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Nitric oxide affects IL-6 expression in human peripheral blood mononuclear cells involving cGMP-dependent modulation of NF-κB activity

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ABSTRACT

Interleukin 6 (IL-6) and nitric oxide (NO) are important mediators of the inflammatory response. We report that in human peripheral blood mononuclear cells (PBMCs), NO exerts a biphasic effect on the expression of IL-6. Using sodium nitroprusside (SNP) and *S*-nitrosoglutathione (GSNO) as NO-donating compounds, we observed that both mRNA and protein levels of IL-6 increased at lower (⩽10 μM) and decreased at higher (>100 μM) concentrations of NO donors. Changes in the expression of IL-6 correlated with changes in the activity of NF-κB, which increased at lower and decreased at higher concentrations of both NO donors as shown by the electrophoretic mobility shift assay (EMSA). The effects of NO on NF-κB activity were cGMP-dependent because they were reversed in the presence of ODQ, the inhibitor of soluble guanylyl cyclase (sGC), and KT5823, the inhibitor of cGMP-dependent protein kinase (PKG). Moreover, the membrane permeable analog of cGMP (8-Br-cGMP) mimicked the effect of the NO donors. These observations show that NO, depending on its concentration, may act in human PBMCs as a stimulator of IL-6 expression involving the sGC/cGMP/PKG pathway.

1. Introduction

Interleukin 6 (IL-6) is pleiotropic cytokine playing an important role in acute and chronic inflammation. It is synthesized by various cell types including T lymphocytes, B lymphocytes, and monocytes [1]. It is also well documented that the development of inflammation is regulated by nitric oxide (NO) and the compounds that release NO (NO donors) are potent regulatory agents in inflammatory processes [2–5]. NO donors have been reported to up- and down-regulate the expression of various inflammatory mediators [6–10]. The action of NO is time-dependent [11,12] and biphasic [10,13–15]. Low concentrations of NO have a stimulatory effect on synthesis of proteins involved in differentiation, apoptosis, or inflammation [7,16], while high concentrations exert opposite effects [17]. The intracellular signaling of NO can be mediated by the second messenger guanosine 3′,5′-cyclic monophosphate (cyclic GMP, cGMP) [5,18–21] and/or be cGMP independent [6,11,17,22]. Similarly to nitric oxide, cGMP has also been shown to play both pro- and anti-inflammatory roles [3–5,10,23,24].

The cGMP-dependent pathway is initiated when NO binds to the heme moiety of cytosolic (soluble) guanylyl cyclase (sGC) and stimulates its enzymatic activity. The generated cGMP signal is in turn transmitted to effector proteins, of which the cGMP-dependent protein kinase (PKG) is the main target in most cells [25]. Soluble guanylyl cyclases and PKG are also detected in lymphoid and myeloid cells of the immune system [18,19,23,26–29].

Nuclear factor κB (NF-κB) plays a key role in the development of inflammation. This transcription factor controls the expression of multiple genes including those encoding proinflammatory cytokines TNF-α, IL-1β, and IL-6 [30–32]. In the cytoplasm, the NF-κB heterodimer (usually consisting of the subunits p50 and p65) is maintained in its inactive form by its association with inhibitory proteins of the IκB family. In response to an activating signal, IκB is phosphorylated by specific kinases and then degraded by the proteasome. The released NF-κB heterodimer translocates to the nucleus, binds to the consensus sites in the target genes and regulates their transcription [30]. The activity of NF-κB can be regulated by various factors including nitric oxide [11,33,34] and thus observed effects of NO on synthesis of proinflammatory cytokines may be mediated by its action on NF-κB. However, data

Abbreviations: EMSA, electrophoretic mobility shift assay; GC, guanylyl cyclase; GSNO, *S*-nitrosoglutathione; IBMX, 3-isobutyl-1-methylxanthine; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase; PBMC, peripheral blood mononuclear cell; sGC, soluble GC; SNP, sodium nitroprusside.

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concerning the role of the NO/cGMP pathway in this process and development of inflammation are often conflicting. Therefore, we designed and conducted a study to establish how NO affects the expression of IL-6 in human peripheral blood mononuclear cells (PBMCs), which are an important source of proinflammatory cytokines [35]. We also define a role for cGMP in mediating the observed effects of NO in these cells.

