PGE_2 accumulation at 24 h (data not shown), indicating that both compounds did not cause direct inhibition of enzyme activity. Moreover, the effect of SNP (1 mM) and GSNO (0.1 mM) on LPS-induced mRNA COX-2 levels was detected by RT-PCR and illustrated in Fig. 3.

Effect of SNP and GSNO on LPS-induced NF-κB activation

Electrophoretic mobility shift assay demonstrated that the activation of NF-κB by LPS was inhibited by NO in a concentration-dependent manner. As shown in Fig. 3, when g1 probe was incubated with nuclear extracts of J774 macrophages, a basal level of NF-κB/DNA binding activity was detected, whereas two retarded bands clearly appeared following stimulation with LPS (1 μg/ml) for 2 h. The binding was not observed by using the g2 probe with a mutation in the NF-κB motif (data not shown). Treatment of cells with SNP (0.25, 0.5 and 1 mM) or GSNO (0.1 mM) caused a significant inhibition of NF-κB/DNA binding activity (by 0.5±2.6%, 39.1±2.4%, 57.2±2.9% and 58.5±2.5%, respectively, *n*=6; Fig. 4). The specificity of NF-κB/DNA binding complex was evident by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabeled g1 probe in the competition reaction. In contrast, a 50-fold molar excess of unlabeled g2 probe or Sp-1 oligonucleotide had no

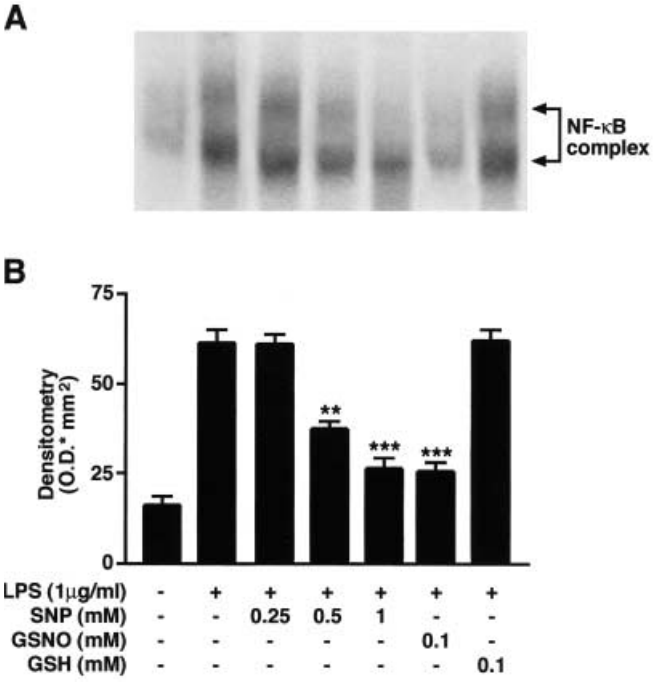

Fig. 4 Representative electrophoretic mobility shift assay (EMSA; **A**) as well as densitometric analysis (**B**) show the effect of SNP (0.25, 0.5, 1 mM), GSNO (0.1 mM) or GSH (0.1 mM) on LPS-induced NF-κB/DNA binding activity. Data in **A** are from a single experiment and representative of three separate experiments, while data in **B** are expressed as means ± SEM of three separate experiments. ***P*<0.001, ****P*<0.0001 vs. control (LPS alone)

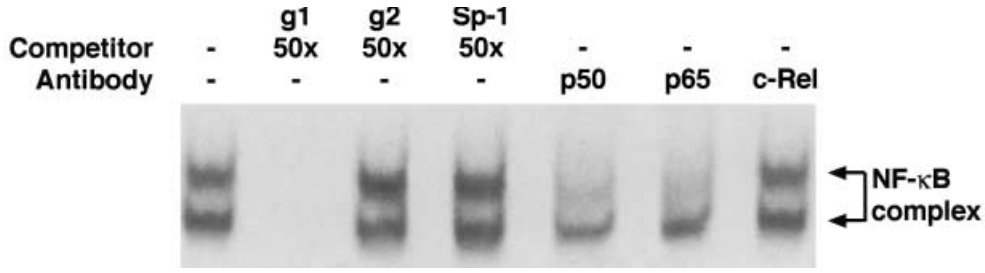

Fig. 5 Nuclear extracts from LPS-treated J774 macrophages were prepared as described in Materials and methods and incubated with ^{32}P-labeled g1 probe. In competition reaction nuclear extracts were incubated with radiolabeled g1 probe in absence or presence of identical but unlabeled oligonucleotides (*g1*, 50×), mutated non-functional κB probe (*g2*, 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (*Sp-1*, 50×). In supershift experiments nuclear extracts were incubated with antibodies against p50 (*p50*), p65 (*p65*) or c-Rel (*c-Rel*) 30 min before incubation with radiolabeled g1 probe. Data illustrated are from a single experiment and representative of three separate experiments

