Nitric oxide induces apoptosis via triggering mitochondrial permeability transition

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Abstract Nitric oxide (NO) induces apoptosis in thymocytes, peripheral T cells, myeloid cells and neurons. Here we show that NO is highly efficient in inducing mitochondrial permeability transition, thereby causing the liberation of apoptogenic factors from mitochondria which can induce nuclear apoptosis (DNA condensation and DNA fragmentation) in isolated nuclei in vitro. In intact thymocytes, NO triggers disruption of the mitochondrial transmembrane potential, followed by hypergeneration of reactive oxygen species, exposure of phosphatidyl serine on the outer plasma membrane leaflet, and nuclear apoptosis. Inhibitors of mitochondrial permeability transition such as bongkrekic acid and a cyclophilin D-binding cyclosporin A derivative, *N*-methyl-Val-4-cyclosporin A, prevent the mitochondrial as well as all post-mitochondrial signs of apoptosis induced by NO including nuclear DNA fragmentation and exposure of phosphatidylserine residues on the cell surface. These findings indicate that NO can cause apoptosis via triggering of permeability transition.

Key words: Apoptosis-inducing factor; Megachannel; Mitochondrial transmembrane potential; Programmed cell death

1. Introduction

Nitric oxide (NO) is a pleiotropic mediator that, among other biological effects, causes apoptosis in a variety of cell types including macrophages, thymocytes, T cells and neurons [1–5]. It is now known that nuclear apoptosis is preceded by the disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$) due to the opening of mitochondrial permeability transition (PT) pores [6–14]. This applies to different cell types (neurons, fibroblasts, B and T lymphocytes, pre-B cells and thymocytes, myelomonocytic cells) and to various apoptosis inducers (growth factor withdrawal, tumor necrosis factor, ceramide, glucocorticoids, positive and negative selection, genotoxic stress, hyperexpression of Bax) (reviewed in ref. [15]). Physiological (sub-micromolar) concentrations of NO potently and reversibly deenergize mitochondria and inhibit several mitochondrial enzymes including aconitase and cytochrome *c* oxidase [16,17]. In addition, peroxynitrate has been shown to induce mitochondrial PT [18,19]. Here we show that mitochondria treated with NO donors release apoptogenic factors when undergoing permeability transition. In contrast, inhibition of PT by specific drugs including bongkrekic acid (BA), as well as by cyclosporin A (CsA) and a non-immunosuppressive CsA derivative, abolishes mitochondria-mediated nuclear apoptosis, both in vitro and in cells. Our results are compatible with the hypothesis that NO induces apoptosis via triggering mitochondrial PT.

2. Materials and methods

2.1. Cells and in vitro culture conditions

Thymocytes from 30–45-day-old BALB/c mice were cultured at a final concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, L-glutamine, HEPES and antibiotics, and cultured in the presence of the indicated dose of *S*-nitroso-*N*-acetylpenicillamine (SNAP), 1,3-morpholinosydnomine (SIN-1), *S*-nitroso-glutathione (GSNO; all from Sigma, St. Louis, MO, USA), bongkrekic acid (BA; 50 μM; gift from Dr. Hans J. Duine, Delft University, The Netherlands), cyclosporin A (CsA; 10 μM), *N*-methyl-Val-4-CsA (SDZ; 10 μM; kindly provided by Sandoz, Basel, Switzerland) and/or *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk; 50 μM; enzyme systems) [20,21]. NO production was determined using the diazotation reaction of Griess, with $NaNO_2$ as standard [22].

2.2. Mitochondrial swelling

Mitochondria were purified from BALB/c mouse livers, on a Percoll gradient [23], and were stored on ice in B buffer (400 mM mannitol, 10 mM PO_4H_2K, 5 mg/ml bovine serum albumin, and 50 mM Tris-HCl, pH 7.2) for up to 4 h. For determination of large amplitude swelling, mitochondria were washed and resuspended in buffer B (100 μg protein/10 μl buffer), followed by addition of 90 μM CFS buffer (300 nM mannitol, 100 mM sucrose, 2 mM NaCl, 2.5 mM PO_4H_2K, 0.5 mM EGTA, 2 mM Cl_2Mg, 5 mM pyruvate, 0.1 mM PMSF, 2 mM ATP, 50 μg/ml creatinine phosphokinase, 10 mM phosphocreatine, 1 mM dithiothreitol, and 10 mM HEPES-NaOH pH 7.4), and recording absorption at 540 nm in a spectrophotometer (model DU 7400) as described [11]. The loss of absorption induced by *ter*-butylhydroperoxide (50 μM) within 5 min was considered 100% large amplitude swelling. Supernatants of mitochondria ($15\,000\times g$, 1 h, 4°C) were obtained after 5 min of incubation at 20°C and tested for apoptogenic activity on isolated nuclei, as described below.

