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Original Research Communication

Nitric Oxide Induces a Decrease in the Mitochondrial Membrane Potential of Peripheral Blood Lymphocytes, Especially in Natural Killer Cells

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ABSTRACT

Increased levels of nitric oxide (NO) at an inflammatory site may affect the biological activity of lymphoid cells. To investigate the effects of NO on the immune system, we measured the mitochondrial membrane potential ($\Delta\psi_m$) of the peripheral blood lymphocytes (PBL) cultured with a chemical NO donor. PBL from healthy volunteers were cultured with NOC18, a NO-generating compound, at various concentrations. The $\Delta\psi_m$ of the PBL was measured by flow cytometry using 3,3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$). NOC18 induced a decrease in the $\Delta\psi_m$ of the PBL in a dose-dependent fashion, induced an increase in the levels of reactive oxygen species (ROS), and caused these cells to undergo apoptosis. Dual-color staining of the $\Delta\psi_m$ and lymphocyte surface markers demonstrated that $CD3^-CD56^+$ natural killer (NK) cells were responsive to NO. Trolox, a vitamin E analog, partially reversed the NO-induced decrease in the $\Delta\psi_m$ of the PBL. We showed that the $\Delta\psi_m$ of peripheral NK cells were decreased by NO, which suggests that abundant NO at an inflammatory site may impair NK cell function. Antiox. Redox Signal. 2, 673–680.

INTRODUCTION

IN INFLAMMATORY CONDITIONS, many cytokines and chemical modulators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and endotoxin are produced (Pullicino *et al.*, 1990; Mayers and Johnson, 1998; Sasada *et al.*, 1999). These inflammatory mediators have been shown to induce nitric oxide (NO) synthase (NOS) expression in endothelial cells (Kilbourn and Belloni, 1990), macrophages (Hibbs *et al.*, 1988), and neutrophils (Wright *et al.*, 1989), which presumably increases the local NO concentration at an inflammatory site. NO is generated from a guanido nitrogen of L-arginine by at least three different isoforms of NOS (Nathan and Xie, 1994), and has multiple biological functions, including vascular relaxation (Garthwaite *et al.*, 1988), promoting the antimicrobial activities of macrophages (Granger *et al.*, 1988), and tumoricidal activities (Stuehr and Nathan, 1989). A low level of NO synthesized by constitutive NOS also acts as a regulator of the blood pressure and platelet aggregation (Moncada *et al.*, 1991). In contrast, the high level of NO produced by inducible NOS (iNOS) upon lipopolysaccharide (LPS) or TNF-α challenge is cytotoxic against pathogens (Nathan and Hibbs, 1991). NO is also known to exert an inhibitory effect on several immune functions,

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such as leukocyte adhesion (Kubes *et al.*, 1991), platelet aggregation (Ignarro, 1990), and lymphocyte proliferation (Hout *et al.*, 1993). The wide variety of effects induced by NO is achieved based on its interactions with target molecules via a high oxidation-reduction (redox) potential (Stamler, 1994).

Increased levels of NO may also affect the biological activity of lymphoid cells. For example, NO-mediated cytotoxicity has been achieved by inhibiting mitochondrial respiration (Bolanos *et al.*, 1995) and DNA synthesis (Kwon *et al.*, 1991). Recent studies have revealed that mitochondria play an important role in the process of apoptosis. *In vitro* studies have shown that the dissipation of the mitochondrial $\Delta\psi_m$ is an early event in apoptotic cell death (Zanzami *et al.*, 1995a, b), and it can be presumed that measured $\Delta\psi_m$ may provide data on the biological activity of living cells. In this study, we investigated the effects of NO on peripheral blood lymphocytes (PBL) by measuring $\Delta\psi_m$ of these cells.

MATERIALS AND METHODS

Reagents

3,3-Dihexyloxacarbocyanine iodide (DiOC6 (3)), propidium iodide (PI), and hydroethidine (HE) were purchased from Molecular Probes, Inc. (Eugene, OR). Carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18) and [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxidel] (carboxy-PTIO) were purchased from Wako Pure Chemicals (Osaka, Japan). Fluorescein-conjugated annexin V (Annexin V-FITC) and phycoerythrin-conjugated annexin V (Annexin V-PE) were purchased from R&D Systems (Minneapolis, MN). The water-soluble analog of vitamin E, 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), was purchased from Aldrich Chemical Co. (Milwaukee, WI). Phycoerythrin-labeled anti-human CD3, anti-human CD19 and anti-human CD56 were purchased from Becton Dickinson (San Jose, CA).