2. Materials and methods

2.1. Cell culture and reagents

Buffy coats were kindly provided by the Station of Blood Donation, 4th Military Hospital, Wrocław, Poland. HEK293 cell line was purchased from ECACC. The cells were grown in DMEM with GlutaMAX (Gibco-BRL) supplemented with 10% fetal bovine serum, penicillin–streptomycin and noromycin and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Fetal bovine serum (FBS) was from GibcoBRL (Karlsruhe, Germany). SDS–PAGE reagents were obtained from Fluka (Buchs, Germany). PKG inhibitor KT5823 was obtained from Calbiochem–Novabiochem (Darmstadt, Germany). *S*-nitrosoglutathione (GSNO), sodium nitroprusside (SNP), 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), 3-isobutyl-1-methylxanthine (IBMX), leupeptin, pepstatin A, PMSF, and other chemicals were from Sigma–Aldrich Co. (Poznan, Poland).

2.2. Expression vectors/recombinant plasmids

The IL-6-luciferase reporter constructs (pGL3-IL6-WT-luc and pGL3-IL6-NFκB-m-luc) were a generous gift from William Farrar (National Institute of Health, Frederick, Maryland). The pHACE PKG-Iα and pHACE PKG-Iβ plasmids were a generous gift from Bo Cen (Columbia University, New York).

2.3. Preparation of human PBMCs

PBMCs were obtained by density centrifugation of buffy coats of healthy voluntary blood donors over Lymphoprep™ (Axis-Shield, Oslo, Norway) as described previously [36].

2.4. Determination of IL-6

Isolated PBMCs were cultured at a concentration of 1×10^6 cells/ml for 4 h at 37 °C in 5% CO_2 in 48-well culture dishes in either the absence (control) or presence of NO donors. Culture supernatants were collected, cleared by centrifugation, and frozen at −70 °C until analysis. IL-6 was determined by microplate ELISA using rat monoclonal antibodies against human IL-6 (Pharmingen, San Diego, CA, USA) and recombinant human IL-6 as a standard (Pharmingen, San Diego, CA, USA) according to the procedure recommended by the manufacturer.

2.5. Induction and measurement of intracellular cGMP

PBMCs were transferred to 48-well cell culture plates (Costar, Corning, NY, USA) and allowed to rest for 30 min at 37 °C before the experiments were performed. Each well contained 5.0 (10^5 cells in a final volume of 0.5 ml. To prevent cGMP hydrolysis, 0.5 mM IBMX was added, and after incubation at 37 °C for 10 min the cells were supplemented with NO donors (SNP, GSNO). After 30 min of incubation, the reaction was terminated, the cells were disintegrated, and the intracellular content of cGMP was determined by the ELISA-based method using rabbit antibodies specific to cGMP [25,26]. All samples were prepared in quadruplicate.

2.6. Nuclear extracts

Nuclear extracts were prepared from PBMCs according to the method described previously [37]. Briefly, PBMCs were washed with ice-cold PBS and disintegrated in ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM sodium ortovanadate, 0.1% NP-40) by gentle pipetting on ice for 15 min. After centrifugation at 12,000*g* for 1 min at 4 °C, the supernatants were removed and the nuclear pellets were resuspended in a 3× packed nuclear volume of ice-cold high-salt buffer B (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM PMSF). The samples were gently vortexed on ice for 30 min, centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatants (nuclear extracts) were saved.

2.7. Electrophoretic mobility shift assay (EMSA)

The double-stranded oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Promega, Madison, WI, USA) representing the NF-κB consensus-binding site was end-labeled with T4 polynucleotide kinase (Promega, Madison, WI, USA) in the presence of [γ-^{32}P] ATP (NEN Life Science, Otwock, Poland). The nuclear extracts (10 μg of protein) were preincubated for 10 min in reaction buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 20% glycerol, and 0.25 mg/ml poly (dI–dC)·poly (dI–dC)) followed by incubation with a ^{32}P-labeled NF-κB probe for 30 min at room temperature. The EMSA was performed in a 4% acrylamide gel in a low-ionic-strength TBE buffer. The dried gels were autoradiographed with storage phosphor screens at −20 °C and the NF-κB complexes were analyzed using a Typhoon 8600 Multi-imaging System software (Molecular Dynamics/Amersham Pharmacia Biotech, Sunnyvale, CA, USA). The resulting images were aquatinted using ImageQuant software (Molecular Dynamics/Amersham Pharmacia Biotech, Sunnyvale, CA, USA). The specificity of the NF-κB-binding protein was controlled by competition with an excess of unlabeled oligonucleotide and by supershift experiments. For the gel supershift assay, nuclear protein was preincubated for 30 min with 1 μl of undiluted polyclonal antibodies to p50 and/or p65 (SCB, Santa Cruz, CA, USA).

