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Activation of Peroxisome Proliferator-Activated Receptor γ by Nitric Oxide in Monocytes/Macrophages Down-Regulates p47^{phox} and Attenuates the Respiratory Burst[1]

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NO appears as an important determinant in auto and paracrine macrophage function. We hypothesized that NO switches monocyte/macrophage function from a pro- to an anti-inflammatory phenotype by activating anti-inflammatory properties of the peroxisome proliferator-activated receptor (PPAR)γ. NO-releasing compounds (100 μM *S*-nitrosoglutathione or 50 μM spermine-NONOate) as well as inducible NO synthase induction provoked activation of PPARγ. This was proven by EMSAs, with the notion that supershift analysis pointed to the involvement of PPARγ. PCR analysis ruled out induction of PPARγ mRNA as a result of NO supplementation. Reporter assays, with a construct containing a triple PPAR response element in front of a thymidine kinase minimal promoter driving the luciferase gene, were positive in response to NO delivery. DNA binding capacity as well as the transactivating capability of PPARγ were attenuated by addition of the antioxidant *N*-acetyl-cysteine or in the presence of the NO scavenger 2-phenyl-4,4,5,6-tetramethyl-imidazoline-1-oxyl 3-oxide. Having established that NO but not lipophilic cyclic GMP analogs activated PPARγ, we verified potential anti-inflammatory consequences. The oxidative burst of macrophages, evoked by phorbol ester, was attenuated in association with NO-elicited PPARγ activation. A cause-effect relationship was demonstrated when PPAR response element decoy oligonucleotides, supplied in front of NO delivery, allowed to regain an oxidative response. PPARγ-mediated down-regulation of p47 phagocyte oxidase, a component of the NAD(P)H oxidase system, was identified as one molecular mechanism causing inhibition of superoxide radical formation. We conclude that NO participates in controlling the pro- vs anti-inflammatory phenotype of macrophages by modulating PPARγ. *The Journal of Immunology*, 2002, 169: 2619–2626.

Inflammation provokes the generation of multiple mediators, including NO. It has been shown that monocytes/macrophages are prominent producers of NO, with the implication that NO is a key component of the antimicrobial and tumoricidal immune response (1). NO not only serves as a cytotoxic molecule to produce cell demise along an apoptotic or necrotic pathway but also gained attention as a regulator of immune function (2, 3). Part of these activities is attributed to the ability to regulate protein expression. Although in many cases the primary targets for NO are uncertain, a number of signal transducers, i.e., transcription factors, are known to be positively or negatively regulated by NO. Examples comprise the redox-sensitive factors NF-κB or AP-1 (4–6). As a consequence, the expression of cytokines, apoptotic proteins, or immediate early genes such as cyclooxygenase-2 are modulated, thus stressing the function of NO as a messenger molecule (7, 8).

The anti-inflammatory properties of the nuclear hormone receptor family known as peroxisome proliferator-activated receptors (PPARs)[3] were recently established (9). Three subtypes, PPARα, PPARβ (also known as PPARδ), and PPARγ have been described so far, originally found in association with obesity, diabetes, and atherosclerosis (10). All of them act as ligand-dependent transcription factors which, upon heterodimerization with the 9-*cis* retinoic acid receptor (RXR), bind to the PPAR response element (PPRE) to modulate target gene expression (11). PPARγ has been shown to be activated by natural agonists such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) or synthetic antidiabetic thiazolidinediones, i.e., ciglitazone, with the outcome to reduce proinflammatory cytokine as well as reactive nitrogen species production in monocytes/macrophages (12, 13). In addition, activation may be achieved by oxidized low-density lipoprotein via the CD36 scavenger receptor (14, 15), which has been linked to the development of atherosclerosis. Experiments performed with cells derived from murine embryonic stem cells that were homozygous for a null mutation in the PPARγ gene questioned the anti-inflammatory properties of PPARγ (16) but verified its role in CD36 expression (17).

Considering the properties of NO as a messenger molecule during innate and adaptive immunity, we sought to correlate NO actions to an anti-inflammatory macrophage phenotype. Therefore, we asked whether NO may activate PPARγ. We demonstrate activation of PPARγ in macrophages by chemically distinct NO donors as well as endogenously synthesized NO by inducible NO synthase (iNOS) induction and link an active PPARγ with an attenuated oxidative burst. Inhibition of superoxide radical formation was due to PPARγ-mediated down-regulation of p47 phagocyte oxidase (p47^{phox}), one important component of the NAD(P)H oxidase enzyme complex. We conclude that anti-inflammatory properties of NO may be transmitted by gene regulation affected by PPARγ activation.

Materials and Methods

Materials

PMA, *N*-acetyl-cysteine (NAC), 2-phenyl-4,4,5,6-tetramethyl-imidazoline-1-oxyl 3-oxide (PTIO), LPS, and 8-bromo-cGMP were purchased from Sigma (Deisenhofen, Germany). Spermine-NONOate (spermine-NO)

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[3] Abbreviations used in this paper: PPAR, peroxisome proliferator-activated receptor; p47^{phox}, p47 phagocyte oxidase; GSNO, *S*-nitrosoglutathione; spermine-NO, spermine-NONOate; PPRE, PPAR response element; NAC, *N*-acetyl-cysteine; PTIO, 2-phenyl-4,4,5,6-tetramethyl-imidazoline-1-oxyl 3-oxide; L-NAME, L-N^G-nitroarginine methyl ester; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; HE, hydroethidine; RXR, retinoic acid receptor; iNOS, inducible NO synthase; ROS, reactive oxygen species.

