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Nitric Oxide Regulation of Human Peripheral Blood Mononuclear Cells: Critical Time Dependence and Selectivity for Cytokine versus Chemokine Expression

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NO is antiproliferative for T cells and other immune cells, but there is debate over whether it influences cytokine expression and if so whether it shows cytokine selectivity. Furthermore, the NO effect may depend on exposure time. To address these issues, we precultured human PBMC with the NO donors S-nitrosogluthathione (a natural storage form of NO) or S-nitroso-N-acetyl-penicillamine for up to 48 h before cell activation and then monitored proliferation and cytokine and chemokine expression. S-nitrosogluthathione or S-nitroso-N-acetyl-penicillamine, but not their non-NO-releasing analogues, inhibited proliferation induced by PHA or IL-2, the effect declining progressively from 48 to 0 h pre-exposure to the mitogen. This was accompanied by reduced PHA-induced IL-2 release and reduced IL-2, IFN-γ, and IL-13 mRNA expression. In contrast, NO did not influence PHA-induced expression of mRNA for the chemokines lymphotactin, RANTES, IFN-γ-inducible protein, macrophage-inhibitory protein-1α, macrophage-inhibitory protein-1β, macrophage chemotactic protein-1, and IL-8 or release of RANTES or IL-8. The NO effects were not toxic and were not accompanied by changes in PHA-induced CD25 expression. We conclude that exposure time to NO is critical to altered PBMC responsiveness and that NO inhibits expression of both Th1 and Th2 cytokines but not chemokines. The Journal of Immunology, 2003, 171: 4809–4815.
recruited to inflammatory sites might be exposed long term to the radical. We show that exposure time to NO is critical for regulation of PBMC proliferation and cytokine expression. Importantly, we found that NO inhibited expression of Th1 and Th2 cytokines equally but had no effect on chemokine expression.

Materials and Methods

**PBMC cultures and proliferation assays**

Human PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Lymphoprep; Invitrogen, Paisley, U.K.). The cells, >99% viable by trypan blue exclusion, were suspended at 10^6/ml in complete RPMI 1640 (Invitrogen) containing 2% TCH serum substitute (ICN, Thame, U.K.); final protein concentration, 0.65 mg/ml protein). The cells were added (100 μl/well) to 96-well round-bottom tissue culture plates (Falcon; Fred Baker, Runcorn, U.K.). As appropriate, freshly dissolved NO donors (GSNO or S-nitroso-N-acetyl-penicillamine (SNAP) (both from Calbiochem, CN Biosciences, Nottingham, U.K.) or control non-NO-releasing compounds GSH or N-acetyl-penicillamine (NAP) (both from Sigma-Aldrich, Poole, U.K.) or control medium were added (50 μl/well). NO donors and control compounds were used at a final concentration of 250 μM, which for GSNO and SNAP had been shown to be effective and non-toxic in preliminary experiments. Finally, 30 μl of either PHA (Sigma-Aldrich; final concentration, 2.5 μg/ml) or recombinant human IL-2 (PeptoTech, London, U.K.; final concentration, 5 ng/ml) were added to give a final volume of 200 μl and 10^5 cells/well. The cells were cultured at 37°C in 5% CO2. Cell proliferation was measured after 72 h of incubation with PHA or 7 days of incubation with IL-2 by cellular incorporation of [3H]Thymidine (0.5 μCi/well) added to the cells for the final 4 h of culture. The cells were harvested (TOMTEC Harvester 96; Receptor Technologies, Banbury, U.K.) onto filters and counted (20 s/well; Wallac 1450 Microbeta Plus; PerkinElmer Life Sciences, Beaconsfield, U.K.). Lactate dehydrogenase (LDH) in cell supernatants was measured by commercial assay (CytoTox 96; Promega, Southampton, U.K.).

