Nitric Oxide Increases Tumor Necrosis Factor Production in Differentiated U937 Cells by Decreasing Cyclic AMP*

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Nitric oxide (NO) increases tumor necrosis factor (TNF) synthesis in human peripheral blood mononuclear cells by a cGMP-independent mechanism. NO has been shown to inhibit adenylate cyclase in cell membranes. Since cAMP down-regulates TNF transcription, we examined the possibility that NO enhances TNF synthesis by decreasing cAMP. U937 cells were induced to differentiate using phorbol myristate acetate (100 nM for 48 h) and then were incubated for 24 h with sodium nitroprusside (SNP) or S-nitroso-N-acetylpenicillamine (SNAP). These NO donors increased TNF production (7.0- and 15.6-fold, respectively, at 500 μM) in a dose-dependent manner (p = 0.002). However, SNP and SNAP did not elevate cGMP levels in U937 cell cultures, and the cGMP analog, 8-bromo-cGMP, had no effect on TNF production. In contrast, SNP (p = 0.001) and SNAP (p = 0.009) decreased intracellular cAMP levels by up to 51.5% over 24 h and, in the presence of a phosphodiesterase inhibitor, blunted isoproterenol-stimulated increases in cAMP by 21.8% (p = 0.004) and 27.6% (p = 0.008), respectively. H89, an inhibitor of cAMP-dependent protein kinase, dose dependently increased TNF production in phorbol myristate acetate-differentiated U937 cells in the absence (6.5-fold at 30 μM; p = 0.035), but not in the presence (p = 0.77) of SNAP. Conversely, the cAMP analog dibutylryl cAMP (Bt2cAMP) blocked SNAP-induced TNF production (p = 0.001). SNP and SNAP (500 μM) increased relative TNF mRNA levels by 57.5% (p = 0.002) and 66.2% (p = 0.001), respectively. This effect was prevented by Bt2cAMP. These results indicate that NO up-regulates TNF production by decreasing intracellular cAMP.

Nitric oxide (NO) is a free-radical gas produced by many cell types (1–4). NO has a diverse repertoire of important functions (5–9) including neurotransmission (5, 10), vasodilatation (11), antiplatelet activity, and immune modulation (12–14). Most of these effects are mediated through a unique cGMP signaling pathway. NO covalently attacks the heme moiety of soluble guanylate cyclase, activating the enzyme, and thereby elevating intracellular cGMP concentrations (15–17). This increase in cGMP subsequently activates certain protein kinases, which phosphorylate target proteins involved in regulation of cell function (17–19). Although the role of cGMP as a NO second messenger is undisputed, some findings have led to speculation about the existence of cGMP-independent signal transduction pathways for NO.

First, NO is a free radical with the ability to react with a variety of enzymes besides soluble guanylate cyclase. NO has been shown to catalyze the covalent binding of NAD to glycer-aldehyde-3-phosphate dehydrogenase (20), oxidize iron-containing proteins such as aconitase or ribonucleotide reductase (21–23), and nitrosylate tyrosine and cysteine residues in a variety of proteins (24–26). Second, some effects of NO cannot be reproduced with cell permeable cGMP analogs. For example, the synthesis of tumor necrosis factor α (TNFα), a proinflammatory cytokine implicated in tissue injury and shock (27), is increased in human peripheral blood mononuclear cells (28) and lipopolysaccharide-stimulated neutrophil preparations (29) by exogenous NO. Although NO increases cGMP concentrations in these cells, cGMP analogs have no effect on TNFα production (28, 29). Collectively, these investigations suggest that NO might use cGMP-independent signaling pathways for some of its cellular functions.

Recently, adenylate cyclase has been added to the list of enzymes that can be modified by NO (30). Treatment of cell membranes with NO decreases cAMP production by inhibiting calmodulin activation of type I adenylate cyclase, presumably through thiol nitrosylation at the calmodulin-binding site (30, 31). Notably, increases in cAMP in leukocytes activate cAMP-dependent protein kinase (PKA). This kinase phosphorylates transcription factors that bind to the cAMP-response element on the TNFα promoter, thereby inhibiting TNFα mRNA transcription (32–35). The effect of NO on type I adenylate cyclase suggests that NO might up-regulate TNFα synthesis in human monocytes by decreasing cAMP concentrations.