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was obtained from Bio-Trend (Cologne, Germany). Hydroethidine (HE) was from Molecular Probes (Leiden, The Netherlands). The polyclonal anti-PPARγ2 Ab and L-N^G-nitroarginine methyl ester (L-NAME) were from Alexis (Grünberg, Germany). The polyclonal anti-PPARα and anti-c-Jun Abs were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Culture supplements and FCS were ordered from Biochrom (Berlin, Germany). 15d-PGJ_2 and ciglitazone were bought from Biomol (Hamburg, Germany). Murine rIFN-γ was from Roche Diagnostics (Mannheim, Germany). The luciferase assay kit was obtained from Promega (Mannheim, Germany) and the β-galactosidase detection kit was from Tropix (Mannheim, Germany). Oligonucleotides were ordered from Eurogentec (Seraing, Belgium). All other chemicals were of the highest grade of purity and commercially available.

GSNO synthesis

S-nitrosoglutathione (GSNO) was synthesized and characterized as previously described (18).

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 and the premonocytic human cell lines U937 and THP1 were maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (complete RPMI). All experiments were performed using complete RPMI.

Flow cytometry of oxygen radical production (HE assay)

Cells were cultured under nonadherent conditions. Following a prestimulation regime, 5×10^5 cells were incubated for 30 min with 1 μM PMA. Thereafter, 3 μM HE was added and incubations went on for 30 min. Cells were harvested, washed with PBS, and resuspended in 200 μl PBS. Flow cytometry analysis was performed using a Coulter Epics XL flow cytometer (Beckman Coulter, Krefeld, Germany) and HE was measured through a 630-nm long pass filter (FL3). Data from 10,000 cells were collected to reach significance.

Nuclear protein extraction

Preparation of crude nuclear extract was basically as described (19). Briefly, following cell activation for the times indicated, 4×10^6 cells were washed in 1 ml of ice-cold PBS, centrifuged at $1{,}000 \times g$ 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 $15{,}000 \times g$ for 30 s. Sedimented nuclei were 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 $15{,}000 \times g$ for 5 min at 4°C. Aliquots of the supernatant, containing nuclear proteins, were frozen in liquid nitrogen and stored at −70°C. Protein was determined using a Bio-Rad II kit (Bio-Rad, Hercules, CA).

EMSAs

An established EMSA method, with slight modifications, was used (20). Nuclear protein (20 μg) was incubated for 20 min at room temperature with 20 μg BSA, 2 μg poly(dI-dC) (from Amersham Biosciences, Freiburg, Germany), 2 μl buffer D (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-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 Abs (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 (4% for supershifts), dried, and visualized (autoradiography using a Fuji x-ray film). Oligonucleotide probes were labeled by a filling reaction using the Klenow fragment (Roche Diagnostics). A total of 1 pmol oligonucleotide was labeled with 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham, Braunschweig, Germany) and cold nucleotides (dATP, dTTP, dGTP; Life Technologies, Eggenstein, Germany), purified on a CHROMA SPIN-10 column (Clontech Laboratories, Heidelberg, Germany), and stored at −20°C until use. Oligonucleotides with the consensus PPRE site (bold letters) were used (21): 5′-GGTAAAGGTCAAAGGTCAAT-3′ and 3′-ATT**TCCAGT**T**TCC AGT**TAGCCG-5′.

PPRE reporter gene assay

The plasmid J3TK pGL3, containing three copies of the human apoAII gene promoter PPRE-containing J site cloned upstream of the thymidine kinase promoter in the pGL3 luciferase expression vector, was kindly provided by B. Staels (Institut National de la Santé et de la Recherche Médicale, Unité 325, Institute Pasteur, Lille, France) (22). Macrophages were transiently transfected using the DEAE-dextran method as previously described (23). Cell selection was unnecessary because the expression of a macrophage-unrelated protein was analyzed. Briefly, 1 day before transfection, cells were seeded in suspension at a density of 1×10^6 cells/ml. A total of 1×10^7 cells were harvested, washed twice with PBS, and incubated for 3 h at 37°C in 1 ml RPMI 1640 supplemented with 50 mM Tris-HCl (pH 7.3), 400 μg DEAE-dextran, 20 μg luciferase reporter construct (J3TK pGL3), and 5 μg CMV-β-galactosidase plasmid as an internal control. To discard the DNA/DEAE-dextran mixture, cells were washed twice with PBS, seeded at a density of 1×10^6 cells/ml, and cultured for 24 h. Afterward cells were stimulated for 15 h with NO donors or 15d-PGJ_2. Cell extracts were assayed for luciferase and β-galactosidase activity. For calculations, luciferase activity was normalized for β-galactosidase by using the following formula: luciferase activity/β-gal activity.

RNA extraction and semiquantitative RT-PCR

RNA was extracted using peqGOLD RNAPure (Peqlab, Erlangen, Germany) according to the distributor's manual. Reverse transcription reactions and PCR for murine and human PPARγ and GAPDH were performed using the Advantage RT-for-PCR kit and the Advantage 2 Polymerase Mix (Clontech Laboratories). The sequences of the primers were as follows: murine PPARγ (480–1228) (24), T_A = 63°C: 5′→3′, ATGGCCATTGAGT GCCGAGTCTG; 3′→5′, GGCTTTTGAGGAACTCCCTGGTCA. Murine GAPDH (47–1048) (25), T_A = 63°C: 5′→3′, ATGGTGAAGGTCGG TGTGAACGG; 3′→5′, TTACTCCTTGGAGGCCATGTAGGGC.