2.8. RNA isolation, reverse transcription, and polymerase chain reaction (RT-PCR)

Total RNA was isolated from all types of cells using the TRIzol® Reagent according to the manufacturer's instructions (Invitrogen). Thereafter, 5 μg of total RNA was incubated with deoxyribonuclease I (MBI Fermentas, Vilnius, Lithuania) and was reverse-transcribed (RT) into cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) with oligo(dT)$_{18}$ primers according to the supplier's instructions. The cDNA was amplified using a PCR Core Kit (Qiagen, Hilden, Germany) and 0.4 μM of primers specific for human *IL-6, sGCα, sGCβ* or hypoxanthine phosphoribosyl transferase (*HPRT*). The sequences and annealing temperatures (T_A) of the primers were: *IL-6*: For: ATGTAGCCGCCCCACACAGA and Rev: ATTTGCCGAAGAGCCCCTCAG (T_A = 60 °C), *sGCα*: For CAGCCATTGCCAAGAAGCAGGAAA and Rev AGGGAGGCATTAACGACACTTCCA (T_A = 60 °C), *sGCβ*: For TGGAAAT TGCTGGCCAGGTTCAAG and Rev AAAGACAGTATCGAGGCATCCGCT (T_A = 60 °C) and *HPRT*: For AGTGATGATGAACCAGGTTA and Rev ATTATAGTCAAGGGCATATC (T_A = 58 °C). The PCR products were separated in 1.5% agarose and visualized under UV light using ethidium bromide. The resulting images were captured and analyzed using Fragment Analysis software (Molecular Dynamics/Amersham Pharmacia Biotech, Sunnyvale, CA, USA). No products were

observed in the control (RT-) samples, in which the reverse transcriptase was omitted.

2.9. Real-time PCR

Total cDNA was used as starting material for real-time RT-PCR quantitation with DyNAmo®HS SYBR Green Kit (Finnzymes) on a real-time PCR system (DNA Engine OPTICON® system; MJ Research). For the amplification of the specific genes the following primers were used; *IL-6*, For AGCCACTCACCTCTTCAGAACGAA, and Rev CAGTGCCTCTTTGCTGCTTTCACA. For mRNA quantification, the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (*HPRT*) was used as a reference point using the following primers, *HPRT* forward, AGCTTGCTGGTGAAAAGGAC, and reverse, TTATAGTCAAGGGCATATCC. Real-time PCR data were analyzed using $2^{-\Delta\Delta CT}$ method as described [38].

2.10. Reporter assays

HEK293 cells (2×10^4 cells/well; 96 well plate) were co-transfected with a luciferase reporter gene plasmid regulated by a wild type IL-6 promoter, or the same promoter containing a mutation in its κb-binding site (IL-6-ΔκB) (80 ng/well), and the expression vectors PKG-Iα and β using Lipofectamine 2000 as described by the manufacturer (Invitrogen). In all cases, 40 ng/well of phRL-TK reporter gene was co-transfected to normalize data for transfection efficiency. After 24 h, cells were stimulated with 8-Br-cGMP or SNP for 8 h. Thereafter, cell lysates were prepared and reporter gene activity was measured using the Dual Luciferase Assay system (Promega) as described [38]. Data was expressed as the mean fold induction ±SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

2.11. Data analyses

Statistical analysis was carried out using the unpaired Student's t test using SigmaPlot 2001 programme. *p*-Values of less than or equal to 0.05 were considered to indicate a statistically significant difference where $*$ or # indicates $p \leqslant 0.05$ and $**$ or ## indicates $p \leqslant 0.01$.