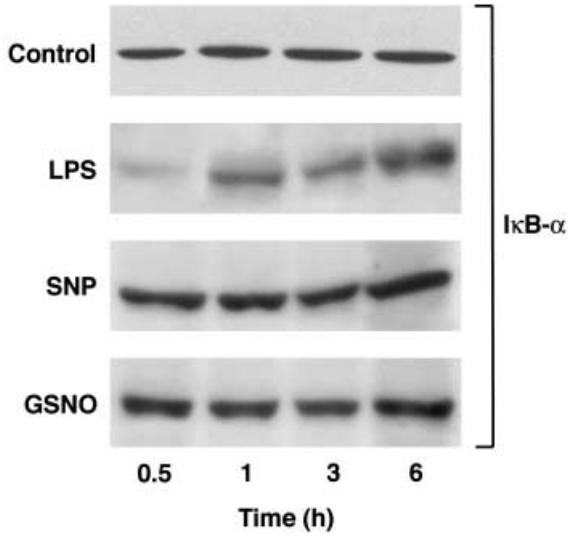

Fig. 6 Western blot analysis shows the effect of SNP (1 mM) and GSNO (0.1 mM) on degradation of IκB-α in J774 macrophages collected at 0.5, 1, 3 and 6 h after LPS (1 μg/ml) challenge. *Control:* basal level of IκB-α band was present in cytosolic fraction of unstimulated cells. *LPS:* IκB-α band has disappeared from the cytosolic fraction at 0.5 h to return to basal level at 1, 3 and 6 h after LPS challenge. *SNP and GSNO:* IκB-α band increased in a time-dependent manner as compared to LPS-stimulated cells. Data illustrated are from a single experiment and representative of three separate experiments

effect on this DNA-binding activity. The composition of the NF-κB complex activated by LPS was determined by using specific antibodies against p50 (p50), p65 (p65) and c-*Rel* (c-Rel) subunits of NF-κB proteins. Addition of either anti-p50 or anti-p65 but not anti-c-Rel to the binding reaction resulted in a marked reduction of NF-κB band intensity, suggesting that NF-κB complex contained predominantly p50 and p65 subunits (Fig. 5).

Effects of SNP and GSNO on degradation of IκB-α and nuclear translocation of NF-κB subunits

The effects of SNP and GSNO treatment on the presence of IκB-α in the cytosolic fraction or p50 and p65 subunits in nuclear fraction were examined by immunoblotting analysis. Stimulation of cells with LPS (1 μg/ml) caused a rapid disappearance of IκB-α from cytosolic fraction at 30 min followed by reappearance after 1 h. Treatment of cells with SNP or GSNO caused a time-dependent induction of IκB-α (Fig. 6). Unstimulated cells expressed a basal level of p50 and p65 in nuclear fraction. Stimulation of cells with LPS (1 μg/ml) increased p50 and p65 level in nuclear fraction. Treatment of cells with SNP (1 mM) and GSNO (0.1 mM) prevented p50 and p65 nuclear translocation (60.6±5.1% and 81.2±3.1%; 72.3±3.1% and 79.6±6.3%, respectively, *n*=3; Fig. 7A,B, respectively).

