Nitric Oxide-Derived Nitrosating Species and Gene Expression in Human Monocytic Cells†

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ABSTRACT: In living cells, NO signaling is mediated by NO-derived metabolites and is therefore dependent on the rate of formation of these so-called reactive nitrogen intermediates (RNIs). We have examined the effects of NO-oxidizing agents, the nitronyl nitroxide PTIO and its less hydrophobic analogue carboxy-PTIO (CPTIO), on the expression of NO-sensitive genes in monocytic U937 and Mono Mac 6 cells. We have observed that pretreatment of cells with PTIO boosted expression of IL-8 and heme oxygenase 1 (HOX) genes to a high level in cells treated with the NO donor DPTA-NO. In contrast, pretreatment of cells with CPTIO significantly inhibited NO-dependent expression of IL-8 and hardly stimulated HOX gene expression by DPTA-NO. The effect of PTIO was abrogated by reduced glutathione, suggesting that upregulation of the IL-8 and HOX genes is dependent on RNI-mediated S-nitrosation of specific regulator(s). The concentration of PTIO required to enhance mRNA level was different for IL-8 and HOX genes. Analysis of 4,5-diaminofluorescein (DAF) nitrosation in the presence of PTIO and DPTA-NO showed that optimal PTIO concentrations required for maximal N_2O_3 synthesis and for highest IL-8 gene expression are similar. Furthermore, we have shown that, besides IL-8 and HOX, PTIO superactivates NO-dependent expression of TNF-α and p21/WAF1 genes. In contrast, the level of MIP-1α, c-jun, and c-fos genes was not changed by the presence of PTIO in U937 cells and was even reduced in Mono Mac 6 cells.

Nitric oxide (NO) is an important signaling molecule with multiple biological functions in a wide variety of tissues (*1*−*5*). NO has a high affinity for iron-containing proteins including cytochromes, cyclooxygenase, soluble guanylate cyclase, and aconitases (*6*−*9*). Yet, its reactivity as well as its impact on cellular responses is largely influenced by the cellular redox environment. A variety of effects attributed to NO are achieved through either thiol oxidation or formation of *S*-nitrosothiols which actually result from the reaction of cellular thiol-containing compounds with the so-called reactive nitrogen intermediates (RNIs).[1] The generation of RNIs occurs during NO oxidation upon reaction with molecular oxygen or reactive oxygen species. The main nitrosating agent is nitrous anhydride N_2O_3 whereas thiol oxidation results from reaction with nitrogen dioxide radical NO_2 or peroxynitrite anion $ONOO^-$ (*10*). In intact cells, the rate of NO oxidation by molecular oxygen is rather slow and mainly confined within the lipophilic area of membranes (*11*). It has been suggested that S-nitrosation of proteins could mediate signaling functions similar to those of protein phosphorylation (*3*). However, phosphorylation of target proteins is a downstream event in signaling cascades, whereas S-nitrosation of sensitive cysteine residues of receptor proteins is an initial switch causing activation of various subordinated pathways. The list of NO-dependent signaling systems is growing and includes G proteins, nonreceptor tyrosine kinases, and master transcription factors such as AP-1, HIF-1α, and NF-κB (*12*−*17*). As a consequence, NO is a potent activator of expression of interleukin 8 (IL-8), heme oxygenase 1 (HOX), ferritin, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1α (MIP-1α), cyclooxygenase-2, the cyclin-dependent protein kinase inhibitor p21/WAF1, the transcription factors c-jun, junB, and c-fos, and several other genes (reviewed in ref *4*). There are several different transducers through which NO can influence cellular signaling systems. Soluble guanylate cyclase is probably the most sensitive NO receptor. However, the cGMP-dependent signaling system is not efficient in U937 cells (*18*), and consequently, this cell line constitutes a good model to study the other RNI-sensitive signaling pathway(s).

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[1] Abbreviations: CPTIO, carboxy-PTIO; DAF, 4,5-diaminofluorescein-2; DPTA-NO, dipropylenetriamine NONOate; DTPA, diethylenetriaminepentaacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, reduced glutathione; HOX, heme oxygenase 1; IL-8, interleukin 8; PBS, phosphate-buffered saline; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; RNIs, reactive nitrogen intermediates; SSC, saline−sodium citrate; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α.

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The aim of the present study was to examine whether RNIs are implicated in upregulation of NO-dependent genes in monocytic cells. For this purpose, we cotreated the human monocytic cell lines U937 and Mono Mac 6 cells with the synthetic NO donor DPTA-NO along with increasing concentrations of PTIO. PTIO is both an NO scavenger and an NO_2• generator. As reported earlier, suboptimal concentrations of PTIO added to NO can mimic nitrosative stress (*19*) by promoting the yield of N_2O_3 from the reaction between NO_2 and the remaining NO. By doing so, NO and its metabolites should be investigated separately, and we can expect to distinguish genes whose expression is modulated by nitrosative stress from those modulated by regular NO signaling. We report that the PTIO-mediated increase in nitrosating species remarkably enhances the mRNA level of a group of proteins including IL-8 and HOX, thus building a specific pattern of gene expression.