We investigated this question using U937 cells, a human monocytic cell line that differentiates into monocyte-macrophase-like cells and produces TNFα when exposed to phorbol myristate acetate (PMA) (36–38). The specific objectives were as follows: 1) to demonstrate that NO up-regulates TNFα production in PMA-differentiated U937 cells and test the cGMP-dependence of this effect; 2) to determine whether NO alters resting or stimulated cAMP concentrations in intact cells; 3) to investigate the effect of inhibitors or activators of PKA on NO-stimulated TNFα production in this system; and 4) to determine if NO-induced changes in TNFα mRNA levels were consistent with a cAMP mechanism.

EXPERIMENTAL PROCEDURES

Reagents and Cells—PMA, S-nitroso-N-acetylpenicillamine (SNAP), 8-bromo-cGMP, dibutylryl cGMP (Bt2cGMP), dibutylryl cAMP

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(Bt$_2$cAMP), H89, and isoproterenol were all purchased from Calbiochem (San Diego, CA). Sodium nitroprusside (SNP) was from Elkins-Sinn (Cherry Hill, NJ). Ficoll-Hypaque, prostaglandin E$_2$ (PGE$_2$), and 3-isobutyl-1-methyl-xanthine (IBMX) were all obtained from Sigma. Dextran was from Amersham Life Science Inc. TRI REAGENT-LS used for RNA isolation was from Molecular Research Center, Inc. (Cincinnati, OH).

Human neutrophils were isolated from the venous blood of normal volunteers by dextran sedimentation and Ficoll-Hypaque density centrifugation as described previously (39). U937 cells obtained from ATCC (Rockville, MD) were cultured in RPMI 1640 supplemented with 25 mM HEPES, 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2% (w/v) L-glutamine (all from Biofluids, Inc., Rockville, MD). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO$_2$. Differentiation was induced by incubating U937 cells with PMA (100 nM) for 48 h. The cells were then washed three times with Hank’s balanced salt solution without Ca$^{2+}$ and Mg$^{2+}$ (HBSS$-$) to remove residual PMA.

Measurement of TNFα Production—Differentiated U937 cells (5 × 10$^5$ cells/ml) were incubated with each of the following reagents for 24 h: increasing concentrations of SNP or SNAP (0–500 µM); 8-bromo-cGMP or Bt$_2$cGMP (0–1000 µM); or Bt$_2$cAMP (0–100 µM) in the presence of varying doses of SNAP (0–500 µM). In another experiment, differentiated U937 cells were pretreated for 6 h with varying concentrations of H89 (0–30 µM), a cell-permeable PKA inhibitor. The cells (5 × 10$^5$ cells/ml) were then washed three times with HBSS$-$ and incubated for an additional 24 h in the absence or presence of SNAP (500 µM). TNFα release into the medium was measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). All reagents were dissolved into RPMI 1640, and tested negative for endotoxin using a limulus amoebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD).

Determination of Total cGMP—At a concentration of 4 × 10$^6$ cells/ml, differentiated U937 cells or freshly isolated human neutrophils were preincubated for 15 min in a shaking water bath at 37 °C with Hank’s balanced salt solution with Ca$^{2+}$ and Mg$^{2+}$ (HBSS$+$) containing 1 mM

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**FIG. 1.** Effect of NO on TNFα production. PMA-differentiated U937 cells were incubated with the indicated concentrations of SNP and SNAP for 24 h. TNFα release into the medium is presented as the mean ± S.E. of four independent experiments, each run in duplicate.

**FIG. 2.** Effect of NO on cGMP production. Human neutrophils and PMA-differentiated U937 cells were preincubated with IBMX (1 mM) for 15 min at 37 °C. Subsequently, the indicated concentrations of SNP or SNAP were added, and the cells were incubated for 2 h. Inset, the effect of SNAP (1 mM) on cGMP at earlier time points. Total cGMP is presented as the mean ± S.E. of three independent experiments, each run in duplicate.
after the addition of SNP or SNAP (0–1000 μM), these cells were incubated for 0–120 min followed by the addition of iced ethanol (65% (v/v) final concentration). The amount of cGMP present in the samples was quantitated by an enzyme immunoassay according to the manufacturer's protocol (Amersham).

**Determination of cAMP**—Differentiated U937 cells (5 × 10^6 cells/ml) were incubated in RPMI 1640 containing increasing doses of SNP or SNAP (0–500 μM) for 24 h. The cells were then scraped and spun for collection of cell pellets. Intracellular cAMP was extracted by lysing the cell pellets in 65% iced ethanol and centrifuging at 2000 × g for 15 min. The resultant supernatants were dried and cAMP was quantitated using an enzyme immunoassay according to the manufacturer's protocol (Amersham).