Effect of SNP and GSNO on LPS-induced NF-IL6 activation

When gA probe was incubated with nuclear extracts of J774 macrophages, complex band intensity increased following stimulation of cells with LPS (1 μg/ml) for 2 h. Treatment of cells with SNP (0.25, 0.5 and 1 mM) or GSNO (0.1 mM) caused a significant inhibition of DNA-binding activity (by 24.7±5.6%, 68.3±8.4%, 78.2±6.1% and 76.1±11.3%, respectively, *n*=6), while the treatment with GSH (0.1 mM) had no effect on DNA-binding activity (Fig. 8). The DNA-protein complexes activated with LPS (1 μg/ml) in the absence or presence of SNP (1 mM) and revealed with gA probe disappeared by using gC radiolabeled probe with a mutation in NF-IL6 consensus sequence whereas they were not affected by using radiolabeled probe gB with a mutation in AP2 motif (Fig. 9). Furthermore, the DNA protein complexes observed with gA radiolabeled probe were completely displaced by addition of a 50-fold molar excess of unlabeled gA and gB, but not gC probe, in the competition reaction. The composition of the NF-IL6 complex activated by LPS was determined by using specific antibodies against anti-C/EBPβ, anti-C/EBPδ, anti-p50, anti-p65 and anti-AP2. Addition of anti-C/EBPβ or anti-p50 but not anti-C/EBPδ, anti-AP2 or anti-p65 to the binding reaction resulted in a marked reduction of NF-IL6 band intensity, indicating that NF-IL6 complex contained predominantly C/EBPβ (Fig. 10).

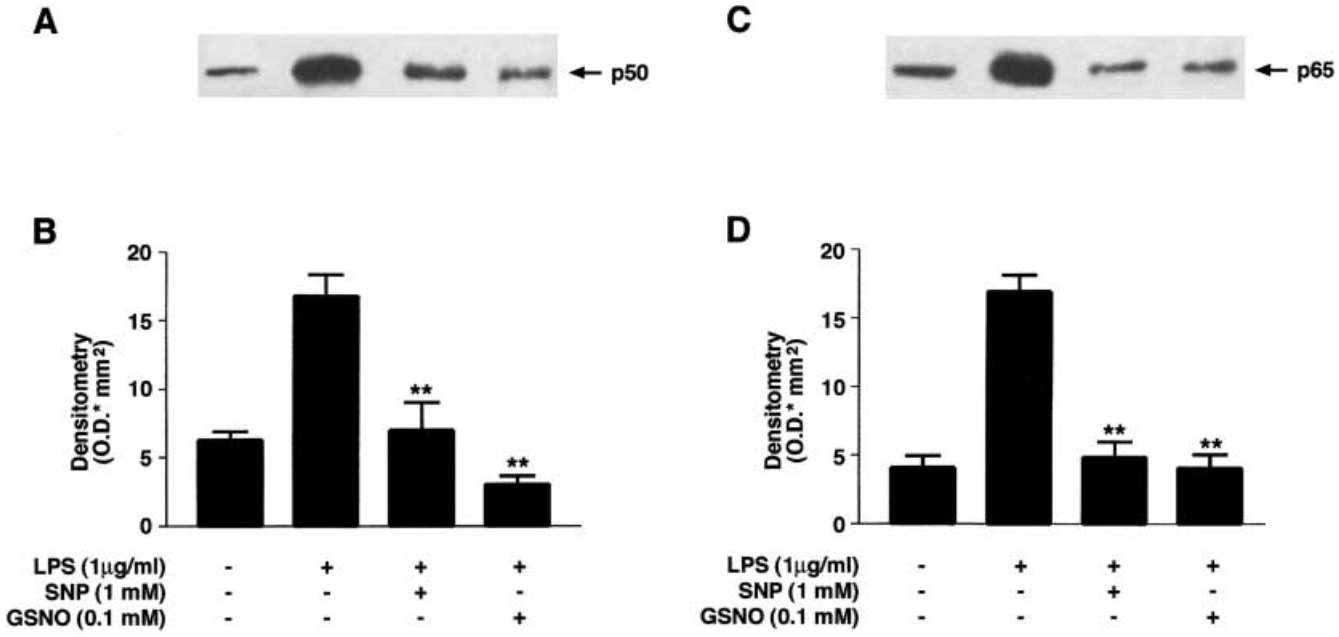

Fig. 7 Representative Western blot of p50 (**A**) and p65 (**C**) as well as the densitometric analysis (**B** and **D**, respectively) show the effect of SNP (1 mM) and GSNO (0.1 mM) on LPS-induced p50 and p65 nuclear translocation. Data in **A** are from a single experiment and representative of three separate experiments, while data in **B** are expressed as means ± SEM of three separate experiments. ***P*<0.01 vs. control (LPS alone)