Isolation and culture of lymphocytes

Heparinized peripheral blood samples were obtained from healthy human volunteers with their informed consent. The lymphocytes were prepared using Ficoll Hypaque gradient centrifugation as described by Böyum (1968). After the cell number was adjusted to 1×10^6 cells/ml, the cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in RPMI-1640 medium containing 10% fetal calf serum (FCS).

Cytofluorometric analysis of the mitochondrial membrane potential, reactive oxygen species, and surface marker

To measure the $\Delta\psi_m$ and reactive oxygen species (ROS) production, the cells were incubated with 1 n*M* DiOC6(3) at 37°C for 20 min, followed by 5 μmol/ml HE at 37°C for 20 min. To deplete completely the $\Delta\psi_m$ as a positive control, some of the cells were incubated with 40 μM of the uncoupling agent CCCP at 37°C for 30 min. For the simultaneous assessment of the $\Delta\psi_m$ and lymphocyte surface markers, the cells were stained by DiOC6(3), followed by phycoerythrin-labeled anti-human CD3, anti-human CD19, and anti-human CD56 antibodies for 30 min on ice. The flow cytometry analysis was performed on a FACSCalibur (Becton Dickinson, San Jose, CA) (Petit *et al.*, 1990; Rottenberg and Wu, 1998). Ten thousand cells per sample were counted for each determination.

Apoptosis analysis

Apoptosis was measured by flow cytometry using the FACSCalibur after concurrent staining with annexin V-FITC and PI, as previously described (Martin *et al.*, 1995; Vermes *et al.*, 1995). The staining with annexin V-PE was used to monitor the phosphatidylserine (PS) externalization in parallel with a measurement of the $\Delta\psi_m$ using DiOC. Apoptosis was also assessed by determining DNA fragmentation (DNA-release assay) after lysing the cells in a hypotonic solution containing 50 μg/ml PI, and then analyzed by flow cytometry as previously described (Nicoletti *et al.*, 1991; Bedner *et al.*, 1999).

RESULTS

NOC18 induced a decrease in the $\Delta\psi_m$ of the PBL in a dose-dependent fashion

In preliminary experiments, we assessed the effects of NOC18, a chemical NO donor (Keefer *et al.*, 1996), on the $\Delta\psi_m$ of the PBL. As shown in Fig. 1, prolonged culture (24 hr) of the PBL in the presence of NOC18 resulted in a decreased $\Delta\psi_m$ in a dose-dependent fashion.

NOC18-induced mitochondrial dysfunction of lymphocyte is mediated by NO

The next study was carried out to determine whether the NOC18-induced decrease in the $\Delta\psi_m$ was NO-specific. Carboxy-PTIO, a NO scavenger, was added to the PBL culture medium together with various concentrations of NOC18. Carboxy-PTIO (1 m*M*) attenuated the decrease in the $\Delta\psi_m$ of the PBL cultured with NOC18, which suggests that the effects of NOC18 were indeed mediated by NO (Fig. 2).

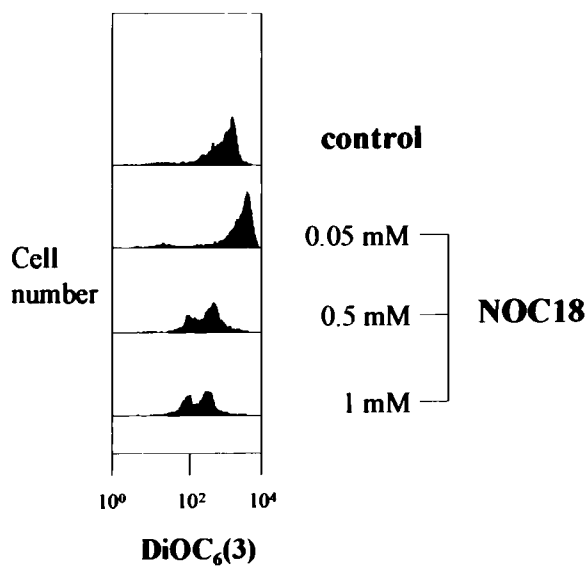

FIG. 1. Effects of NOC18 on the $\Delta\psi_m$ of the PBL. The PBL from healthy donors were cultured in the presence of the indicated concentrations of NOC18 for 24 hr. The $\Delta\psi_m$ of the PBL was then measured by flow cytometry using $DiOC_6(3)$. Data were representative of five different experiments.