3. Results

3.1. Expression of IL-6 is affected by NO donors in PBMCs

We initially examined the expression of IL-6 in response to various amounts of the nitric oxide as released from NO donating molecules. The cells were independently treated with two different NO donors, sodium nitroprusside (SNP) and *S*-nitrosoglutathione (GSNO) and cell supernatants subsequently assayed for protein levels of IL-6 analyzed. Both donors, used at a low dose (10 μM), caused an increase in IL-6 synthesis, while an inhibitory effect was observed at higher (above 100 μM) concentrations (Fig. 1A). In order to assess if the regulatory influence of NO was likely mediated at the level of transcription the effects of both NO donors on the expression levels of IL-6 mRNA were subsequently assessed. Semi-quantitative RT-PCR was used to measure IL-6 mRNA levels and the findings mirrored those observed when measuring IL-6 protein levels in that both donors stimulated IL-6 mRNA expression at low concentration with higher concentrations showing inhibitory effects (Fig. 1B). Quantitative-real time PCR analysis also confirmed these findings using SNP as a NO donor. Given that the main intracellular receptor of nitric oxide is soluble guanylyl cyclase, that synthesizes cGMP, it was important to establish whether cGMP mediates the effects of the NO donors on IL-6 expression.

Firstly the ability of the NO donors to influence cGMP synthesis in PBMCs was examined. The experiments were performed in the presence of a non-selective inhibitor of phosphodiesterases (IBMX) to prevent hydrolysis of the synthesized nucleotide. The level of cGMP in unstimulated (control) PBMCs was low (26 ± 6.8 fmol/10^6 cells/30 min) and considerably increased after treating the cells with either donor of NO (Table 1). SNP and GSNO increased the level of intracellular cGMP over 30-fold. This effect was reduced in the presence of the guanylyl cyclase inhibitor ODQ indicating that both donors released NO which in turn activated the enzyme. Given that NO could elevate cGMP the role of this signaling molecule in regulating IL-6 expression was next examined. Indeed 8-Br-cGMP, a known membrane-permeable analog of cGMP, was shown to stimulate the expression of IL-6 mRNA (Fig. 1C) indicating that cGMP is at least capable of replicating the stimulatory effects of the NO donors. Furthermore cGMP appears to act a mediator of the stimulatory effects of the NO donors since the guanylyl cyclase inhibitor ODQ blocked the stimulatory effects of SNP on IL-6 expression (Fig. 1C). In addition the stimulatory effects of SNP are also blocked by the PKG inhibitor KT5823 and this supports a role for cGMP-activated PKG in mediating the stimulatory effects. Interestingly the inhibitors of guanylyl cyclase and PKG failed to affect the inhibitory effects of the high concentrations of SNP suggesting different mechanisms underlie the stimulatory and inhibitory effects of NO donors on IL-6 expression. Whilst the exact nature of the inhibitory effects of NO on IL-6 is unknown, the high concentrations of NO donors do not induce any cytotoxicity and hence we can exclude non-specific toxicity as a mechanistic basis to the effects.

3.2. The effects of NO donors on IL-6 expression correlate with activity of NF-κB

Since the expression of IL-6 is regulated by the transcription factor NF-κB we next analyzed the influence of both donors on its activity as measured by EMSA. The activity of NF-κB in freshly isolated untreated PBMCs (Fig. 2A) showed some constitutive level, which further increased after treating the cells with phorbol 12-myristate 13-acetate (PMA). The effect of PMA was abolished in the presence of gliotoxin, a known inhibitor of NF-κB activation [32]. Similarly to PMA, SNP at a concentration of 10 μM also caused an increase in NF-κB activity. Specificity of NF-κB complexes detected by EMSA was confirmed by inhibition of the NFκB-DNA complexes with the unlabeled (cold) oligonucleotide containing the NF-κB binding site (specific competitor) whereas the unrelated oligonucleotide (non-specific competitor) was ineffective. The complex that was translocated to the nucleus consisted of p50 and p65 subunits as determined by supershift analysis using antibodies specific against each subunit (Fig. 2A). The effect of SNP on NF-κB activity was dose dependent and showed a biphasic character (Fig. 2B). The activity of NF-κB increased at lower and decreased at higher (above 100 μM) concentrations of SNP. Similar effects were observed when GSNO was applied as the NO donor (Fig 2C).