Annealing temperatures were calculated using the primer design program Oligo (MBI, St. Leon-Rot, Germany). The number of amplification cycles (25 for GAPDH and 30 for PPARγ) was necessary to achieve exponential amplification where product formation is proportional to starting DNA. Products were run on a 1% agarose gel and were ethidium bromide stained. Controls of isolated RNA omitting reverse transcription were used during PCR to guarantee genomic DNA-free RNA preparations (data not shown).

Decoy approach

Cells were exposed to an oligonucleotide containing a PPRE consensus site as specified for the EMSA. Cells were seeded at a density of 1×10^6 cells/well into six-well plates. Oligonucleotides (3 μM) were added 24 h before cell stimulation. Cell stimulation was performed as indicated. Oligonucleotide sequences were identical to those used for EMSA. To guarantee oligonucleotide stability within the cells, oligonucleotides containing a phosphorothioate backbone were applied. For control reasons oligonucleotides with a mutated PPRE site were used (mutated sites in bold letters): 5′-GGTAAAGAACAAAGAACAAT-3′ and 3′-ATTTC**TT**GTTTC**TT**GTTA GCCG-5′.

Griess assay

To this end, nitrite, a stable end product of NO metabolism, was determined in the supernatant of RAW 264.7 cells. Cells were seeded in six-well plates at a density of 1×10^6 cells/well. After incubations for 24 h, cells were exposed to 100 μM GSNO, 3 μM ciglitazone, and 1 μM 15-dPGJ_2, or remained as controls. After 2 h medium was changed and cells were directly stimulated with 1 μg/ml LPS in combination with 10 U/ml IFN-γ for 15 h. The amount of nitrite was determined spectrophotometrically by the Griess assay (Promega, Heidelberg, Germany) according to the manufacturer's instructions. Nitrite concentrations in the supernatants were calculated by comparison with standard concentrations of $NaNO_2$ dissolved in culture medium. Unless otherwise stated, the reported values are the mean (±SD) of three separate experiments, each performed in triplicate.

Northern blot

Total RNA was extracted using peqGOLD RNAPure (Peqlab) according to the distributor's manual. Twenty micrograms of total RNA was used for Northern blotting. The probes for $p22^{phox}$ and $p47^{phox}$ were generated using the human GenBank sequences. The used primer sequences were as follows: Human $p22^{phox}$ (31–605) (26), T_A = 63°C: 5′→3′, GGGGCA GATCGAGTGGGCC; 3′→5′, CGTCGGTCACCGGGATGGG. Human $p47^{phox}$ (131–871) (27), T_A = 63°C: 5′→3′, TCTACCGGCGCTTCAC CGAGA; 3′→5′, CGTCTTGCCCCGACTTTTGCA.

Subsequently, cDNA was cloned into a pDrive cloning vector (Qiagen, Hilden, Germany). After *Eco*RI (Roche Diagnostics) restriction cleavage and gel extraction, probes were radiolabeled with [α-^{32}P]dCTP using the

Rediprime II random prime labeling system (Amersham). Blots were also probed with a 28S RNA probe to assess equal loading.

Statistical analysis

Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student *t* test. Otherwise representative data are shown.

Results

Activation of PPARγ by NO-releasing compounds and iNOS induction

In a first set of experiments we evaluated activation of PPARγ by gel shift analysis. Experimentally, monocytes/macrophages were dose-dependently exposed to chemically distinct NO donors. As shown in Fig. 1*A*, GSNO stimulated binding of PPARγ to radioactively labeled oligonucleotides in U937 cells. Activation was pronounced at 100 μM, somewhat lower at 200 μM, and virtually absent at doses of 50 μM as well as concentrations at or above 500 μM GSNO. Controls show minor or no PPARγ activation. To exclude any cell type artifact we repeated these studies in THP1 cells (Fig. 1*B*). In THP1 monocytes GSNO, at a concentration of 100 μM, evoked PPARγ activation, with lower or higher doses being inactive.

In extending studies we demonstrated that a chemically distinct NO donor such as spermine-NO activated PPARγ as well, with the notion that 50 μM was the most effective concentration (data not shown). Similar results were obtained using the murine macrophage-like cell line RAW 264.7 (data not shown). It appeared that activation of PPARγ was achieved at low concentrations of NO donors that elicited neither necrosis nor an apoptotic phenotype.

We went on to verify the identity of the PPAR-oligonucleotide complex by supershift analysis (Fig. 1*C*). This was achieved by adding PPARγ vs PPARα Abs to the binding assay followed by EMSA analysis. Specificity was further verified using an unrelated anti-c-Jun Ab in parallel. While the PPARγ antiserum shifted the protein-oligonucleotide complex to a higher m.w., the PPARα Ab did not. The c-Jun Ab was ineffective as well. Conclusively, the γ-isoform of PPARs is activated in response to NO.

The biological significance shown for NO donor-mediated effects was verified in experiments using RAW 264.7 macrophages (Fig. 1*D*). After LPS/IFN-γ treatment, an established iNOS-inducing regimen in these cells, activation of PPARγ was clearly visible by EMSA analysis (Fig. 1*D*, *lane 3*). Interestingly, inhibition of iNOS by L-NAME markedly reduced activation of PPARγ (Fig. 1*D*, *lane 2*), thus pointing to the involvement of endogenously generated NO in PPARγ activation.

In the following set of experiments we looked for the time-dependent activation of PPARγ by exposing U937 cells to GSNO or spermine-NO (Fig. 2*A*) and RAW 264.7 macrophages to spermine-NO (Fig. 2*B*). With 100 μM GSNO we elicited an immediate onset of the PPARγ response after 30 min. The strongest activation was achieved after 1–2 h, with a decreased responsiveness after 4 h.