To investigate the effect of NO on agonist-stimulated cAMP responses, differentiated U937 cells (5 × 10^6 cells/ml) were pretreated for 15 min with HBSS− containing 500 μM SNP or SNAP in the presence of IBMX (1 μM). Adenylate cyclase agonists, isoproterenol (1 μM), or PGE₂ (10 μM) were then added and total cAMP was extracted at several time points (0–15 min) and quantitated as described above.

**Ribonuclease Protection Assay (RPA)**—Differentiated U937 cells (5 × 10^6 cells/ml) were incubated under the following conditions for 3.5 h: RPMI 1640 alone or with 8-bromo-cGMP (100 μM), Bt₂cAMP (100 μM), SNP (500 μM), SNAP (500 μM), or Bt₂cAMP (100 μM) and SNAP (500 μM). RNA was isolated using TRI REAGENT-LS. The entire open reading frame of human TNFα cDNA, in the PAW711 plasmid, was kindly provided by Dr. Alice M. Wang (40). A 253-base pair TNFα gene fragment, obtained by digestion with the enzymes AvaI and HincII (Stratagene, La Jolla, CA), was subcloned into the pGEM3Z vector (Promega, Madison, WI). The recombinant pGEM3Z plasmid was subsequently sequenced to confirm the presence of the TNFα fragment insert. Single-strand antisense mRNA probe labeled with 35S was prepared by in vitro transcription using SP6 polymerase. RPA was performed with a RPA II kit (Ambion Inc., Austin, TX) using 50 μg of total RNA according to the manufacturer's instructions. Results obtained were expressed as percentages of the concurrently run β-actin controls.

**Statistics**—All data are expressed as mean ± S.E. All p values are two-sided. To analyze dose-response curves in each experiment, the data was first summarized using the nonparametric Sen-Theile estimate of regression slope (41). From these dose-response slope estimates, one sample t tests (against the hypothesis of H₀: slope = 0) were done to determine whether the observed dose-response was significant. To determine if the TNFα response to doses of SNAP was monotonically decreasing as the Bt₂cAMP dose increased, in addition to using the same technique as described above, we also used Page's nonparametric test (42) for ordered alternatives in a 2-way ANOVA layout (the first factor was the increasing doses of Bt₂cAMP, the second factor was experiment). To evaluate the effects of NO on the isoproterenol-induced cAMP response in PM-differentiated U937 cells, the area under the curve was determined for each condition/experiment. Then, nominal p values were computed for each of the two comparisons of interest: isoproterenol versus isoproterenol/SNP, and isoproterenol versus isoproterenol/SNP. Last, these nominal p values were adjusted by multiplying each by 2, to take into account the multiple comparisons (Bonferroni adjustment) (43). When comparing relative TNFα mRNA levels, we used paired t tests.

**RESULTS**

**Effect of NO on TNFα Production by PM-differentiated U937 Cells**—First, we confirmed previous reports (37, 38) that PM-differentiated U937 cells produce TNFα (Fig. 1). Next, we demonstrated that exogenous NO donors, SNP or SNAP, increased TNFα release from PM-differentiated U937 cells over a 24-h incubation period in a dose-dependent manner (p = 0.002 for both). At the highest concentrations examined (500 μM), SNP and SNAP increased TNFα production 7.0- and 15.6-fold, respectively (Fig. 1).

**Effect of NO on Total cGMP in U937 Cell Cultures**—Production of cGMP by PM-differentiated U937 cells in response to

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**Fig. 3. Effect of cGMP analogs on TNFα production.** PMA-differentiated U937 cells were incubated with the indicated concentrations of 8-bromo-cGMP (A) or Bt₂cGMP (B) for 24 h in separate experiments. TNFα released into the medium is presented as the mean ± S.E. of three independent experiments, each run in duplicate.

**Fig. 4. Effect of NO on intracellular cAMP concentrations.** PMA-differentiated U937 cells were incubated with the indicated concentrations of SNP or SNAP for 24 h. Intracellular cAMP is presented as the mean ± S.E. of seven independent experiments, each run in duplicate.
exogenous NO donors was assessed using human neutrophils as a positive control (Fig. 2). After 2 h incubation in the presence of IBMX (1 mM), neither SNP nor SNAP elevated total cGMP in U937 cells ($p > 0.1$). In contrast, and as expected (28), either SNP or SNAP increased total cGMP production by human neutrophils in a dose-dependent manner ($p = 0.012$ and $p = 0.004$, respectively).