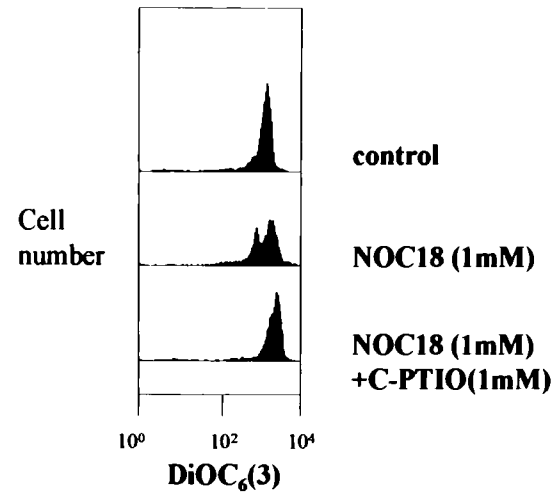

FIG. 2. Effects of carboxy-PTIO on the NOC18-induced dissipation of the $\Delta\psi_m$. The PBL were pretreated with carboxy-PTIO for 5 min, and then NOC18 (1 m*M*) was added to the culture. The $\Delta\psi_m$ of the PBL was measured by flow cytometry using $DiOC_6(3)$ after 2 hr. Data were representative of three different experiments.

NOC18 induced an increase in the production of ROS

NOC18 also induced a reproducible increase in the ROS production in a dose-dependent fashion, as shown in Fig. 3.

NOC18 causes apoptosis in the PBL

Because the dissipation of the mitochondrial $\Delta\psi_m$ has been shown to be an early event in apoptotic cell death, we investigated whether the prolonged culture (24 hr) of the PBL in the presence of NO may cause apoptotic cell death. Flow cytometric analysis using double-staining with PI and Annexin V demonstrated that PI-negative and annexin V-positive cells were increased in the cultured PBL treated with NO for 24 hr in a dose-dependent fashion (Fig. 4). Furthermore, we assessed the extent of apoptosis in the cultured PBL treated with NO by determining DNA fragmentation (DNA-release assay) using flow cytometry. As shown in Fig. 5, DNA fragmentation was increased in the PBL cultured with NO for 24 hr.

The decrease in the $\Delta\psi_m$ was found mainly in NK cells

After the demonstration that the $\Delta\psi_m$ of the PBL was decreased by NO, further studies

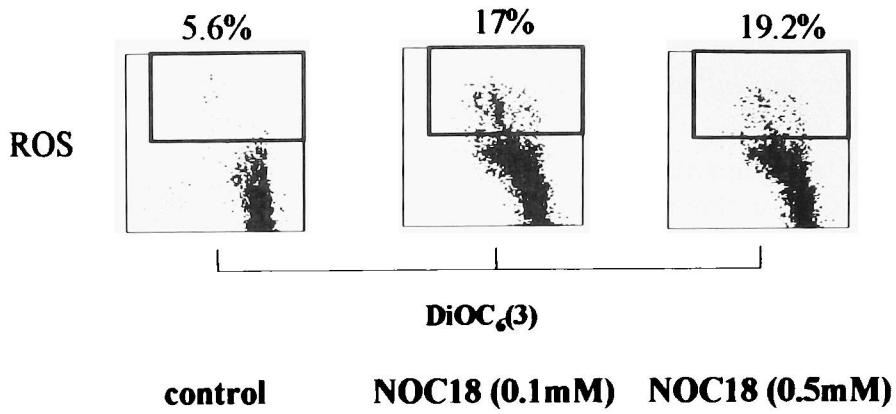

FIG. 3. Increased ROS production was found in the PBL cultured in the presence of NO. The ROS production from the PBL treated with NOC18 for 24 hr was measured by flow cytometry using hydroethidine described as Materials and Methods. Data were representative of three different experiments.

were carried out to determine which lymphocyte population was responsible for the decrease in the $\Delta\psi_m$ of the PBL. Dual-color immunostaining for the $\Delta\psi_m$ and lymphocyte cell surface markers (CD3, CD19, and CD56) were employed to resolve this issue. A representative sample from Fig. 6 shows that about one-half of the $CD56^+$ NK cells showed low levels of DiOC6, and that a large percentage (93%) of the $CD3^+$ cells belonged to the DiOC6(3)-high cells. The $CD19^+$ cells treated with NOC18 (0.1 m*M*) showed little change (data not shown). This suggests that the majority of lymphocytes with a low $\Delta\psi_m$ were $CD3^-CD56^+$ NK cells. Thus, NK cells seemed to be more affected by NO.