MATERIALS AND METHODS

Cell Cultures. U937 cells were from ATCC. They were cultured under 5% CO_2 atmosphere in RPMI 1640 supplemented with 25 mM HEPES, pH 7.3, 5% fetal calf serum (Invitrogen), 2 mM glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). Culture medium was supplemented with 10% fetal calf serum and 2 mM sodium glutamate. Mono Mac 6 cells were from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunsweig, Germany (*20*). They were cultured under 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM sodium glutamate, 1 mM sodium pyruvate, 8 μg/mL bovine insulin (Sigma), nonessential amino acids (1:100) (Gibco), penicillin (100 units/mL), and streptomycin (100 μg/mL). PTIO and other modulators of NO effects were added to serum-containing culture medium 15 min before DPTA-NO.

Cell Treatments and Reagents. Cells were grown in fresh culture medium for 16 h before the experiments. Synthetic NO donor dipropylenetriamine NONOate (DPTA-NO) (Cayman Chemicals) was dissolved in phosphate-buffered saline (PBS) (pH 7.3) and immediately added to culture medium to final concentrations as indicated. The NO scavengers PTIO and carboxy-PTIO (CPTIO) (ICN) were dissolved in ethanol. In these experiments, the final ethanol concentration in culture medium did not exceed 0.05%. Reduced glutathione (GSH) (Sigma) was dissolved in PBS, and pH was adjusted to 7.3 by addition of 1 M NaOH. Cells were lysed, and total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Each experiment was performed at least three times.

Northern Blot Analysis. Ten micrograms of total cellular RNA was separated by electrophoresis in denaturing 1.2% agarose−formaldehyde gel and transferred to Hybond N membrane (Pharmacia Biotech). Equal RNA loading and membrane transfer were confirmed by hybridization with a GAPDH radiolabeled probe. Membranes were hybridized overnight at 42 °C with ^{32}P-labeled probes and first washed in 2 × SSC and 0.1% SDS at room temperature, then washed in 0.5 × SSC and 0.1% SDS at room temperature, and finally washed in 0.1 × SSC and 0.1% SDS at 42 °C. Hybridization signals were quantified with a PhosphorImager.

cDNA Probes and Plasmids. Gene fragments were amplified from human cDNA by using the following primers: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-GACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTAG-3′ (reverse); IL-8, 5′-ATGACTTCCAAGCTGGCCGTG-3′ (forward) and 5′-CTCAGCCCTCTTCAAAAACTTCTC-3′ (reverse); HOX, 5′-CCTTGTTGACACGGCCATGACCAC-3′ (forward) and 5′-AGTAGACCAAGGCCACAGTGCCG-3′ (reverse); TNF-α, 5′-GCCTACAGCTTTGATCCCTG-3′ (forward) and 5′-CAGCCTATTGTTCAGCCTCC-3′ (reverse); p21/WAF1, 5′-TTAGCAGCGGAACAAGGAGT-3′ (forward) and 5′-CAGTACAGGGTGTGGTCCCT-3′ (reverse). Plasmids containing cDNA fragments of c-jun, c-fos, and MIP-1α genes were from the University of Toronto Microarray Center (Toronto, Canada). Specific inserts were amplified using universal primers. The resulting PCR products were verified by sequencing. To synthesize cDNA radiolabeled probes, the PCR products were purified using a Qiagen kit according to the manufacturer's instructions and used as a template. cDNA inserts were labeled with [α-^{32}P]dCTP by a random-priming method using a commercial labeling kit (Invitrogen) according to the manufacturer's instructions. The [α-^{32}P]dCTP was from Amersham Biosciences, Orsay, France.

DAF Fluorescence Instrumentation and Analysis. The production of N_2O_3 was measured by formation of the highly fluorescent N-nitrosated derivative of 4,5-diaminofluorescein-2 (DAF) (Calbiochem). Stock solution of DAF (2 mM) was prepared in DMSO. The incubation mixture contained 100 μM DAF, the metal chelator DTPA (50 μM), the NO donor DPTA-NO and PTIO as indicated, and PBS up to 10 μL. Reaction was maintained in a water bath at 36 °C for 10 min and stopped by addition of 100 μL of 50 μM Tris buffer. Measurements were performed on a Perkin-Elmer Victor ^{2}D fluorometer (200 μL volume, 37 °C) with excitation at 485 nm and emission at 535 nm.