Spermine-NO evoked PPARγ activation after 30 min and promoted the strongest response after 1 h, with a declining responsiveness toward the control level at 2 and 4 h as shown for U937 (Fig. 2*A*) and RAW 264.7 cells (Fig. 2*B*). To confirm specific PPARγ activation we conducted costimulation experiments with the PPARγ agonists ciglitazone and 15d-PGJ_2 in combination with the NO donor GSNO. As shown for U937 cells in Fig. 2*C*, ciglitazone, used at a concentration of 3 μM, itself led to PPARγ activation. This was significantly increased with the simultaneous addition of 100 μM GSNO. Similar results were obtained using 15d-PGJ_2 in combination with GSNO (data not shown).

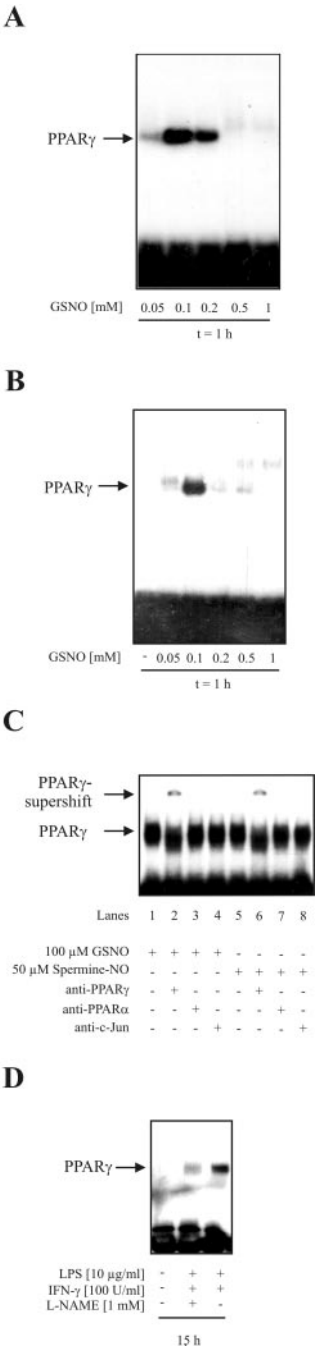

FIGURE 1. Activation of PPARγ in response to NO-releasing compounds. Activation of PPARγ was analyzed by EMSA as described in *Materials and Methods*. *A*, U937 cells were dose-dependently stimulated with GSNO (50–1000 μM) for 1 h. *B*, THP1 cells were stimulated with increasing concentrations of GSNO (50–1000 μM) for 1 h. For controls cell stimulation was omitted. *C*, Supershift analysis of the active PPAR complex was performed as described in *Materials and Methods*. RAW 264.7 macrophages were stimulated with 100 μM GSNO or 50 μM spermine-NO for 1 h. For supershift analysis a PPARγ2 Ab (*lanes 2* and *6*), a PPARα Ab (*lanes 3* and *7*), or a c-Jun Ab (*lanes 4* and *8*) was included. Incubations without supershift Abs are shown in *lanes 1* and *5*. *D*, RAW 264.7 cells were stimulated for 15 h with a combination of 10 μg/ml LPS and 100 U/ml IFN-γ with or without 1 mM L-NAME. For controls cell stimulation was omitted. Data are representative of three similar experiments.

A well-established signal transduction pathway for NO is activation of the soluble guanylyl cyclase with the formation and action of cGMP (28, 29).

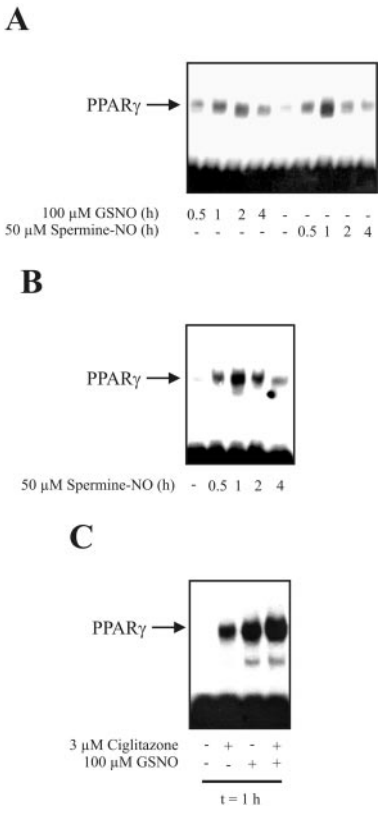

FIGURE 2. Time-dependent activation of PPARγ by NO donors. Activation of PPARγ was analyzed by EMSA as described in *Materials and Methods*. *A*, U937 cells were stimulated with 100 μM GSNO or 50 μM spermine-NO for 30 min up to 4 h. *B*, RAW 264.7 macrophages were exposed to 50 μM spermine-NO for 30 min up to 4 h. *C*, U937 cells were stimulated with 100 μM GSNO or 3 μM ciglitazone for 1 h alone or in combination. For controls cell stimulation was omitted. Data are representative of three similar experiments.

To analyze for the potential involvement of cGMP in conferring activation of PPARγ in our system, we exposed THP1 as well as RAW 264.7 cells to the lipophilic cyclic GMP analog 8-bromo-cGMP (1 mM) for 30 min up to 4 h (Fig. 3). Unlike NO, 8-bromo-cGMP did not reproduce PPARγ activation, thus pointing to a cGMP-independent NO signaling mechanism.

In continuation of the study we asked whether NO promoted PPARγ transactivation, besides enhancing DNA binding. We used the luciferase reporter assay to demonstrate transcriptional activation of PPARγ in response to NO donors and 15d-PGJ_2, which represents a commonly accepted control activator (Fig. 4). At most effective concentrations GSNO and spermine-NO evoked a 2-fold activation in RAW 264.7 cells, while 15d-PGJ_2 elicited an ~4-fold increase of luciferase activity. The response toward NO and 15d-PGJ_2 was determined 15 h after cell stimulation.