Trolox, a vitamin E analog, protects lymphocytes from NO toxicity

As one of the models of cytotoxity caused by NO has been reported to be lipid peroxidation, we investigated whether Trolox, a potent inhibitor of lipid peroxidation (Phoenix *et al.*, 1989), could reverse the dissipation of the mitochondrial $\Delta\psi_m$ induced by NO. Figure 7 shows that Trolox could partially attenuate the decrease in the mitochondrial $\Delta\psi_m$.

DISCUSSION

Previously, we demonstrated that the number of iNOS-expressing cells correlated with

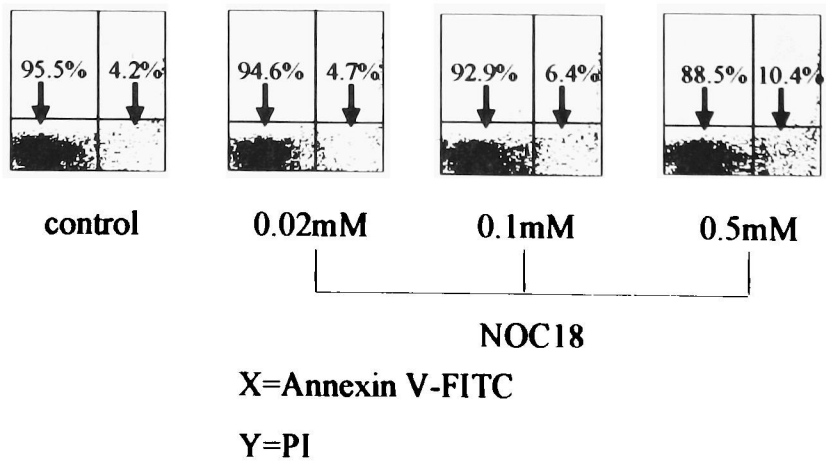

FIG. 4. Dual-color staining for anexine-V and PI. In a dose-dependent fashion, anexine-positive and PI-negative cells were increased in the PBL cultured with NOC18 for 24 hr. Data were representative of three different experiments.

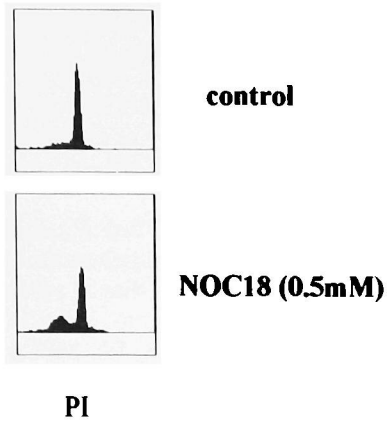

FIG. 5. Staining for apoptosis with PI was also observed in the PBL cultured with NOC18 for 24 hr. Apoptosis was also detected by flow cytometry using PI as described in Materials and Methods. Data were representative of three different experiments.

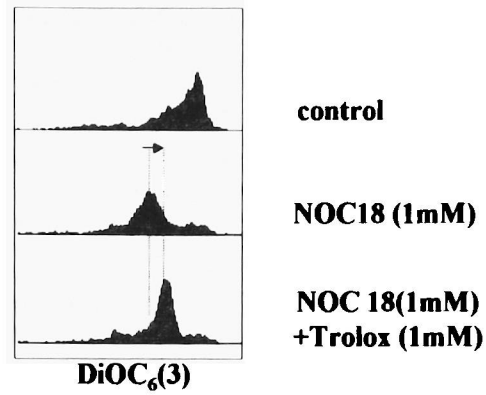

FIG. 7. Trolox, a vitamin E analog, prevented the dissipation of the $\Delta\psi_m$ induced by a high concentration of NO in the PBL. The PBL were cultured with NOC18 alone or NOC18 plus Trolox for 24 hr. The $\Delta\psi_m$ was measured by flow cytometry using $DiOC_6(3)$. Data were representative of three different experiments.

the histopathological severity of acute appendicitis. Increased levels of NO at an inflammatory site may therefore affect the biological activity of lymphoid cells. In an animal model of multiple organ dysfunction syndrome caused by endotoxin, NOS inhibitors attenuated the liver dysfunction observed (Thiemermann *et al.*, 1995), suggesting that NO is one of the key components in the progression of inflammation. NO has been shown to induce cell death via apoptosis in various cell lines (Blanco *et al.*, 1995; Fehsel *et al.*, 1995; Shimaoka *et al.*, 1995; Xie *et al.*, 1997; Ushmorov *et al.*, 1999).