To exclude the possibility that cGMP was quickly degraded despite the presence of a phosphodiesterase (PDE) inhibitor, thereby masking an increase in cGMP production, total cGMP was also measured at earlier time points (0, 1, 3, 5, 10, and 15 min) in the presence of IBMX (1 mM) after exposure to SNAP (1 mM). SNAP increased total cGMP in neutrophils which reached a maximum at 15 min. However, this NO donor had no effect on cGMP production in PMA-differentiated U937 cells (Fig. 2, inset). Furthermore, in separate experiments, SNP or SNAP had no effect on total cGMP in naive U937 cells, or in U937 cells differentiated with retinoic acid with or without 1α,25-dihydroxyvitamin D$_3$ (data not shown).

Effect of cGMP Analogs on TNFα Production by PMA-differentiated U937 Cells—Cell-permeable analogs of cGMP, 8-bromo-cGMP, or Bt$_2$cGMP were incubated with PMA-differentiated U937 cells for 24 h. The release of TNFα into the medium was not altered by 0–1 mM of either 8-bromo-cGMP (Fig. 3A, $p = 0.33$) or Bt$_2$cGMP (Fig. 3B, $p = 0.66$).

Effect of NO on cAMP Levels in PMA-differentiated U937 Cells—To determine if NO altered cAMP levels, intact PMA-differentiated U937 cells were incubated in the presence of increasing concentrations of SNP or SNAP for 24 h. Either SNP or SNAP decreased intracellular cAMP levels (Fig. 4) in PMA-differentiated U937 cells in a dose-dependent manner ($p = 0.001$ and $p = 0.009$, respectively). Compared with culture medium alone, SNP or SNAP at the highest concentrations examined (500 μM) decreased intracellular cAMP by 37.8 and 51.5%, respectively.

To confirm that NO decreases cAMP levels in PMA-differentiated U937 cells, the effect of NO on agonist-stimulated cAMP responses was examined in the presence of IBMX (1 mM). As shown in Fig. 5A, SNP or SNAP (500 μM) blunted isoproterenol-stimulated increases in total cAMP by 21.8% ($p = 0.004$) and 27.6% ($p = 0.008$), respectively. Furthermore, the inhibitory effects of SNP ($p = 0.045$) or SNAP ($p = 0.011$) were also demonstrated using PGE$_2$ (10 μM) instead of isoproterenol to activate adenylate cyclase (Fig. 5B). This experiment was performed in HBSS to avoid activation of Ca$^{2+}$/calmodulin-dependent PDE I (44, 45).

Effect of Inhibitors or Activators of PKA on TNFα Production by PMA-differentiated U937 Cells—Finding that NO but not cGMP increased TNFα production, and that NO decreased intracellular cAMP levels, we next examined the effect of cell-permeable agents that either inhibit (H89) or activate (Bt$_2$cAMP) PKA on TNFα production. Preincubation of PMA-differentiated U937 cells with H89 increased TNFα production (6.5-fold at 30 μM) in a dose-dependent manner (Fig. 6, $p = 0.035$). The addition of SNAP elevated TNFα production and eliminated the dose effect of H89 ($p = 0.77$). Conversely,
Fig. 6. Effect of H89, a PKA inhibitor, on TNFα production. PMA-differentiated U937 cells were pretreated for 6 h with H89, washed with HBSS, and then incubated for an additional 24 h with or without SNAP (500 μM). TNFα released into the medium is presented as the mean ± S.E. of three independent experiments, each run in duplicate.

Fig. 7. Effect of Bt2cAMP on NO-induced TNFα production. PMA-differentiated U937 cells were incubated with 0–100 μM Bt2cAMP in the presence of the indicated concentrations of SNAP for 24 h. TNFα released into the medium is presented as the mean ± S.E. of three independent experiments, each run in duplicate.

Bt2cAMP blocked SNAP-induced TNFα production (Fig. 7). With increasing concentrations of Bt2cAMP (0–100 μM), the dose-dependent effect of SNAP on TNFα production was abolished (p = 0.001). Cell viability by trypan blue exclusion was not decreased at the concentrations of Bt2cAMP employed (0–100 μM, data not shown).