3.3. The activation of NF-κB by NO donors in PBMCs depends on the cGMP pathway

The main intracellular receptor of nitric oxide is soluble guanylyl cyclase which synthesizes cGMP. Therefore, it was important to establish whether cGMP mediates the changes in NF-κB activity caused by the NO donors. We thus explored the regulatory effects of the inhibitors of the two key enzymes of the cGMP signaling pathway, namely sGC and PKG. Inhibitors of both enzymes

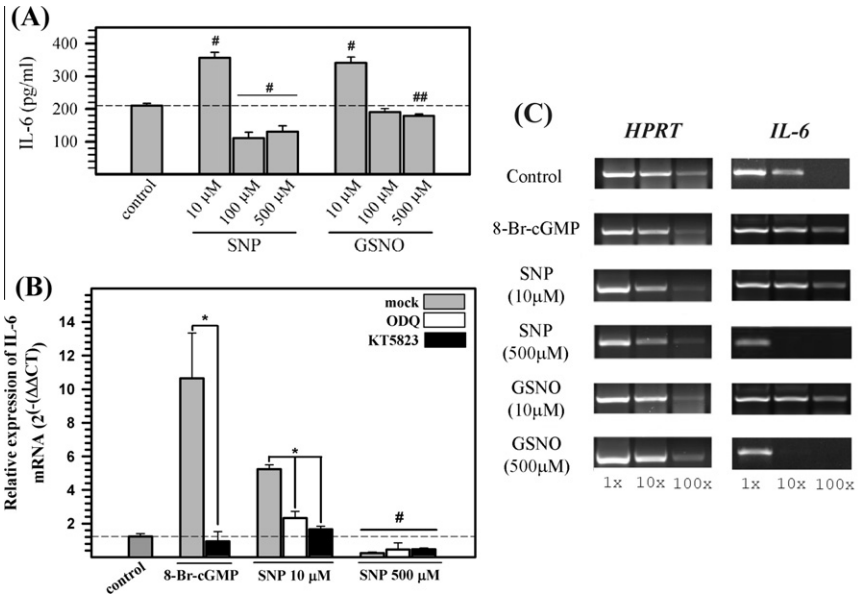

Fig. 1. NO donors regulate the expression of IL-6 expression in a dose-dependent manner. (A) PBMCs were treated with the indicated concentrations of SNP or GSNO and 10 μM 8-Br-cGMP and the expression of IL-6 was subsequently assayed by the ELISA-based method. Data are presented as mean ± SE of three independent experiments. (B) PBMC were treated with 8-Br-cGMP, SNP or GSNO for 4 h. Thereafter, total RNA was isolated, converted to first-strand cDNA. Various dilutions (1×, 10× or 100×) of cDNA were used as template for semi-quantitative RT-PCR. HPRT was used as a housekeeping gene and the data is representative of three independent experiments. (C) Freshly isolated PBMC were pretreated with ODQ (1.0 μM) or KT5823 (0.2 μM) for 20 min. Thereafter cells were treated with the indicated concentrations of 8-Br-cGMP, SNP or GSNO for 12 h and total RNA was isolated, converted to first-strand cDNA and used as a template for quantitative IL-6 real-time RT-PCR as described under "Section 2". Values were normalized relative to HPRT and data are presented relative to unstimulated cells. $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ statistical significances related to control as described in Section 2.

Table 1
Synthesis of cGMP by soluble GC in response to NO donors.

Treatment	cGMP (fmol/10^6 cells)
None	26.0 ± 6.8
SNP	833.5 ± 68.5
SNP + ODQ	133.4 ± 30.0
GSNO	1743.6 ± 109.1
GSNO + ODQ	133.0 ± 40.5

PBMCs were treated with 10 μM SNP or GSNO in the presence of 0.5 mM IBMX in either the absence or presence of 1 μM ODQ and then the intracellular content of cGMP was determined according to the procedure described in the Section 2. Results are expressed as the mean ± SD of three independent experiments.

prevents the activation of NF-κB by NO in PBMCs. As shown in Fig. 3A, cells treated with NO donors in the presence of either ODQ or KT5823 (inhibitor of PKG) showed decreased activation of NF-κB in comparison with cells treated with NO donors alone. In addition the inhibitory effects of the high concentrations of SNP on NFκB are also blocked by the inhibitors indicating a key role for cGMP and its effector enzyme in mediating the regulatory effects of NO donors on NFκB. Indeed the ability of cGMP to regulate NFκB was directly confirmed by showing that 8-Br-cGMP, a membrane-permeable analog of cGMP could activate NF-κB in PBMCs and this was inhibited by PKG inhibitor KT5823 (Fig. 3B).