We concluded that NO stimulated binding of PPARγ to the DNA and concomitantly caused transactivation of a PPAR-responsive reporter system.

Specificity of the NO response

Considering the possibility that PPARγ is transcriptionally regulated by NO we analyzed the mRNA level by RT-PCR in RAW 264.7 macrophages (Fig. 5). Neither GSNO (100 μM) nor spermine-NO (50 μM), both supplied up to 4 h, revealed any variations in the mRNA content of PPARγ after normalization with the GAPDH standard.

Excluding a transcriptional regulation of PPARγ in response to NO delivery, we assumed a direct activation mechanism to be more likely. Along that line we asked whether antioxidants such as NAC may interfere with PPARγ activation. This was tested by preincubating RAW 264.7 cells for 1 h with 1 mM NAC before the addition of 100 μM GSNO or 50 μM spermine-NO, supplied for 1 h (Fig. 6*A*). The efficacy of NAC was demonstrated in gel shift assays, when activation of PPARγ by GSNO and spermine-NO was largely attenuated in the presence of the antioxidant. Moreover, NAC suppressed the transactivation capacity of PPARγ (Fig. 6*B*). For these experiments 1 mM NAC was supplied for 1 h in front of NO donors, followed by determination of luciferase activity 15 h later.

The 2-fold activation, normally seen with NO donors, was completely absent with NAC being present, implying that oxidative or nitrosative modifications are involved in PPARγ activation. It was our further intention to pinpoint NO as the molecule setting into motion the activation cascade that culminated in PPARγ activation. Therefore, we used the NO scavenger PTIO (Fig. 6*B*). The

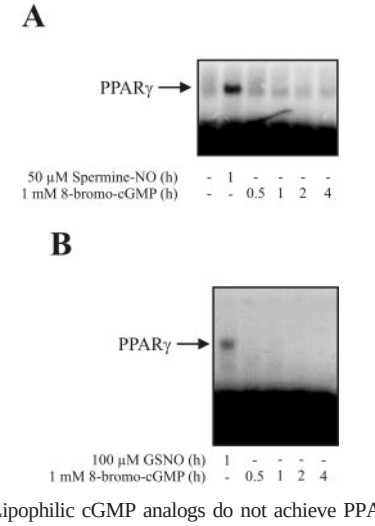

FIGURE 3. Lipophilic cGMP analogs do not achieve PPARγ activation. *A*, THP1 cells were stimulated with 50 μM spermine-NO for 1 h or 1 mM 8-bromo-cGMP for 30 min up to 4 h. *B*, RAW 264.7 macrophages were exposed to 100 μM GSNO for 1 h or 1 mM 8-bromo-cGMP for 30 min up to 4 h. Activation of PPARγ was followed by EMSA as described in *Materials and Methods*. Data are representative of three similar experiments.

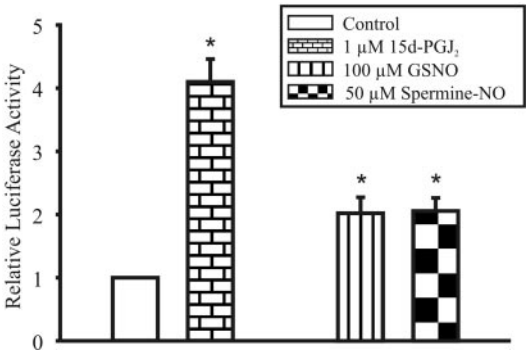

FIGURE 4. NO-evoked PPARγ reporter gene activation. RAW 264.7 macrophages were cotransfected with the J3TK-plasmid and a plasmid encoding β-galactosidase. Luciferase and β-galactosidase expression were analyzed after both activities had been normalized as described in *Materials and Methods*. Cell stimulation was for 15 h with 100 μM GSNO, 50 μM spermine-NO, 1 μM 15d-PGJ_2, or vehicle (control). Data are means ± SD of three individual experiments (*, $p \leq 0.05$ vs control).

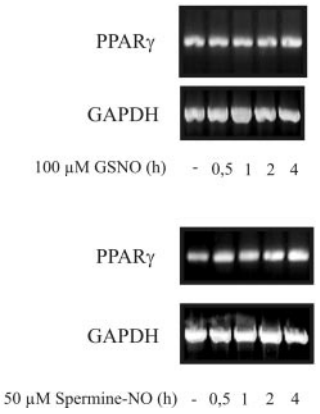

FIGURE 5. mRNA expression of PPARγ in response to NO donors. Expression of PPARγ mRNA was analyzed by RT-PCR. RAW 264.7 cells were stimulated with 100 μM GSNO or 50 μM spermine-NO for the times indicated. Semiquantitative RT-PCR was performed as described in *Materials and Methods*. Data are representative of three similar experiments.