In the present study, we demonstrated that a high concentration of NO depressed the $\Delta\psi_m$ and induced the production of ROS in NK cells. The $\Delta\psi_m$, an electrical potential difference across the inner mitochondrial membrane, is generated as a result of coupling the transport of electrons with the spatially oriented uptake and release of H^+. In a series of experiments, the early stages of the apoptotic process were characterized by the collapse of the $\Delta\psi_m$, which preceded the stage of nuclear disintegration (Zanzami *et al.*, 1995a, b). The dissipation of the $\Delta\psi_m$ in these cells was presumed to indicate that they were in a "pre-apoptosis" state (Susin *et al.*, 1998; Jacoto *et al.*, 1999; Zamzami and Kroemer, 1999). Thus, the dissipation of the $\Delta\psi_m$ in NK cells exposed to increased concentrations of NO may also indicate that these cells were in a "pre-apoptosis" stage. This is further supported by the result that ROS production, the stage that follows the dissipation of the $\Delta\psi_m$ in the process of apoptotic cell death, was dependent on the duration of culture with NO and on the concentration of NOC18 used (data not shown). The result that Annexin V-positive and PI-negative cells were increased in the cultured PBL treated with NO in a dose-dependent fashion also supports the contention that NO induces apoptosis in the PBL.

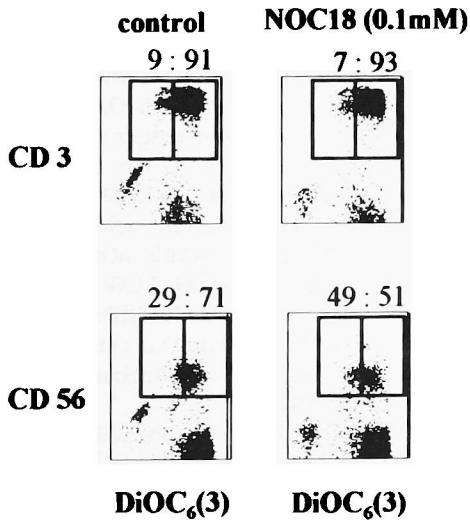

FIG. 6. Dual-color staining for $DiOC_6(3)$ and lymphocyte surface markers of the PBL cultured with NOC18 for 24 hr. The $CD56^+$ NK cells were responsible for the decreased $\Delta\psi_m$ of the PBL treated with NOC18. Data were representative of three different experiments.

The mechanism by which NO decreases the $\Delta\psi_m$ in the PBL is still unclear. It has been shown that NO can inhibit the activity of respiratory complexes I, II, III (Cassina and Radi, 1996; Lizasoain *et al.*, 1996), and IV (Bolanos *et al.*, 1994), and can promote the degradation of cardiolipin and lipids, which results in the opening of PT pores followed by a depolarization of the inner mitochondrial membrane and massive ROS production (Zanzami *et al.*, 1995a, b; Balakirev *et al.*, 1997). It is presumed that the NO-induced mitochondrial dysfunction in the observed NK cells might be at least partially mediated by the same mechanisms described above. This is also supported by the result that Trolox, a potent inhibitor of lipid peroxidation (Phoenix *et al.*, 1989), partially restored the decrease in the $\Delta\psi_m$ of the PBL cultured in the presence of NO.

In conclusion, the $\Delta\psi_m$ of peripheral NK cells was decreased by NO, which presumably indicates that NO abundant at an inflammatory site may impair NK cell function.

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ABBREVIATIONS

Annexin V-FITC, Fluorescein-conjugated annexin V; Annexin V-PE, phycoerythrin-conjugated annexin V; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxidel; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; $DiOC_6(3)$, 3,3-dihexyloxacarbocyanine iodide; FCS, fetal calf serum; IL-1β, interleukin-1β; iNOS, inducible NOS; $\Delta\psi_m$, mitochondrial membrane potential; LPS, lipopolysaccharide; NO, nitric oxide; NOC18, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene; PBL, peripheral blood lymphocytes; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; Trolox, 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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