3.4. 8-Br-cGMP and NO donors target the NFκB binding site in the IL-6 promoter

In order to relate the regulatory effects of the NO/cGMP/PKG pathway on NFκB to IL-6 expression we explored the effects of the various molecules on activation of the IL-6 promoter and the importance of the NFκB-bidning site in this promoter to mediating these effects. To this end we used the IL-6 full length promoter and IL-6 NF-κB deletion mutant luciferase reporter gene constructs (Fig. 4A). The experiments with the IL-6 promoter constructs were conducted in HEK293 cells and thus it was initially important to confirm that these cells express sGC. RT-PCR was used to confirm expression of both subunits (α and β) of sGC (Fig. 4B). Next, HEK293 cells were co-transfected with the luciferase reporter constructs and PKG-Iα or PKG-Iβ. We found that transfection of HEK293 cells with the PKG-I increased the cGMP-dependent activation of the IL-6 reporter genes but not IL-6-ΔκB (Fig. 4C and D). Interestingly, we found that transfection of HEK293 cells with PKG-Iα and treatment with the 8-Br-cGMP or 10 μM SNP lead to stronger activation of the full-length IL-6 reporter gene, whilst the PKG-Iβ was less effective (Fig. 4C and D). Taken together, these data clearly demonstrate that a NO donor like SNP can elevate cGMP to promote IL-6 gene induction in a PKG and NF-κB dependent manner.

Interestingly the high concentration of SNP (500 μM) showed inhibitory effect on both kinases and this correlates closely with the inhibitory effects of high concentration of SNP on NFκB indicating that the latter is a key target for mediating the complex effects of NO on IL-6 expression.

4. Discussion

Among the multiple physiological processes regulated by nitric oxide are also those related to the development of inflammation [2,39]. Published data show that NO may play a dual role in this process and exerts either a stimulatory or an inhibitory effect on the synthesis of proinflammatory cytokines [11,22,40].

Taking into account the possible pro- and anti-inflammatory effects of NO, we examined whether and how NO donors affect the expression of proinflammatory IL-6 and the activity of the transcription factor NF-κB in human peripheral blood mononuclear cells (PBMCs). The action of NO donors was dose-dependent and showed biphasic character. At low