Discussion

A role for NO on the COX pathway has been extensively studied in recent years by several research groups. However, the molecular mechanisms by which NO modulates COX-2 expression are not yet elucidated (Clancy et al. 2000; Di Rosa et al. 1996; Salvemini 1997). The results of our study demonstrate that SNP and GSNO, two different NO donors, down-regulate LPS-induced COX-2 protein expression and PGE_2 production. The effect is likely to be mediated by the release of NO, and not another metabolite, since the two compounds are structurally unrelated. In addition, the parent compound of GSNO, GSH, which is unable to release NO, had no effect on COX-2 protein expression and PGE_2 production. Inhibition by GSNO was more effective than that exhibited by SNP. In fact, the LPS-induced COX-2 protein expression and PGE_2 production were abolished by 0.1 mM GSNO compared to 1 mM SNP. The inhibition of PGE_2 production by NO was not dependent on direct inhibition of COX-2 activity since both compounds, when added to the cells 12 h after LPS challenge, did not affect the enzyme catalytic activity, suggesting the possibility that they may act at transcriptional level. A recent study has defined the responsive elements on the murine COX-2 gene promoter and has identified NF-κB and NF-IL6 as the primary transcription factors regulating COX-2 expression after TNF-α stimulation (Yamamoto et al. 1995). Several other reports have described the key role of NF-κB and NF-IL6 activation in

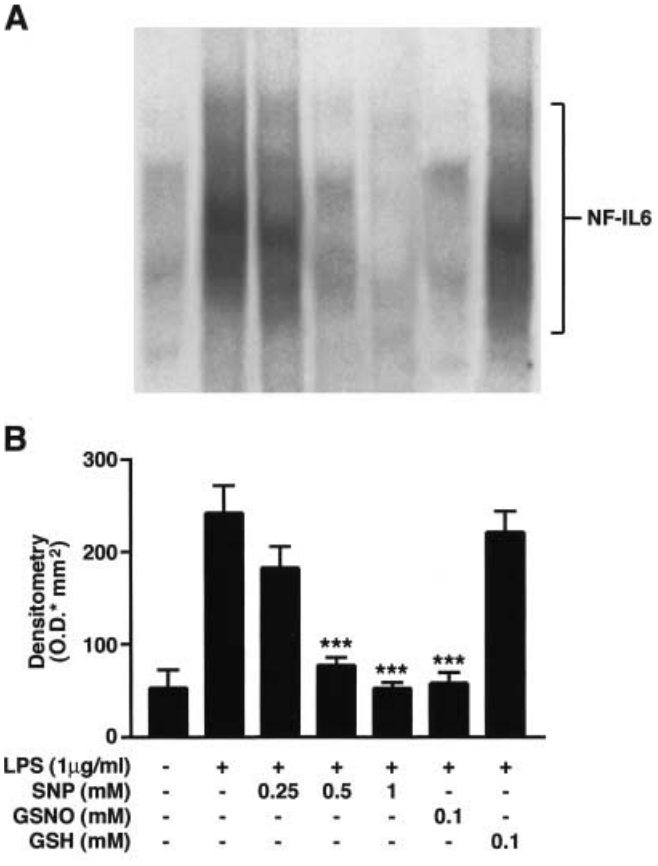

Fig. 8 Representative EMSA (**A**) as well as densitometric analysis (**B**) show the effect of SNP (0.25, 0.5, 1 mM), GSNO (0.1 mM) or GSH (0.1 mM) on LPS-induced NF-IL6/DNA binding activity. Data in (**A**) are from a single experiment and representative of three separate experiments, while data in (**B**) are expressed as means ± SEM of three separate experiments. ****P*<0.0001 vs. control (LPS alone)