RESULTS

Analysis of PTIO-Mediated N_2O_3 Generation. To investigate the response of monocytic cells to N_2O_3-mediated nitrosation, we combined DPTA-NO, a synthetic NO donor, and PTIO that is widely used as an NO scavenger (*21*, *22*). We took advantage of the ability of PTIO and its water-soluble analogue CPTIO to promote NO_2 and N_2O_3 formation (*19*, *21*, *23*, *24*) according to the reactions:

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO + (C)PTIO \rightarrow NO_2 + (C)PTI \quad (2)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (3)$$

Therefore, PTIO mimics the reaction of NO oxidation by molecular oxygen. The reaction between NO and oxygen is second order with respect to NO [$k = (2.2-2.9) \times 10^6$ M^{-2} s^{-1}], and consequently the rate of NO_2 formation is dependent on the square of NO concentration. Under physiological conditions, it is held that the rate of NO oxidation by molecular oxygen and NO_2 synthesis are low (*10*, *11*) and expected to be enhanced in the presence of PTIO or CPTIO.

In the first set of experiments, we quantified N_2O_3 generated by the reaction between NO released from DPTA-NO (0.2 or 0.5 mM) and PTIO in the presence of 100 mM DAF. N-Nitrosation of DAF by N_2O_3 yields triazofluorescein,

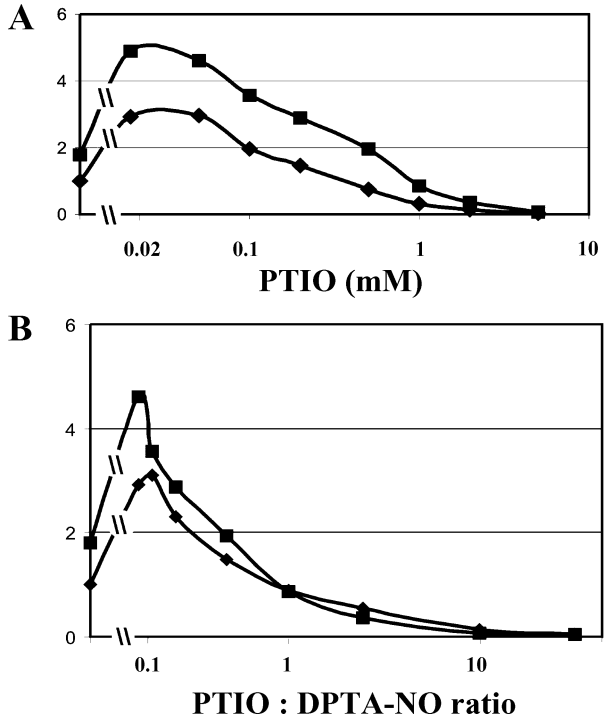

FIGURE 1: Concentration effect of PTIO on DAF nitrosation by DPTA-NO. DPTA-NO at the concentration of 0.2 mM (◆) or 0.5 mM (■) was added to a PBS-buffered solution containing DAF (100 μM), the metal chelator DTPA (50 μM), and PTIO as indicated. (A) Dependence of DAF nitrosation on PTIO concentration. (B) Dependence of DAF nitrosation on PTIO:DPTA-NO ratio. The reaction was carried out at 36 °C for 10 min. Fluorescence changes were monitored at $\lambda_{ex/em}$ of 485/535 nm. The basal fluorescence level related to NO oxidation by molecular oxygen in the absence of PTIO was assigned a value of 1. Data represent the mean of duplicate determinations from two separate experiments.

a highly fluorescent compound (*19*). As shown in Figure 1, PTIO addition enhanced the DAF-generated fluorescent signal in a dose-dependent manner. The optimal PTIO concentration to achieve maximal DAF nitrosation was 0.05 mM. Under these conditions, the DAF-generated fluorescent signal was about three times stronger than that measured in the absence of PTIO and which is due to NO autoxidation only. At PTIO concentrations higher than 0.1 mM, the fluorescent signal constantly declined, pointing to the primary formation of NO_2 that is unable to generate triazofluorescein upon reaction with DAF. According to data shown in Figure 1, maximal DAF nitrosation (i.e., N_2O_3 formation) was achieved when the PTIO:DPTA-NO molar ratio was 1:10 or below. When this ratio was higher than 2:1, we observed an N_2O_3 production lower than that in the absence of PTIO due to the scavenging of most of the NO released by DPTA-NO.