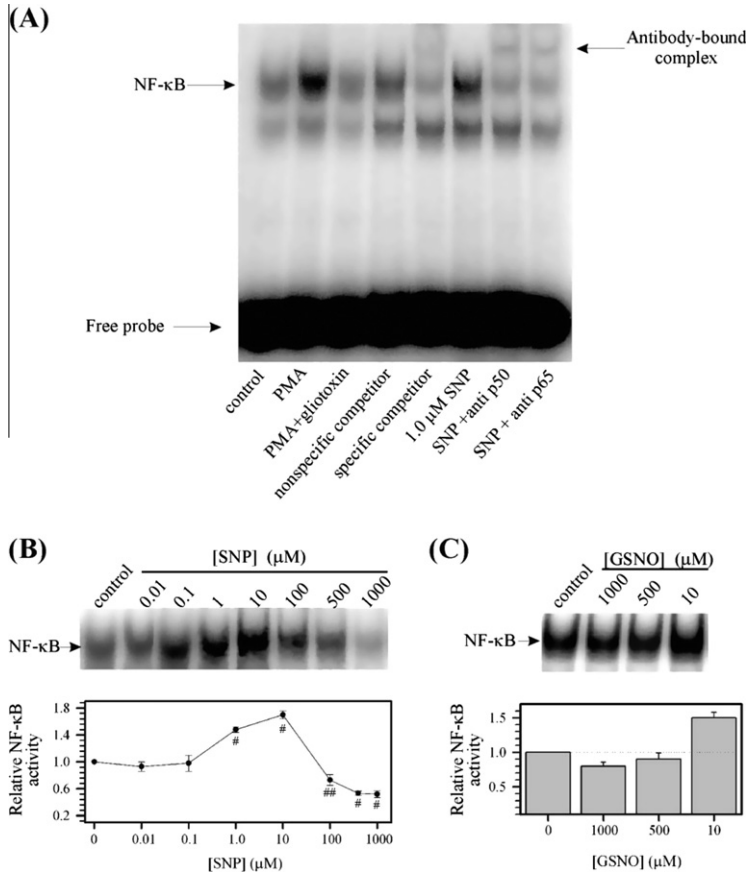

Fig. 2. NO donors exert a biphasic effect on NF-κB activity in PBMCs. Cells were treated with the indicated concentrations of SNP or GSNO for 60 min and then NF-κB binding to a DNA oligonucleotide was measured by EMSA. Relative NF-κB activity was determined by densitometry of the corresponding bands and is expressed as the fold of the NF-κB activity in the control sample. Values represent means ± SE from at least three independent experiments. (A) Specificity of the assay was verified on unstimulated PBMC using unlabeled oligonucleotides corresponding to NF-κB or AP-1 binding sites and the super electrophoretic mobility shift assay (supershift) with antibodies against the p50 and p65 subunits of NF-κB. PMA (10 ng/ml) was used as a stimulator and gliotoxin (1 μg/ml), as an inhibitor of NF-κB activation. (B) Dose-dependent effect of SNP on NF-κB binding activity; $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ statistical significances related to nontreated as described in Section 2. (C) Effect of low and high concentrations of GSNO on NF-κB activity.

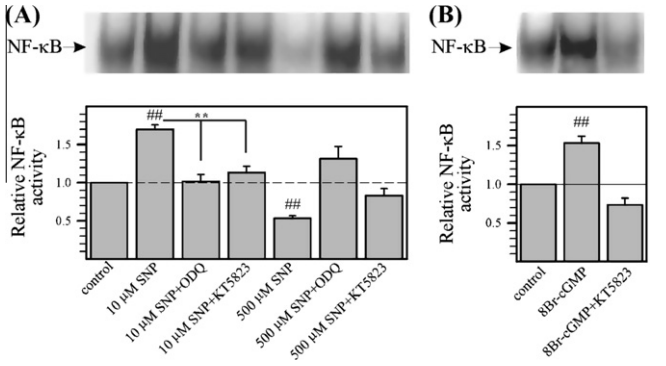

Fig. 3. NO modulates NF-κB activity in PBMCs by the sGC/cGMP/PKG pathway. (A) Cells were treated with low and high concentrations of SNP in either the presence or absence of sGC inhibitor (1.0 μM ODQ) and PKG inhibitor (0.2 μM KT5823). (B) Cells were treated with 10 μM 8-Br-cGMP in the absence and presence of PKG inhibitor. Nuclear extracts were analyzed by EMSA for binding to the probe containing the NF-κB consensus sequence; ** or $^{\#\#}p < 0.01$ statistical significances as described in Section 2, all other details are as described in the legend to Fig. 1.

concentrations, they elevated but at higher concentrations inhibited both IL-6 expression and NF-κB activity. The stimulatory and inhibitory effects of NO donors on NF-κB were mediated by the cGMP-dependent pathway containing soluble GC and cGMP-dependent protein kinase (PKG) as key elements, because the effects were no longer apparent in the presence of specific inhibitors of either enzyme. Interestingly whilst our studies indicate that GC and PKG mediate the stimulatory effects of NO on IL-6 expression, the inhibitory effects of NO on IL-6 expression are likely manifested by a distinct mechanism since such negative regulatory effects are not blocked by inhibitors of sGC and PKG.

PKG has been detected in various immune cells [26–29], where it was shown to be responsible for the effects of cGMP [18,19,28]. We observed that inhibition of PKG abrogated the effects of SNP on the activity of NF-κB. Previous reports demonstrated that the sGC/cGMP/PKG signaling pathway participates in stimulating NF-κB activity in adult feline myocardium [20] and in human pulmonary epithelial cells [33]. Our experiments show that this pathway regulates NF-κB activity also in human PBMCs. Cyclic GMP, generated in response to a low concentration of NO donor, activates PKG that stimulates NF-κB using a yet unknown pathway. Earlier published data indicate that PKG may phosphorylate IκB and thus cause the degradation of this inhibitory subunit and the activation of NF-κB [20]. Another possibility was raised by He and Weber [41], who showed that PKG is able to directly phosphorylate p65 in the NF-κB heterodimer, increasing its transcriptional activity. The fact that NO donors exert their stimulatory effect at low doses is also in agreement with experiments that showed the expression of endothelial nitric oxide synthase in murine macrophages [42]. This type of NO synthase provides NO at a low level and induces proinflammatory response in macrophages through the activation