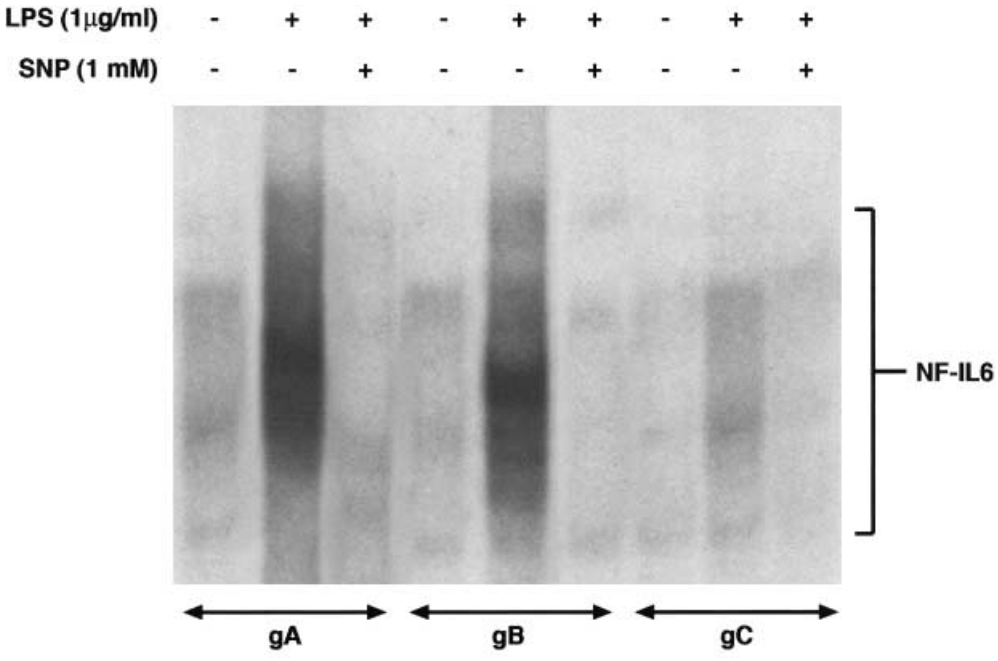

Fig. 9 J774 macrophages were incubated with medium alone or LPS (1 μg/ml) in the presence or absence of SNP (1 mM) for 2 h. The nuclear extracts, prepared as described in Materials and methods, were incubated with ^{32}P-labeled gA probe (wild type), gB probe (mutation for AP2) or gC (mutation for NF-IL6). Data illustrated are from a single experiment and representative of three separate experiments