**Production of T cell lines and proliferation assays**

Alloreactive T cells were produced by stimulating HLA-DR4+ PBMC (5 x 10^5/ml) with irradiated (5000 rad) HLA-DR4/B7-transfected L cell fibroblasts (5 x 10^5/ml) in 2 ml of complete RPMI 1640 containing 5% human serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in six-well culture plates at 37°C. IL-2 (10 U/ml; Roche Diagnostics, Lewes, U.K.) was added every 3 days. After 14 days, the cells were restimulated in the same way, and this cycle was repeated four times. The HLA-DR4+/B7-responsive T lymphocytes were expanded further by restimulation (1:5) with irradiated (5000 rad) HLA-DR4+ PBMC. After 3 days, IL-2 (10 U/ml) was added, and culture was continued for a total of 14 days. Aliquots of T cells were then frozen in liquid nitrogen, and cultures were re-established as required. The alloreactive T cells were thawed rapidly and cultured 1:5 with irradiated (5000 rad) HLA-DR4+ stimulator PBMC in complete RPMI 1640 containing 125 ng/ml anti-CD3 Ab (AstraZeneca). IL-2 (10 U/ml) was added after 2 days and thereafter every 2–3 days. The cells were then restimulated 1:25 every 14 days with irradiated (5000 rad) HLA-DR4+ PBMC, and IL-2 (10 U/ml) was added every 2–3 days. For experimental use, at day 10 of the cycle, the T cells were transferred to a round-bottom 96-well culture plate (10^5 cells in 100 μl/well), and GSNO, SNAP or control medium (50 μl) were added for 72 h. The cells were then stimulated with 50 μl of a mixture of various concentrations of anti-CD3, anti-CD28 (BD Biosciences, San Jose, CA), and cross-linking rabbit anti-mouse IgG (Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 24 h. Cell proliferation was measured by incorporation of [3H]thymidine during the final 20 h of culture (details as above for PBMC).

**Cytokine and chemokine assays**

Cell supernatants were removed from PBMC cultures 48 h after stimulation with PHA and assayed in duplicate by commercial ELISA for IL-2, IL-4, IFN-γ (Immunodiagnostic Systems, Boldon, U.K.), RANTES, and IL-8 (R&D Systems, Abingdon, U.K.) according to the manufacturer’s instructions. In every case, samples were diluted appropriately to give readings on the linear range of standard curves. Experiments were performed four times, each time with PBMC from a separate donor.

**Fluorescence flow cytometry**

Expression of the T cell marker CD3, the cell activation marker CD25 and the apoptosis marker annexin V was measured by fluorescence flow cytometry. After culture with NO donors or control compounds and stimulation with PHA as above, PBMC (0.5 x 10^5 cells in 0.5 ml) were sedimented by centrifugation and suspended in 50 μl of PBS at 0°C. The cells were double-stained for 30 min in the dark at 0°C with PE-anti-CD3 (4 μl)/FITC-anti-CD25 (2 μl), or PE-anti-CD3/FITC-anti-annnexin V (4 μl) (all Abs from CalTag-Medsystems, Towcester, U.K.). FITC- or PE-labeled isotype-matched control Abs (CalTag-Medsystems) were used as internal controls and to set analysis parameters. The cells were washed twice in ice-cold PBS and suspended in 0.5 ml of CellFix (BD Biosciences) for analysis (EPIC-XL, flow cytometer and associated software; Coulter Electronics, Luton, U.K.).

**RNase protection assays**

PBMC (10^6/ml in RPMI 1640 plus 2% TCH) were preincubated for 48 h with or without GSNO or SNAP (250 μM) before stimulation with PHA for a further 48 h. The cells were then centrifuged for 5 min at 400 x g, the cell pellets suspended in TRizol (Life Technologies, Paisley, U.K.), and RNA was extracted according to the manufacturer’s instructions. 32P-Pradioabeled antisense RNA probes were synthesized from RiboQuant cDNA template kit hCK-1, incorporating probes for IL-2, IL-4, IL-9, IL-13, IFN-γ, and GAPDH, and kit hCK-5, incorporating probes for lymphotactin (Ltn), RANTES, IFN-γ-inducible protein (IP-10), macrophage inhibitory protein (MIP)-1α, MIP-1β, macrophage chemotactic protein-1 (MCP-1), IL-8, IL-10, and GAPDH using T7 RNA polymerase according to the manufacturer’s instructions (BD Pharmingen, Cambridge, U.K.). Probe solutions were hybridized to 10–20 μg of total RNA at 56°C overnight followed by digestion with RNase A and T1 (BD Pharmingen). The solutions were then treated with protease K to remove RNases, and the RNA was purified by phenol-chloroform extraction and ethanol precipitation. Each RNA sample was loaded onto a 6% polyacrylamide urea gel, run at 38 mA in 0.5x Tris-borate-EDTA buffer alongside the undigested probe as size marker. Gels were exposed overnight to autoradiography film (Kodak XAR-5) at ~70°C with intensifying screens. Densitometric analysis was conducted using the Image program (National Institutes of Health, Bethesda, MD).