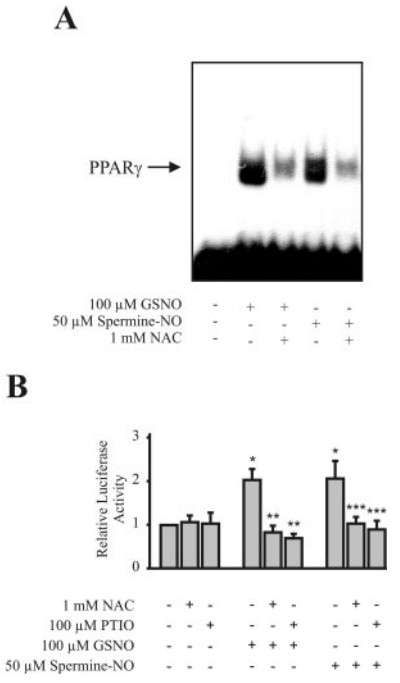

FIGURE 6. NAC and PTIO attenuate PPARγ activation in response to NO. *A*, RAW 264.7 macrophages were incubated for 1 h with 1 mM NAC before the addition of 100 μM GSNO or 50 μM spermine-NO. EMSA was performed after 1 h as described in *Materials and Methods*. For controls cell stimulation was omitted. Data are representative of three similar experiments. *B*, RAW 264.7 macrophages were cotransfected with the J3TK plasmid and a plasmid encoding β-galactosidase. Luciferase and β-galactosidase expression were analyzed after both activities had been normalized as described in *Materials and Methods*. As indicated, cells were prestimulated for 1 h with 1 mM NAC or 100 μM PTIO, or remained as controls, before the addition of 100 μM GSNO, 50 μM spermine-NO, or vehicle (control) for 15 h. Data are means ± SD of three individual experiments (*, $p \leq 0.05$ vs untreated sample; **, $p \leq 0.05$ vs GSNO-treated sample; ***, $p \leq 0.05$ vs spermine-NO-treated sample).

NO scavenger, preincubated at a dose of 100 μM for 1 h, completely attenuated PPARγ-evoked luciferase activity in response to GSNO and spermine-NO. Control determinations showed no interference of PTIO or NAC on the basal PPARγ response.

NO attenuated the oxidative burst in macrophages via activation of PPARγ

Decoy oligonucleotides can be used to scavenge and thereby to inactivate transcription factors (30, 31). Using this experimental approach we provided evidence that NO donors attenuated reactive oxygen species (ROS) formation via PPARγ activation (Fig. 7). As shown in Fig. 7*A*, oxidation of HE was elicited in response to 1 μM PMA in RAW 264.7 macrophages. This was measured by flow cytometry and is shown by the rightward shift of the HE signal (Fig. 7*A*). Prestimulation with 100 μM GSNO (Fig. 7*B*) or 50 μM spermine-NO (Fig. 7*C*) for 15 h eradicated ROS formation because NO donors attenuated the HE shift in response to PMA.

The presence of PPRE decoy oligonucleotides attenuated the down-modulatory behavior of GSNO and allowed to regain HE oxidation after PMA addition (Fig. 7*D*). The soft shoulder seen in the trace from PPRE decoy oligonucleotides/GSNO/PMA-treated cells may refer to a population of macrophages that have not been reached by oligonucleotides, therefore still attenuating ROS formation. The specificity for NO in diminishing the oxidative burst was further demonstrated when coincubations of PTIO, GSNO, and PMA allowed ROS generation (Fig. 7*E*). In line with the observation that cGMP does not activate PPARγ (Fig. 3), prestimulation with 1 mM 8-bromo-cGMP turned out to be ineffective in inhibiting ROS formation following PMA addition (Fig. 7*F*).

We conclude that NO, delivered by the breakdown of GSNO or spermine-NO, activates PPARγ by a cGMP-independent mechanism that in turn attenuates the proinflammatory signal of the macrophage oxidative burst.

NO-mediated inhibition of superoxide radical production is due to PPARγ-dependent down-regulation of p47^{phox}

Modulation of gene expression in response to PPARγ activation is known and associated with, e.g., inhibition of iNOS expression (13). To verify our system we used RAW 264.7 macrophages, known to express iNOS in response to LPS/IFN-γ treatment. To establish that NO-activated PPARγ may attenuate iNOS expression, we treated cells for 2 h with 100 μM GSNO, changed medium, and stimulated macrophages with 1 μg/ml LPS in combination with 10 U/ml IFN-γ for 15 h. Thereafter nitrite was determined by the Griess assay as the metabolic end-product of iNOS-generated NO. Prestimulation of the cells with GSNO attenuated nitrite production by 50% compared with cells treated with LPS/IFN-γ only (27 ± 9 vs 14 ± 7 μM nitrite). To make the contribution of PPARγ more likely, PPARγ agonists such as 15d-PGJ_2 or ciglitazone were used. Prestimulation for 2 h with either 15d-PGJ_2 or ciglitazone decreased LPS/IFN-γ-mediated NO production by ~70% (27 ± 9 vs 9 ± 5 μM nitrite for 15d-PGJ_2 or 11 ± 7 μM nitrite for ciglitazone).

We now focused on components of the NAD(P)H oxidase system, known to be involved in the superoxide radical formation, to identify the molecular mechanism responsible for attenuating the oxidative burst. We analyzed the expression pattern of different members of this multifactor complex on RNA levels by Northern blotting. Using this experimental system we demonstrate as depicted for U937 cells that NO-mediated PPARγ activation leads to p47^{phox} down-regulation. As shown in Fig. 8, *upper panel*, transcription of p47^{phox} was clearly reduced in response to 50 and 100 μM GSNO. To verify the role of PPARγ activation concerning this effect, we applied 15d-PGJ_2 and ciglitazone, two known specific PPARγ agonists, to the cells with a similar outcome. Expression of p47^{phox} was significantly diminished. p22^{phox}, another component of the NAD(P)H oxidase system, was not altered by