Expression of IL-8 and HOX Genes in U937 Cells Exposed to NO. To test the sensitivity of U937 monocytic cells to NO, we examined IL-8 and HOX mRNA levels by a Northern blot analysis. As shown in Figure 2, exposure to 0.5 mM DPTA-NO for 4 h caused a significant increase in both IL-8 and HOX mRNA levels. DPTA-NO stimulated the expression of these genes in a time- and dose-dependent manner (Figure 3). Within 2 h after addition of 0.5 mM DPTA-NO to U937 cells, the IL-8 mRNA level was 7.7-fold higher than the basal value. Accumulation of IL-8 mRNA peaked after 6 h (25.7-fold increase) and thereafter

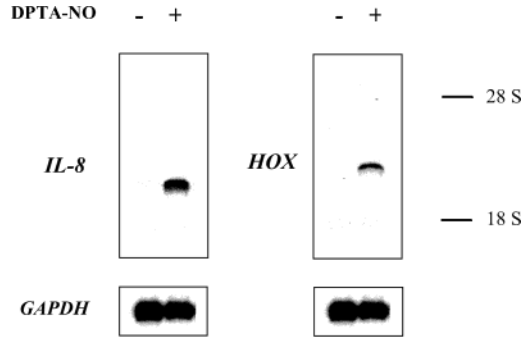

FIGURE 2: Level of IL-8 and HOX mRNA by DPTA-NO in the human monocytic U937 cell line. Cells were treated with 0.5 mM DPTA-NO for 4 h. Total RNA (10 μg/lane) was extracted from DPTA-NO-exposed and control cells and analyzed by Northern blot. Radiolabeled specific cDNA was used as a probe for IL-8 and HOX mRNA (top panel) and for GAPDH mRNA (bottom panel). The positions of 28S and 18S rRNAs are marked on the right. The results are representative of at least three independent experiments.

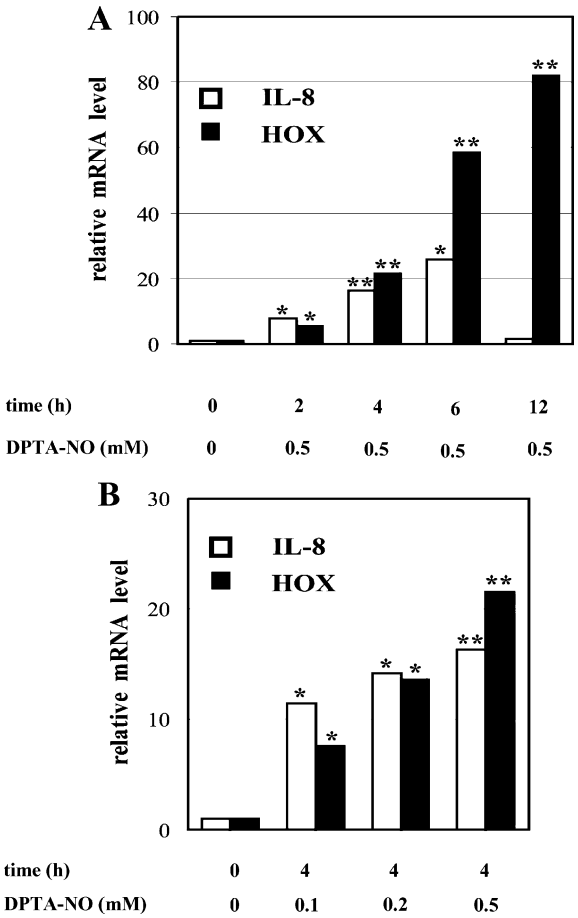

FIGURE 3: Time course (A) and concentration dependence (B) of IL-8 and HOX mRNA accumulation in U937 cells treated with DPTA-NO. Cells were incubated for 4 h in the presence or absence of the indicated amounts of DPTA-NO. Total RNA (10 μg/lane) was extracted and assayed by Northern blotting. Hybridization signals were quantified with a PhosphorImager. Data were normalized to corresponding GAPDH mRNA levels and represent the mean of at least three independent experiments [*, $p < 0.05$; **, $p < 0.005$ vs control (no DPTA-NO)]. The basal content of IL-8 and HOX mRNA in control cells was assigned a value of 1.

dropped, returning to the basal level 12 h after DPTA-NO addition in contrast to the HOX mRNA level, which continued to rise 12 h after exposure to DPTA-NO. Dose−effect experiments showed that DPTA-NO was more effec-

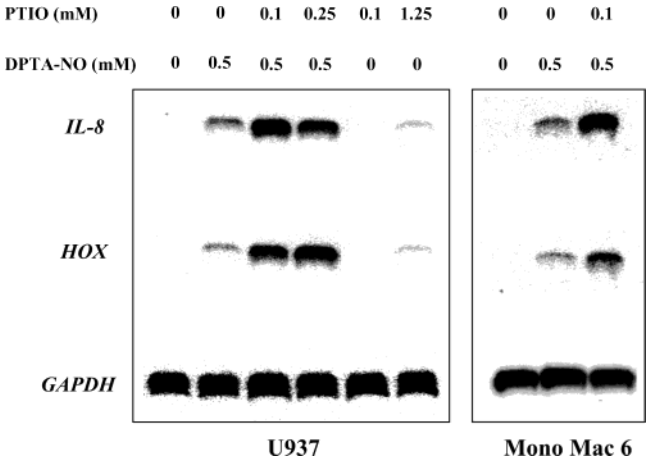

FIGURE 4: Effect of PTIO on the NO-induced expression of IL-8 and HOX mRNA. U937 or Mono Mac 6 cells were cultured for 4 h in the presence or absence of 0.5 mM DPTA-NO with and without PTIO as indicated. Total RNA (10 μg/lane) was extracted and analyzed by Northern blot as described in the legend to Figure 2. The results are representative of at least three independent experiments.