**Results**

**Nitrite production by GSNO, SNAP, and activated PBMC**

To monitor nitrite production by NO donor compounds, GSNO and SNAP (250 μM), were incubated in complete RPMI 1640 at 37°C in 5% CO2, and supernatant nitrite was measured by Griess assay. SNAP and GSNO both generated nitrite at a steady rate for 24 h; thereafter, the rate of nitrite synthesis decreased, and nitrite levels reached a plateau by 48 h (Fig. 1A). The levels of nitrite produced reached levels comparable with those generated by human A549 epithelial cells (B. M. Brooks and C. A. Gibney, unpublished observations) and rat peritoneal macrophages (33). In additional experiments, GSNO (250 μM) was added to PBMC with PHA (2.5 μg/ml) or IL-2 (5 ng/ml), and supernatant nitrite levels were measured after 3 or 7 days, respectively. GSNO alone induced nitrite production; PHA or IL-2 had no effect on nitrite production with or without GSNO present (Fig. 1B). The detected nitrite levels did not reach the molar concentration of the donors (250 μM) because not all NO is converted to nitrite; NO also forms nitrate and nitrosylates thiols that are abundant in serum. The apparent discrepancy between nitrite levels generated by cell-containing and cell-free preparations may reflect the different balance of these products in each system.
Effects of NO on proliferation of PBMC and a T cell line

The NO donor compounds GSNO and SNAP and their respective non-NO-donating chemical controls GSH and NAP were added (250 μM) to PBMC 4, 24, or 48 h before or simultaneous with (time 0) addition of the T cell mitogens PHA or IL-2. Cell proliferation was measured 72 h after PHA stimulation or 7 days after IL-2 stimulation. GSNO but not GSH (Fig. 2, A and C) and SNAP but not NAP (Fig. 2, B and D) produced a statistically significant time-dependent inhibition of proliferation induced by PHA (Fig. 2, A and B) or IL-2 (Fig. 2, C and D). In all cases, the inhibitory effects of GSNO or SNAP were most pronounced at 48 h pre-stimulation and declined progressively with reduced incubation time. At 48 h, GSNO produced 68 and 63% inhibition of proliferation induced by PHA (Fig. 2, A and B) or IL-2 (Fig. 2, C and D), respectively (all statistically significant over six independent experiments). When the NO donors were added simultaneously with the mitogens, the inhibition fell to 29–37%, but this was statistically significant only for SNAP (Fig. 2, B and D). GSNO and SNAP were nontoxic to PBMC under these conditions, as determined by trypan blue exclusion and LDH release.

GSNO and SNAP (250 μM) inhibited proliferation of a human alloreactive T cell line by ~90% when added optimally 3 days before cell stimulation with soluble anti-CD3 plus anti-CD28 Ab (Fig. 3).

Effect of NO on cytokine release by PBMC

PBMC experiments were performed as above except that cell supernatant fractions were removed 48 h after PHA stimulation for cytokine measurements. As shown in Fig. 4, stimulation with PHA led to release of IL-2, IFN-γ, and IL-4 to 30-, 10-, and 6-fold background levels, respectively. In absolute terms, IFN-γ was produced at the highest levels, IL-2 was intermediate, and IL-4 was produced at low levels (~4000, 120, and 6 pg/ml, respectively, over six experiments). Preincubation of the cells with GSNO or SNAP for 48 h strongly inhibited IL-2 release (74 and 79% inhibition, respectively; Fig. 4A). The degree of inhibition declined with reduced incubation times down to 4 h, whereas simultaneous

FIGURE 1. Nitrite production by SNAP, GSNO, and activated PBMC. A, SNAP or GSNO (250 μM) were incubated alone in complete RPMI 1640, and supernatant nitrite was measured at time intervals as indicated. B, In the presence or absence of GSNO (250 μM), PBMC were stimulated with or without PHA (2.5 μg/ml) or IL-2 (5 ng/ml), and supernatant nitrite was measured after 3 or 7 days, respectively. Results are means ± SEM of four experiments.

FIGURE 2. Effects of GSNO (A and C) or SNAP (B and D) and their respective control compounds GSH and NAP on PBMC proliferation induced by PHA (A and B) or IL-2 (C and D). PBMC were preincubated with the compounds (250 μM) for various periods (0–48 h) as indicated before stimulation with PHA or IL-2. 0 represents cells costimulated with NO and mitogen. Cell proliferation was measured as 4-h uptake of [3H]thymidine 72 h after PHA stimulation or 7 days after IL-2 stimulation and is presented as percent of positive control (PHA or IL-2 alone). [3H]Thymidine uptake was 100-fold background by cells stimulated with PHA-stimulated cells and 10-fold background in cells stimulated by IL-2. Results are means ± SEM for six independent experiments, each performed in quintuplicate. p < 0.001 for effects of GSNO on PHA, SNAP on PHA and IL-2; p = 0.017 for GSNO on IL-2 by one-way ANOVA; **, p < 0.01; *, p < 0.05 for comparisons with no-drug control by two-tailed paired Student’s t test.