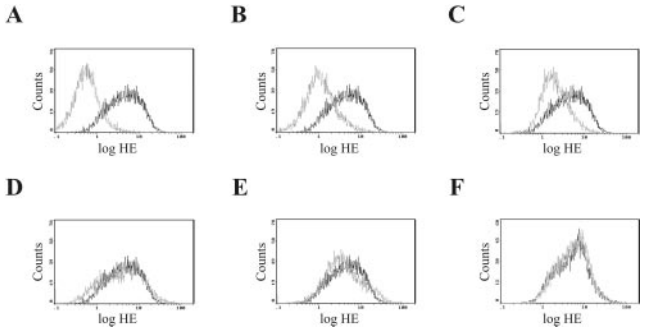

FIGURE 7. Oxygen radical production in macrophages under the influence of NO and PPARγ. ROS production was analyzed in RAW 264.7 macrophages by flow cytometry using 3 μM HE as the redox-sensitive dye as described in *Materials and Methods*. Cell stimulation was achieved with 1 μM PMA and data are representative of three or more similar experiments. Manipulations are as follows: *A*, controls (gray) vs PMA stimulation (black); *B*, PMA stimulation (black) vs PMA with 100 μM GSNO being preincubated for 15 h (gray); *C*, PMA stimulation (black) vs PMA with 50 μM spermine-NO being preincubated for 15 h (gray); *D*, PMA response (black) vs a treatment with PPRE decoy oligonucleotides, 100 μM GSNO, and PMA as described in the text (gray); *E*, PMA treatment (black) vs PMA with 100 μM PTIO and 100 μM GSNO being preincubated for 16 and 15 h (gray); *F*, PMA response (black) vs PMA with 1 mM 8-bromo-cGMP being preincubated for 15 h (gray).

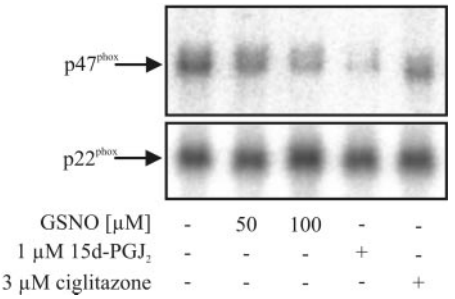

FIGURE 8. Down-regulation of p47^{phox} in response to NO-dependent PPARγ activation. Regulation of p22^{phox} and p47^{phox} expression was analyzed on total RNA level by Northern blotting as described in *Materials and Methods*. Cells were treated with 50 or 100 μM GSNO, 1 μM 15d-PGJ_2, or 3 μM ciglitazone for 24 h or remained as controls. The blots were hybridized first with a probe specific for p47^{phox} (*upper panel*) and second with one specific for p22^{phox} (*lower panel*). Data are representative of three similar experiments.

NO exposure or by PPARγ agonist addition compared with untreated controls (Fig. 8, *lower panel*). To assess equal loading, filters were hybridized with a 28S RNA probe (data not shown).

We assume that NO-dependent PPARγ activation leads to attenuation of at least p47^{phox} expression, one necessary component of the NAD(P)H oxidase, responsible for oxidative burst generation.

Discussion

In this work we presented evidence that NO-releasing compounds activated the anti-inflammatory properties of the transcription factor PPARγ and concomitantly attenuated the oxidative burst in monocytes/macrophages, likely due to down-regulated p47^{phox} expression. Activation of PPARγ was confirmed by gel shift analysis, reporter gene assays, and a decoy oligonucleotide approach. The involvement of NO was substantiated by using the NO scavenger PTIO and the antioxidant NAC with the notion that lipophilic cGMP analogs were unable to reduce superoxide formation. Attenuation of p47^{phox} expression was shown by Northern blotting. To verify the biological significance of these results obtained with NO donors, RAW 264.7 macrophages were stimulated with LPS/IFN-γ to endogenously produce NO as a result of iNOS induction. As shown by gel shift analysis, PPARγ was activated in response to endogenously synthesized NO, because blocking iNOS significantly attenuated PPARγ activation. We conclude that iNOS-generated NO suffices in provoking DNA binding of PPARγ. Taking the lipid-soluble nature of NO into account, it appears attractive to hypothesize that cells in the direct neighborhood to NO-producing cells are affected with the outcome of an impaired proinflammatory signaling cascade in those target cells.

Reactive oxygen- and nitrogen-derived species are implicated as effector molecules in the immune system, serving major functions during immunological host defense, mainly as a result of macrophage and neutrophil activation (32). However, reactive oxygen and nitrogen species operate as modulators of signal transducing pathways as well, thus characterizing them as intra- and intercellular messenger molecules (33). In line, redox-controlled transcription factors, oxidative susceptible thiol groups, or redox-sensitive phosphorylation events allow to channel the action of reactive species into established intracellular communication systems (2, 34).

The observation that NO-provoked PPARγ-activation is unrelated to cGMP signaling appears in line with several recent reports on direct NO/target interactions. For example, activation of c-Ha-Ras (p21) via nitrosylation of cysteine 118 has recently been shown by using electrospray ionization mass spectrometry (34), up-regulation of transcription factors such as NF-κB has been noted in a NO-dependent manner in a murine model of hemorrhagic shock (6), and protein tyrosine phosphorylation is achieved by NO. Moreover, the transcription factor HIF1α turned out to be NO responsive (35), and NO is needed to activate tyrosine kinase 2 and to tyrosine phosphorylate STAT4 (36). In this work we show, by using chemically distinct NO donors, that PPARγ is activated by NO. This is substantiated by using a NO scavenger to attenuate PPARγ activation. The use of NAC to abrogate the NO signal may aim toward its NO scavenging ability or its antioxidant properties. In any case, the interference of NAC appears rational. Although our studies excluded cGMP to activate PPARγ, molecular details on NO action remain unknown so far. In this respect, extending examinations are needed to address various possibilities, such as a direct interaction of NO with PPARγ, phosphorylation events, or NO-evoked generation of a PPARγ activator. Of note, DNA binding of PPARγ appeared fast and was noticed 1–2 h after the addition of NO. The rapid response points to activation of a preformed transcription factor rather than a mechanism involving enhanced PPARγ expression. This assumption is strengthened by our observation that mRNA of PPARγ remained unchanged in response to NO.