tive in inducing IL-8 and HOX genes when used at 0.5 mM than at 0.1 or 0.2 mM (Figure 3B). Subsequent experiments were therefore performed using 0.5 mM DPTA-NO.

On the basis of the experiments described above and on previous data *(19*, *21*, *24*), we took advantage of the capacity of the NO scavenger PTIO to oxidize NO in N_2O_3 and NO_2, to analyze the role of various higher oxides of NO in the regulation of NO-dependent genes. By oxidizing NO to NO_2, suboptimal concentrations of PTIO mimic the reaction between NO and molecular oxygen (*19*). As shown in Figure 4, PTIO addition at concentrations of 0.1 or 0.25 mM caused a significant increase in the expression of IL-8 and HOX genes as compared to that achieved in cells incubated with DPTA-NO alone. PTIO-mediated upregulation of these two genes occurred to about the same extent in U937 and Mono Mac 6 cell lines (not shown). In control experiments, U937 cell treatment with PTIO alone caused either negligible (at the concentration of 0.1 mM) or slight (at the concentration of 1.2 mM) upregulation of the mRNA levels of the genes studied (Figure 4). Moreover, PTI, a reaction end product of PTIO and NO, had no significant boosting effect on HOX mRNA level when added to U937 cells along with DPTA-NO (not shown).

The concentration of PTIO required to increase mRNA level was different for IL-8 and HOX genes. U937 cells treated with 0.5 mM DPTA-NO for 4 h exhibited the highest level of IL-8 mRNA at a PTIO concentration of 0.1 mM (Figure 5A). In contrast, the dependence of HOX gene expression on PTIO concentration seemed to be biphasic. The first rise of HOX mRNA level was achieved at a PTIO concentration of 0.1 mM (Figure 5B). Then, within the range of 0.1−0.5 mM PTIO, the further increase in HOX mRNA level was modest, whereas at concentrations between 0.5 and 1 mM a second rise was manifest, reaching a 10-fold increase.

As shown in Figure 6, the boosting effect of 0.25 mM PTIO on IL-8 gene expression was rather weak after 4 h incubation but reached 8-fold after 6 h. With regard to HOX, 6-fold and 9-fold increases in HOX mRNA level due to PTIO were reached after 4 and 6 h, respectively. We also treated

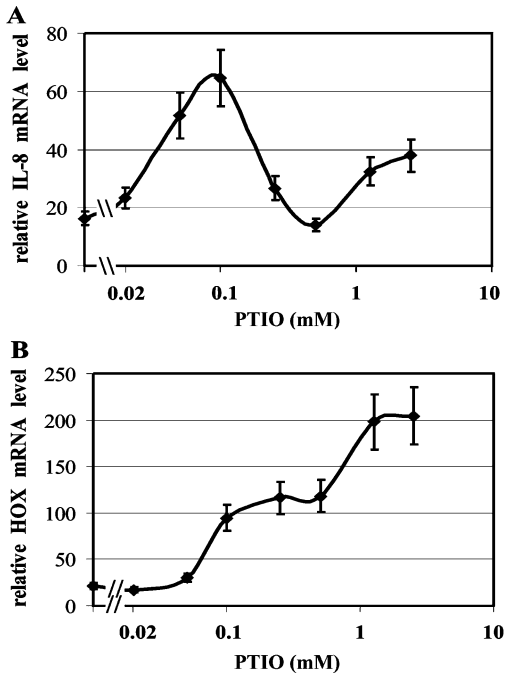

FIGURE 5: Concentration dependence of IL-8 and HOX mRNA accumulation in U937 treated with PTIO at various concentrations. Cells were incubated for 4 h in the presence of 0.5 mM DPTA-NO with or without PTIO as indicated. Total RNA (10 μg/lane) was extracted and analyzed by Northern blotting for IL-8 mRNA (A) or HOX mRNA (B) expression. Hybridization signals were quantified with a PhosphorImager. Data were normalized to GAPDH mRNA levels and represent the mean of at least three independent experiments ± SD. The basal content of IL-8 and HOX mRNAs in control cells was assigned a value of 1.