Effect of NO and Cyclic Nucleotide Analogs on TNFα mRNA Levels in PMA-differentiated U937 Cells—Relative TNFα mRNA levels were measured using a RPA to investigate the effects of NO, cGMP, and cAMP on TNFα mRNA transcription (Fig. 8). SNP or SNAP increased TNFα mRNA levels by 57.3% (p = 0.045) and 66.2% (p = 0.001), respectively. An analog of cAMP, Bt2cAMP, decreased TNFα mRNA levels (p = 0.002), and prevented the effect of SNAP (Bt2cAMP versus Bt2cAMP and SNAP; p = 0.93). In contrast to the NO donors, 8-bromo-cGMP had no effect on TNFα mRNA levels (p = 0.52).

DISCUSSION

We demonstrated that NO increased TNFα production in PMA-differentiated U937 cells by decreasing intracellular cAMP levels, indicating that NO uses cAMP, rather than cGMP, as a second messenger for some of its cellular effects. This conclusion is based on these findings: 1) two structurally dissimilar NO donors increased TNFα production in a dose-dependent manner; 2) both SNP and SNAP increased cGMP concentrations in human neutrophil cultures, but had no effect on cGMP concentrations in PMA-differentiated U937 cell cultures; 3) cell-permeable analogs of cGMP, 8-bromo-cGMP and Bt2cGMP, did not alter TNFα production by PMA-differentiated U937 cells; 4) SNP or SNAP not only decreased intracellular cAMP in a dose-dependent manner, but also blunted isoproterenol- and PGE2-stimulated cAMP responses in PMA-differentiated U937 cells; 5) an inhibitor of PKA, H89, increased TNFα release in the absence but not in the presence of SNAP; 6) conversely, an activator of PKA, Bt2cAMP, abolished the effect of SNAP on TNFα production; and 7) finally, NO donors and Bt2cAMP but not 8-bromo-cGMP caused changes in relative TNFα mRNA levels that were consistent with a cAMP mechanism for the observed effects of NO. Collectively, these experiments demonstrate that NO-induced up-regulation of TNFα production in this human cell line uses cAMP, not cGMP, as its second messenger.

Many of the known effects of NO have been attributed to its ability to generate cGMP through its action on soluble guanylate cyclase (15–18). However, we were unable to demonstrate that NO donors increase cGMP in either naive or differentiated U937 cells. It seems unlikely that our inability to detect NO-stimulated increases in cGMP was due to degradation of cGMP. U937 cells have extremely low cGMP hydrolytic activity and do not contain the cGMP-specific PDE isoenzyme (PDE V) (45, 46). Furthermore, our experiments were conducted in the presence of a potent, nonselective PDE inhibitor. These data indicate that U937 cells lack NO-sensitive soluble guanylate cyclase. Moreover, membrane permeable cGMP analogs, 8-bromo-cGMP and Bt2cGMP, were unable to mimic the effect of NO on TNFα production, a finding that has also been reported in human peripheral blood mononuclear cells and neutrophil preparations (28, 29). These results further suggest that NO regulates TNFα production in PMA-differentiated U937 cells by a cGMP-independent mechanism.

The ability of NO to decrease intracellular cAMP levels and blunt isoproterenol- and PGE2-stimulated cAMP responses provide direct evidence for our speculation that NO increases TNFα production in PMA-differentiated U937 cells by decreasing cAMP levels. Decreases in intracellular cAMP can result either from its reduced synthesis by adenylate cyclase or from increased catabolism due to increased PDE activity (47). The result that NO decreased cAMP concentrations in the presence of IBMX, a nonspecific PDE inhibitor, support the hypothesis that changes in cAMP levels were due to decreased synthesis, rather than increased catabolism by PDE. Interestingly, cGMP can either increase cAMP hydrolysis by activating PDE II (45, 46) or decrease cAMP hydrolysis by inhibiting PDE III (48, 49). Increased or decreased cAMP hydrolysis mediated by cGMP is unlikely in our experiments since U937 cells lack PDE II ac-
tivity (45, 46), a PDE inhibitor was used, and as shown here, U937 cells do not produce cGMP in response to a NO signal. However, in other cell types that contain NO-sensitive soluble guanylate cyclase, the ability of NO to decrease cAMP production may be masked by decreased cAMP hydrolysis via cGMP-mediated inhibition of PDE III activity (48–51).