FIGURE 3. Effects of GSNO and SNAP on proliferation of an alloreactive T cell line. T cells were preincubated with GSNO or SNAP (250 μM) for 72 h before activation with anti-CD3 (1 μg/ml) plus anti-CD28 (concentrations as shown) plus anti-mouse IgG cross-linking Ab (5 μg/ml). Proliferation was measured as [3H]thymidine uptake between 4 and 24 h poststimulation. Results are means ± SEM of triplicate assays from a representative experiment.
addition of GSNO with PHA significantly enhanced IL-2 release. GSNO and SNAP had no significant effect (one-way ANOVA) on PHA-induced release of IFN-γ (Fig. 4B) and IL-4 (Fig. 4C). GSH or NAP had no effect on release of any of these cytokines (Fig. 4).

Effect of NO on expression of cytokine mRNA

PHA-activated PBMC expressed strong message for the cytokines IL-2, IL-9, IL-13, and IFN-γ but weak message for IL-4 (Fig. 5A). Pre-exposure of the cells to GSNO or SNAP for 48 h inhibited PHA-induced expression of mRNA for IL-2, IL-9, IL-13, and IFN-γ (Fig. 5A), and these effects were statistically significant for IL-2, IL-13, and IFN-γ over four experiments after densitometric analysis of mRNA bands (Fig. 5B).

Effect of NO on expression of chemokine mRNA and release of RANTES and IL-8 protein

PHA-activated PBMC expressed mRNA for the chemokines Ltn, RANTES, IP-10, MIP-1α, MIP-1β, MCP-1, and IL-8 (Fig. 5C). Pre-exposure of the cells to GSNO or SNAP for 48 h before stimulation did not influence the PHA-induced expression of any of these species of chemokine mRNA (Fig. 5, C and D). Likewise, the NO donors had no effect on PHA-induced release of RANTES and IL-8 protein (Fig. 6).

Effect of NO on CD25 expression by PBMC

PBMC were preincubated for 48 h with or without GSNO, GSH, SNAP, or NAP before activation by PHA. Three days after PHA stimulation, corresponding to the time point at which proliferation was measured (see Fig. 2), the cells were double-stained for CD3/CD25 and CD3/annexin V. Flow cytometry revealed that the PBMC were ~70% positive for CD3 and that neither PHA nor any of the NO donor or control compounds influenced expression of this T cell marker (Table I). As expected, PHA led to a sizeable increase in both median CD25 fluorescence and percent of cells positive for CD25 in both total PBMC and CD3⁺ cell populations, but CD25 expression was not influenced by any of the NO donors or control compounds (Table I). No induction of annexin V was seen under any of the experimental conditions (Table I).

Discussion

NO inhibits proliferation of T cells and other immune cells, but questions remain as to whether this is associated with altered cytokine expression and, if so, whether certain cytokines/chemokines are selectively affected. To address these issues from a novel standpoint, we studied in detail the time dependence of NO effects on human PBMC proliferation and cytokine production and examined expression of a wide range of species of cytokine and chemokine mRNA. We found that the inhibitory effect of NO on PHA- and IL-2-induced human PBMC proliferation and IL-2 production is dependent on exposure time. The degree of inhibition of proliferation declined with reduced NO exposure times such that when the cells were costimulated with NO plus PHA or NO plus IL-2, the NO donors had little (SNAP) or no (GSNO) significant effect. In parallel with its effect on proliferation, NO significantly inhibited PHA-induced release of IL-2 in a time-dependent manner. IFN-γ secretion appeared to be reduced by NO, although less so than IL-2, but this did not reach statistical significance. IL-4 release was not affected by NO, although this cytokine was detected at considerably lower levels than IL-2 and IFN-γ. In parallel, NO significantly inhibited PHA-induced expression of mRNA for IL-2, IL-13, and IFN-γ and, consistent with the cytokine measurements, IL-2 mRNA was proportionately reduced to a greater extent (~70%) than IFN-γ mRNA (32–48% inhibition). We are unable to comment on NO effects on IL-4 mRNA because this transcript was barely detected in PHA-stimulated cells.