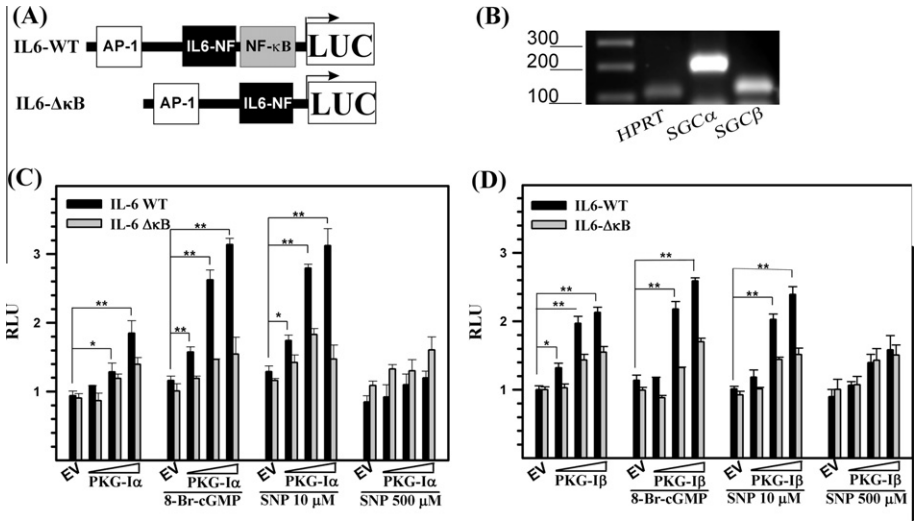

Fig. 4. The regulation of the IL-6 promoter activation by the cGMP/PKG pathway is dependent on the NF-κB. (A) Histograms of the IL-6 promoter luciferase constructs with the regulatory binding sites for IL6–NF-κB, IL6-IL6NF and IL6-AP1. The transcription start site is indicated with the arrows. The IL-6 promoter constructs illustrated on panel A were used for transfection and luciferase assay shown on panels C and D. (B) The mRNA of s*GCs* was detected using RT-PCR. *HPRT* is the reference gene. The first line contains DNA mass markers. Their molecular sizes in bp are shown on the left side of the gel. (C and D) HEK293 cells were co-transfected with vectors encoding a reporter gene for the full length IL-6 promoter (black) and IL-6 NF-κB deletion mutant (grey), and increasing amounts of the expression vectors encoding PKG-Iα or β (1, 10, 100 ng) as indicated. After 24 h cells were stimulated with 8-Br-cGMP (50 μM) or SNP for 8 h followed by harvesting of the cell lysates and assessment of luciferase gene reporter activity. The results presented are representative of at least three independent experiments; $^{*}p < 0.05$; $^{**}p < 0.01$ statistical significances as described in Section 2.

of NF-κB [42]. Another cellular effect of low doses of NO is its protective role against apoptosis [43]. Since the anti-apoptotic role of low concentrations of NO has been reported for several cell types, our observations suggest that the NO/cGMP/PKG pathway might at least in part be responsible for the anti-apoptotic effect of NF-κB activation described for human leukocytes [32]. Further studies are needed to explore this possibility in details.

Others described that NO inhibited the activity of NF-κB when high levels of NO donors were used [5,10]. Again, our results showed that at high concentrations of both SNP and GSNO a diminished expression of IL-6 correlated with decreased NF-κB activity. Inhibitory, effects of higher doses of NO-donating compounds on NF-κB activity, mediated at least in part by cGMP, was also recently reported for human mast cells [5]. However, cellular production of NO is controlled, in part, by NF-κB itself [44]. In particular, deletion of κB sites from the NOS2 promoter prevents the cytokine-induced increase in NOS2 transcription [45]. The primary molecular mechanism by which high NO inhibits NF-κB signaling is via *S*-nitrosylation of several different NF-κB proteins including IκB kinase β and p50 [46]. Particularly, p50 and p65 are *S*-nitrosylated under conditions of nitrosative stress and is associated with a decrease in NF-κB (p50–p65) DNA binding [46,47].

In summary, our findings indicate that the NO/sGC/cGMP/PKG pathway is involved in the regulation of IL-6 expression in human PBMCs through the regulation of NF-κB activity. Thus NO/cGMP-dependent signaling appears to be an important regulatory pathway in the inflammatory process. At its early stage, it may accelerate the process by stimulating the synthesis of proinflammatory cytokines, while at a later stage, when the cells are fully activated and NO is supplied in excess, it rather counteracts the undesired development of inflammation.

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