Activation of PPARγ by NO is contrasted by inhibition seen at higher concentrations of NO donors. An attenuated DNA binding ability of PPARγ at higher doses of GSNO appears in some agreement with studies of Kröncke et al. (37). They observed reduced DNA binding activities of zinc finger transcription factors, among them the RXR, at high doses of NO donors (≥0.5 mM). Taking into consideration that PPARγ heterodimerizes with RXR before DNA binding, one may envision an attenuated promoter binding activity of PPARγ at elevated NO concentrations. The importance of RXR in transcription factor complex activation/inhibition will be an important issue of future experiments. As seen in our study, at concentrations >200 μM GSNO we lost the ability of NO to stimulate DNA binding or to cause transactivation of PPARγ (data not shown).

Activation of PPARγ by NO results in desensitization of macrophages with the consequence of an attenuated oxidative burst. A cause-effect relationship is established by the successful use of a decoy oligonucleotide approach that allowed to regain an oxidative signal despite the presence of NO. Decoy oligonucleotide approaches have been shown to block transcriptional activity with high efficacy by scavenging active transcription factors (31). PPARγ is known for its anti-inflammatory properties and is reported to exert a negative effect on proinflammatory cytokine and/or iNOS expression in macrophages, such as RAW cells (12, 13). The identified anti-inflammatory property of PPARγ is in contrast to the work of Chawla et al. (16), who showed in macrophages derived from murine embryonic stem cells that were homozygous for a null mutation in the PPARγ gene, that inhibitory effects on cytokine production and inflammation may be receptor independent. Concerning this data we assume a concentration-dependent, PPARγ-independent effect, which might not occur using lower doses of the applied PPARγ-specific and unspecific agonists.

On a molecular basis PPARγ has recently been shown to antagonize coactivators such as the CREB-binding protein from interacting with its cognate target gene, thereby attenuating up-regulation of, e.g., iNOS (13), or blocking TNF-α formation (12). Having these reports in mind, our decoy approach may imply either that transactivation of PPARγ with concomitant formation of a PPARγ-responsive gene product interferes with the oxidative burst or that active PPARγ complexes and thereby removes another factor that presumably is required to express constituents of the NAD(P)H oxidase. In any case, PPARγ decoy oligonucleotides will interfere with gene expression with the limitation that unknown transcription factors/coactivators, distinct from PPARγ, may be targeted by this approach. Taking advantage of published data that iNOS expression is inhibited by PPARγ activation (13), we provided evidence that our system is in line with published results. Prestimulation for 2 h with GSNO inhibits LPS/IFN-γ-mediated nitrite production by ~50%. However, complete inhibition was not achieved and control experiments performed with specific PPARγ agonists revealed ~70% inhibition. This may depend on the presence of IFN-γ, which antagonizes PPARγ-provoked inhibitory mechanisms (38). Having demonstrated that PPARγ targets established systems, e.g., NO formation, we provided further evidence on PPARγ in attenuating ROS formation. Results came from Northern blotting experiments, showing inhibition of $p47^{phox}$ expression in response to NO-releasing compounds as well as PPARγ-specific agonists; we assume that attenuated $p47^{phox}$ expression as one component of the NAD(P)H oxidase system accounts for reduced ROS formation (39). These observations are in some analogy with the results of Inoue et al. (40), demonstrating inhibition of $p22^{phox}$ expression in response to PPARγ agonists in primary endothelial cells. Regulation of NAD(P)H oxidase components has been described in human cultured monocytes, where decreased $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$ expression in response to diminished binding of the transcription factor PU.1 to the corresponding promoter sites reduced the ability to produce microbicidal oxidants (41). Endogenous production of NO seems insufficient to down-regulate $p47^{phox}$ (data not shown). Rather, up-regulation of $p47^{phox}$ upon LPS/IFN-γ stimulation was shown (42). PPARγ-mediated effects are counter-regulated by IFN-γ (38). Further studies will need to analyze the involvement of $p47^{phox}$ promoter regions in conferring this opposing effect of LPS/IFN-γ vs NO.

Inhibition of the oxidative burst by NO has been known for some time. In neutrophils NO blocked O_2^- formation with some indication that assembly of the NAD(P)H oxidase is affected (43). Along that line, NO attenuated the oxidative burst in murine microglia as well (44). In analogy to our studies, this was unrelated to cGMP signaling or a simple scavenging of O_2^-, thus implying activation of PPARγ as a rational explanation for these observations.

Macrophages are key players during the innate immune response. Immunological activation of macrophages is achieved by cytokines and bacterial components (45). Cell activation results in the release of various proinflammatory cytokines and reactive nitrogen as well as oxygen species (46). The excessive release of these mediators results in the development of whole body inflammation, which is closely related to the clinical symptoms of sepsis or septic shock (47). During sepsis the early hyperinflammatory phase is counterbalanced by an anti-inflammatory response, characterized by monocyte deactivation (48). It appears attractive to hypothesize whether the formation of NO in macrophages not only represents an early cytotoxic signal but also shifts the balance toward an anti-inflammatory response via activation of PPARγ, which would be in line with recent reports describing NO as an inhibitor of proinflammatory cytokine expression (7, 49, 50). In further studies it will be essential to compare the cytokine profile of activated macrophages to that of a deactivated macrophage phenotype, to study the impact of NO on this cytokine balance, and to elucidate the role of PPARγ during this activation-deactivation transition.

Acknowledgments

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