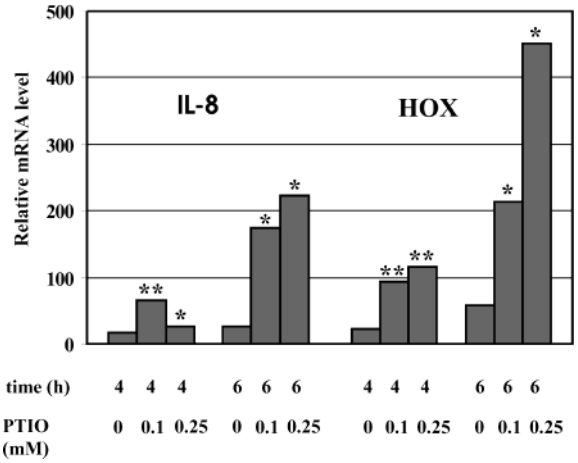

FIGURE 6: Time course and concentration dependence of IL-8 and HOX mRNA accumulation in U937 cells treated with DPTA-NO in the presence of PTIO. Cells were incubated for 4 or 6 h with 0.5 mM DPTA-NO in the presence or absence of the indicated amounts of PTIO. Total RNA was extracted and analyzed by Northern blotting as described in the legend to Figure 5. Data represent the mean of at least two independent experiments [*, $p < 0.05$; **, $p < 0.005$ vs control (no PTIO)]. The content of IL-8 and HOX mRNA in cells untreated with PTIO was assigned a value of 1.

cells with DPTA-NO alone or with PTIO or in the presence of reduced glutathione (GSH), a physiological thiol compound known as a potent RNI scavenger and herein a nitrosation suppressor.

As shown in Figure 7, 10 mM GSH completely inhibited the expression of the HOX gene by DPTA-NO alone

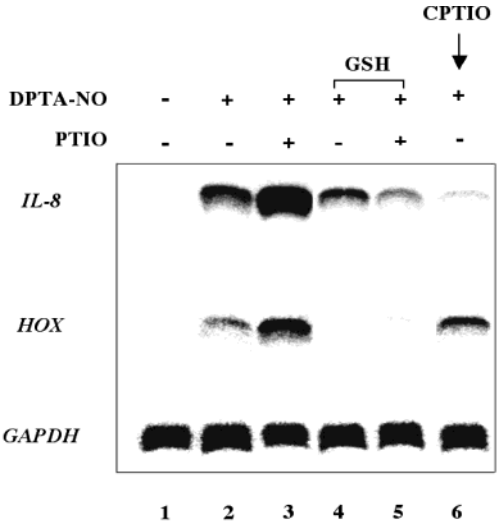

FIGURE 7: Effect of PTIO, GSH, or CPTIO on the NO-induced IL-8 and HOX mRNA expression in U937 cells. Cells were cultured for 4 h with 0.5 mM DPTA-NO either with or without CPTIO (0.25 mM), PTIO (0.1 mM), or GSH (10 mM). Total RNA (10 μg/lane) was extracted and assayed by Northern blotting. Radiolabeled specific cDNA was used as a probe for IL-8 and HOX mRNA (top panel) and for GAPDH (bottom panel). The results are representative of at least three independent experiments.

(compare lane 4 to lane 2) and reduced it to a large extent when PTIO was added along with DPTA-NO (compare lane 5 to lane 3). With regard to IL-8, GSH was far more efficient at inhibiting gene expression when PTIO was added with DPTA-NO (lane 5). In contrast to PTIO, its water-soluble analogue CPTIO, at the concentration of 0.25 mM, significantly suppressed NO-dependent IL-8 expression and only slightly (about 1.5-fold) stimulated HOX expression (Figure 7).

Sensitivity to PTIO of Several Genes in U937 and Mono Mac 6 Cells. To extend our study beyond IL-8 and HOX genes, we also examined the responsiveness of several other NO-dependent genes, namely, c-jun, c-fos, MIP-1α, TNF-α, and p21/WAF1, to PTIO. Again, the expression of these genes exhibited distinct sensitivity to PTIO. NO-dependent expression of TNF-α was upregulated (about two to three times) by PTIO in both cell lines. In U937 cells, NO-dependent expression of p21/WAF1 gene was also increased (about three times) in the presence of PTIO (Figure 8). Although DPTA-NO-mediated alteration was slightly more potent for IL-8 and HOX genes than for TNF-α and p21/WAF1 genes, in general, the additional stimulatory effect of 0.1 mM PTIO was similar for all of these genes, thus arguing for a common upstream regulating step. In contrast, the rate of the NO-dependent expression of MIP-1α, c-jun, and c-fos genes was not changed by the presence of 0.1 mM PTIO in U937 cells and was even reduced in Mono Mac 6 cells (Figure 8).