In contrast to its effects on cytokines, under the same conditions and in the same starting populations of cells, NO had no effect on PHA-induced expression of mRNA for the chemokines Ltn, RANTES, IP-10, MIP-1α, MIP-1β, MCP-1, and IL-8 and on the release of RANTES and IL-8. We conclude that NO targets some signaling component, as yet unidentified, that is unique or more important to cytokine than chemokine expression.

Our PBMC preparations comprised 70% CD3⁺ T cells and therefore contained up to 30% monocytes. We used the mitogens IL-2 and PHA which in long term cultures almost certainly activate indirectly cell types other than T cells, including monocyes and NK cells. Furthermore, most of the species of cytokine that we
studied are not produced exclusively by T cells, and monocytes or other differentiated cell types may be a significant source of these. Because IL-2 is a specific T cell marker, we conclude that NO does indeed regulate T cell function, but the regulatory effects of NO on expression of other cytokines may be due to its targeting of other cell types. Likewise, the failure of NO to modify chemokine expression by PBMC may reflect resistance to NO not only of T cells but also of other cell types. In parallel with its inhibition of PBMC proliferation, NO inhibited growth of a T cell line activated by anti-CD3 and anti-CD28 Abs.

Under the experimental conditions used, NO was nontoxic because it did not induce release of LDH, increase trypan blue uptake or influence expression of CD3, CD25, annexin V or levels of mRNA for the housekeeping gene GAPDH. Thus, NO does not induce a global shutdown of PBMC function but acts selectively on expression of certain induced genes (IL-2, IL-13, IFN-γ) but not others (CD25, chemokines). In this context, NO is known to inhibit the DNA binding activity of transcription factors such as NFκB, AP-1, c-Myb, signal peptide-1, and early growth response factor-1 by S-nitrosylating cysteine-rich regions in these proteins (31, 35–37). However, these transcription factors are involved in control of many genes and are not selective for individual cytokines. Therefore, although it seems plausible that NO may exert selective effects on gene expression according to its preferential targeting of specific transcription factors, the identity of these is not yet known fully. The delayed effect of NO is certainly consistent with protein nitrosylation or gene regulation.

On the question of the cytokine selectivity of NO, Wei et al. (21) reported that NO inhibits secretion of IL-2 and IFN-γ by cloned murine Th1 cells and that genetic deletion of the NOS-2 gene in mice leads to enhanced Th1 cell responses and decreased Th2-mediated immunity (22). In our studies, NO inhibited expression of mRNA for both the Th1 cytokine IFN-γ and the Th2 cytokine IL-13 in PBMC. Thus, we are unable to concur with the hypothesis that NO amplifies Th2 responses in humans and that this may underlie IgE-mediated diseases such as asthma (23). In this respect, our results agree more closely with those of Bauer et al. (25).

Our observation that PBMC regulation by NO is dependent on exposure time is consistent with the effect that one would expect from a radical that is produced in a sustained rather than a transient manner. NO is generated slowly by NOS-2 in immune and inflammatory cells, reflecting mRNA and protein synthesis, but once under way NO is produced at sustained and high levels (1, 2, 27). Hence, one would expect that cells targeted by NOS-2-derived NO would respond relatively slowly, to reflect the kinetics of NO synthesis (reviewed in Ref. 2). In the present study, it was therefore crucial that cells were precultured with NO for extended time periods before stimulation. Indeed, the NO donors used (GSNO and SNAP) mimicked closely the kinetics and levels of NO production by NOS-2. Under these conditions, we show unequivocally that NO inhibits the induced expression of IL-2, IL-13, and IFN-γ in parallel with reduced cell proliferation. Our finding that long term
exposure to NO is necessary for T cell regulation is consistent also with our findings in mast cells; here too, extended incubation times with NO of 24–48 h are required for inhibition of IgE/Ag-induced degranulation (32, 33).

In conclusion, pre-exposure of human PBMC to NO inhibits proliferation, IL-2 release, and expression of IL-2, IL-13, and IFN-γ mRNA. However, NO did not influence expression of several species of chemokine mRNA or release of RANTES and IL-8. Thus, NO may regulate T cell-dependent processes but is not Th1/Th2 selective. Furthermore, it is unlikely to influence recruitment of inflammatory cells (eosinophils, neutrophils, monocytes, and further T cells) that is chemokine dependent. Importantly, for regulation of T cell activation, an extended period of exposure to NO is critical, consistent with the slow but sustained production of NO by NO-2 during immune and inflammatory reactions.

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