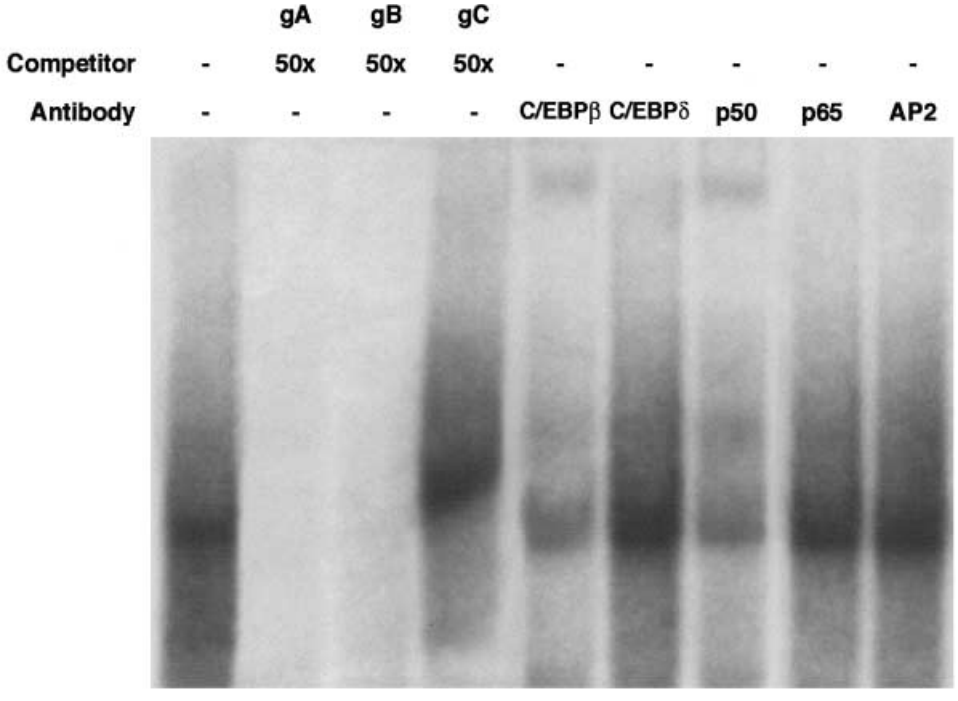

Fig. 10 Nuclear extracts from LPS-treated J774 macrophages were prepared as described in Materials and methods and incubated with ^{32}P-labeled gA probe. In competition reaction nuclear extracts were incubated with radiolabeled gA probe in absence or presence of the same unlabeled probe (*gA*, 50×) or probe containing mutation for AP2 (*gB*, 50×) or NF-IL6 (*gC*, 50×) binding site. In supershift experiments nuclear extracts were incubated with antibodies against C/EBPβ (*C/EBPβ*), C/EBPδ (*C/EBPδ*), p50 (*p50*), p65 (*p65*) and AP2 (*AP2*) 30 min before incubation with radiolabeled gA probe. Data illustrated are from a single experiment and representative of three separate experiments

the regulation of COX-2 expression, although their relative importance in the COX-2 gene regulation varies in different cell types (Sirois and Richards 1993; Sirois et al. 1993; Yamamoto et al. 1995). Moreover, we have previously shown that treatment of J774 cells with two structurally unrelated NF-κB inhibitors suppressed LPS-induced COX-2 protein expression and prostanoid generation (D'Acquisto et al. 1997). Our data clearly show that both SNP and GSNO inhibit NF-κB and NF-IL6/DNA binding activity. Previous studies have shown that exogenous NO donors inhibit NF-κB/DNA binding (delaTorre et al. 1997; Matthews et al. 1996; Park et al. 1997; Umansky et al. 1998; Yamamoto et al. 1995). Exogenous NO has been shown to stabilise NF-κB by inhibiting the dissociation of the IκB inhibitor and simultaneously increasing IκB mRNA levels in human vascular endothelial cells (Peng et al. 1995; Spiecker et al. 1997). Other studies have shown that ex vivo biochemical modification of NF-κB p50 inhibits DNA binding as determined by the gel shift assay (delaTorre et al. 1997; Matthews et al. 1996).

Particularly, *S*-nitrosylation of the redox-sensitive NF-κB p50 C62 residue was associated with the inhibition of p50 binding to its consensus DNA target sequence (Matthews et al. 1996). Moreover, it has been demonstrated that inhibition of endotoxin-mediated NO synthesis alters NF-κB p50 DNA binding and that NO synthesis results in *S*-nitrosylation of p50 with a functional decrease in DNA binding in murine macrophages (delaTorre et al. 1999). Our results, in agreement with those of Peng et al. (1995) and Spiecker et al. (1997), indicate that SNP and GSNO prevent LPS-induced IκB-α degradation and increase IκB-α protein levels. Furthermore, the nuclear translocation of p50 and p65 subunits in LPS-stimulated J774 macrophages was prevented by SNP and GSNO at the same concentrations that blocked proteolytic degradation of IκB-α, reinforcing the hypothesis that these compounds inhibit NF-κB activation through induction and stabilisation of IκB-α. In addition, we demonstrated that SNP and GSNO also inhibit LPS-induced NF-IL6 activation, suggesting, for the first time, a modulatory role of NO on this transcription factor. It has previously been shown that point mutation of NF-κB and NF-IL6 binding site in interleukin 8 (IL-8) promoter decreased the NO-stimulated IL-8 expression in a melanoma cell line (Andrew et al. 1995). However, in this report the effect of NO on NF-IL6 was shown by using a luciferase reporter gene construct. Our finding, achieved by gel shift assay, is of particular relevance since it expands not only the number of potential bioregulatory targets of NO but also suggests a possible modulatory role of NO on several genes controlled at transcriptional level by NF-IL6. In conclusion, our results suggest that the inhibition of COX-2 induction by NO occurs through the prevention of NF-κB and NF-IL6 activation. However, the involvement of other regulatory elements, such as CRE, present in mouse (Fletcher et al. 1992), rat (Sirois et al. 1993) and human (Kosaka et al. 1994) COX-2 gene promoter, can not be ruled out. Nevertheless, delineation of the mechanism(s) of negative transcriptional regulation of COX-2 promoter by NO remains to be shown. Further experiments performed with gene reporter assay are needed to establish which transcription factors are functionally inactivated by NO. In any case, the down-regulation of COX-2 gene expression by exogenous or endogenous NO may represent a negative regulatory mechanism in modulating the sustained PGs production that occurs in many pathological events.

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