DISCUSSION

Although NO itself is able to interact mostly with other radicals and metalloproteins, higher oxides of NO, often referred to as reactive nitrogen intermediates (RNIs), influence a broader range of intracellular compounds. In this study, we investigated the gene response of monocytic cells to N_2O_3-mediated nitrosation, and to this end, we combined

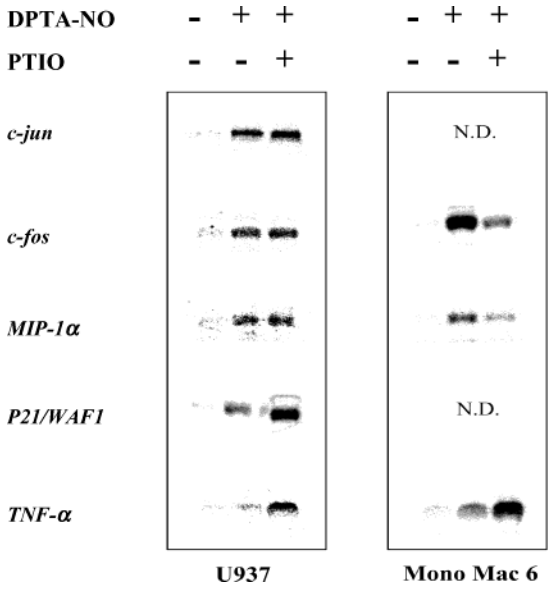

FIGURE 8: Effect of PTIO on NO-stimulated gene expression. U937 or Mono Mac 6 cells were incubated for 4 h in the presence or absence of 0.5 mM DPTA-NO with and without 0.1 mM PTIO. Total RNA (10 μg/lane) was extracted from DPTA-NO-exposed and control cells and analyzed by Northern blot. ^{32}P-Labeled specific cDNAs were used as probes for MIP-1α c-jun, c-fos, TNF-α, and p21/WAF1 mRNA, (top panel). Equal RNA loading and transfer efficiency were verified by hybridization with radiolabeled cDNA probe specific for GAPDH mRNA. The results are representative of at least two independent experiments. N.D. = not determined.

DPTA-NO, a synthetic NO donor, and PTIO. To analyze gene expression, we performed Northern blot analyses, and accordingly, transcriptional alteration was not distinguished from mRNA turnover. It is worth noting that previous reports indicate that NO-dependent upregulation of the expression of IL-8 and HOX genes is mediated both by transcriptional activation and by mRNA stabilization (*22*, *25*). The main RNI targets are reactive thiol-containing peptides and proteins, i.e., cysteine residues located in the vicinity of basic and acidic amino acids (*3*, *26*, *27*). Two types of thiol modifications on NO-sensitive sensors are of prime importance to trigger subsequent intracellular signaling pathways: (1) the reaction of sulfhydryl groups with nitrogen dioxide NO_2 or its highly reactive dimer dinitrogen tetraoxide N_2O_4, which leads to their oxidation, and (2) the reaction with nitrous anhydride N_2O_3, which generates *S*-nitrosothiols.

$$NO_2 + RSH \rightarrow NO_2^- + RS\cdot \quad (4)$$

$$RS\cdot + RS\cdot \rightarrow RSSR \quad (5)$$

$$N_2O_3 + RSH \rightarrow NO_2^- + RSNO \quad (6)$$

Consequently, according to reactions 3 and 6, the excess of NO over NO_2 is a requirement for RSNO synthesis. In our experiments, this requirement was fulfilled when cells were treated with low concentrations of PTIO. The data presented here indicate that strengthening of NO oxidation by optimal concentrations of PTIO leads to a marked increase in the expression levels of IL-8, HOX, and some other NO-sensitive genes. It is worth noticing that optimal PTIO:DPTA-NO ratios required for maximal N_2O_3 synthesis (evaluated by the rate of DAF nitrosation) and for high expression of IL-8 gene were similar. This is a strong

argument in favor of the role of N_2O_3 in IL-8 gene expression. Meanwhile, maximal activation of HOX gene expression required higher concentrations of PTIO, suggesting that both products generated from the reaction between NO and PTIO, i.e., N_2O_3 and NO_2, are implicated. The demonstration that nitrosating species are involved in IL-8 and HOX gene regulation was strengthened by further experiments in which cells were exposed to both DPTA-NO and PTIO in the presence of the RNI quencher GSH. The presence of GSH in culture medium caused complete inhibition of HOX gene expression and significant reduction of PTIO-mediated increase in IL-8 gene expression (Figure 7).