As already noted, NO has been shown to inhibit calmodulin-dependent adenylate cyclase activity in isolated cell membranes by oxidizing cysteine residues at the calmodulin-binding site (30). Other studies have shown that calcium ionophores potentiate cAMP responses in human peripheral blood mononuclear cells and neutrophils stimulated with isoproterenol and PGE₂, and this potentiation was inhibited by calmodulin inhibitors (52–54). These observations indicate that the calmodulin-dependent adenylate cyclase subtype that is inhibited by NO is present in human leukocytes. Although this suggests a possible mechanism for NO modulation of intracellular cAMP levels, other possibilities exist. Substitution of cysteine residues for other amino acids in the β,β-adrenergic receptor markedly shifts the dose-response curve to the right for isoproterenol-stimulated increases in intracellular cAMP concentrations (55). This suggests that NO could reduce agonist-stimulated cAMP responses by decreasing receptor affinity through the nitrosylation of key cysteine-containing domains.

Inhibitors and activators of PKA were used to further explore the possibility that NO was using a cAMP-dependent signaling pathway. H89, a specific cell-permeable inhibitor of PKA (56), dose-dependently increased TNFα release. This finding demonstrates in PMA-differentiated U937 cells that basal cAMP levels and the resulting degree of PKA activation are inhibitory to TNFα synthesis. Interestingly, H89, at the doses tested, did not further up-regulate SNAP-induced TNFα production, suggesting that PKA was maximally inactivated by the decrease in cAMP levels caused by NO. In contrast, Bt2cAMP only slightly suppressed basal TNFα release, but completely abolished SNAP-induced increases in TNFα production. This cAMP analog can permeate cell membranes and is resistant to hydrolysis by PDE (57), enabling it to persist in cells cultures and mimic prolonged elevations of intracellular cAMP. Toxicity caused by the butyrate moiety of the Bt2cAMP molecule was unlikely to be responsible for this effect, since the concentrations of Bt2cAMP used were relatively low and Bt2cGMP (up to 1 mM), which also contains a butyrate moiety did not alter TNFα production. Furthermore, cell viability by trypan blue exclusion was not decreased by Bt2cAMP. Together, these results reinforced our conclusion that NO increases TNFα production in PMA-differentiated U937 cells by decreasing cAMP levels.

Similarly, NO donors were also found to increase relative TNFα mRNA levels and Bt2cAMP completely prevented this effect. An analog of cGMP, 8-bromo-cGMP, had no effect. These results are consistent with a cAMP mechanism acting at the level of transcription for the observed effects of NO on TNFα production in PMA-differentiated U937 cells. Evidence is also available that cAMP can down-regulate TNFα expression at a post-transcriptional level in monocytes and macrophages (58–60).

Previously, NO was demonstrated to increase TNFα mRNA levels in HL-60 cells (61), but we found no change in TNFα mRNA levels in human neutrophil preparations (28). These inconsistent findings may be ascribed to the different methods employed to measure mRNA levels. In our present study, we measured TNFα mRNA levels using a RPA, which may be quantitatively more reliable than the reverse transcription polymerase chain reaction assay used in our previous experiments with neutrophils (28). Furthermore, differentiated U937 cells may contain more copies of TNFα mRNA than neutrophils. Besides the cAMP mechanism, our data do not exclude the possibility of additional mechanisms for the up-regulation of TNFα production by NO. For example, NO could activate or induce other transcription factors, such as NF-κB (29).

In conclusion, the present study indicates that NO increase TNFα production in PMA-differentiated U937 cells by decreasing intracellular cAMP. To our knowledge, this is the first demonstration in intact cells that NO signal transduction can use cAMP rather than cGMP to regulate cell function.

REFERENCES
1. Pollock, J. S., Förstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H. H. W., Nakane, M., and Murad, F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10480–10484
2. Safe, S. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D’Angelo, D. D., Lynch, K. R., and Peach, M. J. (1992) J. Biol. Chem. 267, 15274–15276
3. Busse, R., and Mülisch, A. (1990) FEBS Lett. 275, 87–90
4. Förstermann, U., Kleinert, H., Gath, I., Schwarz, P., Closs, E. J., and Dunn, N. J. (1995) Adv. Pharmacol. 34, 171–186
5. Brodt, D. S., Hwang, P. M., and Snyder, S. H. (1990) Nature 347, 768–770
6. Shibuki, K., and Okada, D. (1991) Nature 349, 326–328
7. Nathan, C. (1992) FASEB J. 6, 3051–3064
8. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
9. Lowenstein, C. J., and Snyder, S. S. (1992) Cell 70, 705–707
10. Garthwaite, J., Charles, S. L., and Chese-Williams, R. (1988) Nature 336, 385–388