Release of NO from diazenium diolates is predictable. On the basis of absorbance of DPTA-NO at 250 nm, we observed that the rate of NO generation was linearly dependent on DPTA-NO concentration and declined in culture medium down to 40% of the initial level after 4 h incubation. In long-lasting cell treatments, the rate of NO generation and its scavenging by PTIO are constantly changing. In fact, the rate of NO_2 synthesis and in turn the NO_2:NO ratio are mainly dependent on the initial level of PTIO. As shown in Figure 6, when cells were incubated with 0.5 mM DPTA-NO in the presence of 0.25 mM PTIO for 4 h, the boosting factor with regard to IL-8 gene expression was barely higher than 1 after 4 h, but strikingly, it exceeded 8 after 6 h. At first glance, such a sharp increase is surprising. However, it is consistent with our experiments showing that PTIO-mediated DAF nitrosation occurred at a PTIO:DPTA-NO molar ratio of about 1:10. As 1 mol DPTA-NO releases 2 mol of NO and since the expected amount of PTIO consumed is equimolar, PTIO decays twice as fast as DPTA-NO (reaction 2). We therefore assume that PTIO concentration had to be reduced below a certain threshold after 4 h to yield optimal N_2O_3 concentrations and boost IL-8 gene expression. In brief, it is likely that a low rate of NO_2 generation is a requirement for N_2O_3 synthesis following reaction between NO_2 and NO (reaction 3) and for subsequent nitrosation of thiol residues by N_2O_3 (reaction 6). On the contrary, higher PTIO concentrations (>0.5 mM) are favorable for higher NO_2 output and subsequent oxidation of sensitive SH groups and nitration of tyrosine residues (*19*, *21*).

It has been reported that, in different cell types, S-nitrosation of thiol groups and tyrosine nitration mediates NO-dependent control of G protein $p21^{Ras}$, Src family nonreceptor protein tyrosine kinases, and several ion channels (*12*, *13*). It is striking that all of these key regulatory systems are associated with the plasma membrane. This is consistent with the view that NO oxidation and RNI generation are about 10^3 times faster in biological membranes than in aqueous solutions since both NO and O_2 are lipophilic molecules and concentrate in the lipid phase of membranes (*11*, *19*). As mentioned above, concentration factor is very important for determining the rate of RNI synthesis in reactions involving NO oxidation. It is generally held that N_2O_3 formation is a prerequisite for the generation of *S*-nitroso proteins, e.g., the transcription factor HIF-1α (*28*). Moreover, it was previously shown that PTIO stimulates NO-dependent S-nitrosation of a critical cysteine residue of ornithine decarboxylase via N_2O_3 (*25*). Similarly, stimulation of NO oxidation by CPTIO leads to activation of purified matrix metalloproteinase 8 (neutrophil procollagenase) (*24*). In this report, we extend these data and suggest that the incubation of intact cells in the presence of PTIO, by increasing NO_2 and N_2O_3 generation, causes a subsequent increase in nitrosation of plasma membrane-associated gene regulators. Since PTIO is a membrane-impermeable compound, a reasonable assumption is that the RNIs produced were able to penetrate the plasma membrane and react with local sensors. Some genes examined in this study, such as genes encoding the transcription factors c-Jun and c-Fos as well as the cytokine MIP-1α, appear to be insensitive to PTIO superactivation (Figure 8). It is widely accepted that expression of the NO-sensitive genes is controlled by different intracellular signaling pathways (*4*). We thus presume either that the upstream regulators which trigger the expression of PTIO-resistant genes are too far from the plasma membrane, being therefore inaccessible to RNIs generated by the reaction with PTIO, or that S-nitrosation of these sensors is not required for their functional activation.

Overall, PTIO effects were similar for both monocytic cell lines studied. On a functional level, enhanced S-nitrosation mimicked here by PTIO seems to lead to a general increase in monocytic cell proinflammatory and cytostatic responses. Thus, TNF-α is a member of the inflammatory cytokine family, and IL-8 is a chemokine with the properties of neutrophil activator, regulator of leukocyte survival, and mediator of angiogenesis (*29*−*31*). The primary function of HOX is to catalyze the oxidative degradation of heme to generate equimolar amounts of biliverdin, carbon monoxide, and iron. Since biliverdin is an antioxidant whereas heme is a powerful pro-oxidant, HOX upregulation mediates cellular protection against oxidative damage (*32*). The cyclin-dependent protein kinase inhibitor p21/WAF1 fulfills a crucial role in cell cycle regulation. Expression of the p21/WAF1 gene is under the control of the transcription factor p53, integrating multiple signals including NO-dependent stimuli, into growth arrest and pro-apoptotic signaling that ultimately determine cell fate (*33*−*35*). In contrast, PTIO treatment did not influence the NO-dependent expression of c-jun and c-fos genes that are involved in cell cycle progression.

In conclusion, speeding up NO oxidation by PTIO has allowed us to discriminate between different genes in relation to their responsiveness to RNI, most likely N_2O_3. This approach supports the idea that the chemical nature of the NO-derived species, which are generated under aerobic conditions, determines the "wake-up call" sent to specific sensor proteins. Lastly, at a more global level, this method may prove useful in identifying, sorting, and localizing master controllers of NO-sensitive genes.

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