2.3. Cell free system of apoptosis

Nuclei from mouse liver were purified on a sucrose gradient, as described [24], and were resuspended in CFS buffer (neg. control) or in mitochondrial supernatants. DNA fragmentation of nuclei was assessed using terminal desoxyribonucleotidyltransferase to incorporate fluorescein-12-dUTP into nuclei, following the manufacturer's protocol (TUNEL method, Boehringer-Mannhein). Briefly, nuclei were incubated 1 h at 37°C with 50 μl of staining buffer: 0.3 nmol FITC-dUTP, 3 nmol dATP (1 μl), 50 nmol $CoCl_2$, 5 U terminal desoxynucleotidyltransferase and 10 μl reaction buffer 5× (sodium cacodylate 1 M, bovine serum albumin 1.25 mg/ml and Tris-HCl 125 mM, pH 6.6). After incubation the nuclei were washed twice with PBS and were resuspended in 500 μl PBS and analyzed cytofluorimetrically as described [25].

2.4. Cytofluorimetric analyses of whole cells

Cells were incubated during 15 min at 37°C with the potential-

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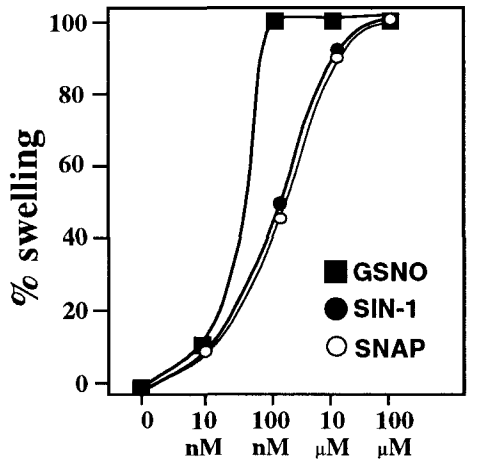

Fig. 1. Effect of different concentrations of NO donors on mitochondrial large amplitude swelling. Liver mitochondria were incubated with variable doses of NO donors while assessing absorbtion at 540 nm as in Fig. 2A. The loss of absorption induced by *ter*-butylhydroperoxide (50 μM) within 5 min was considered as 100% of large amplitude swelling.

sensitive dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; 40 nM; Molecular Probes), hydroethidine (HE; 2 μM; Molecular Probes); the vital dye ethidium bromide (EthBr, 200 ng/ml) and/or an FITC-annexin V conjugate (1:400 dilution; 1 μg/ml, 15 min at 4°C; Brand Applications, Maastricht, The Netherlands), as described [6,8,10,26]. Labeling with FITC-annexin V was performed after removal of FCS by washing cells twice in HEPES buffer (10 mM HEPES-NaOH, pH 7.4; 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) [27]. After incubation cells were immediately analyzed in an Epics Profile (Coulter) cytofluorometer (excitation 488 nm). Ethidium (Eth, the oxidation product of HE) and EthBr fluorescence was recorded in FL3; $DiOC_6(3)$ and FITC-annexin V in FL1. Hypoploidy was determined by staining of ethanol-fixed cells with propidium iodide, as described [26].

3. Results and discussion

3.1. NO induces the mitochondrial release of apoptogenic factors concomitant with permeability transition

Isolated mitochondria undergo rapid colloidosmotic swelling indicative of PT [28,29] in response to three different NO donors (Fig. 1). This effect is observed with an ED_{50} of approximately 25 nM (GSNO) to 100 nM (SIN-1, SNAP), corresponding to an effective ED_{50} of NO of about 10 nM. NO-induced swelling was efficiently inhibited by specific substances such as BA, CsA, and the non-immunosuppressive CsA derivative *N*-methyl-4-valine-CsA (SDZ), which are known to inhibit PT [28,29] (Fig. 2A). We have previously shown that PT leads to the liberation of apoptogenic factors, that is factors which, in a cell free system, provoke signs of nuclear apoptosis (chromatin condensation and DNA fragmentation) [11,25]. As shown in Fig. 2B, NO treatment of isolated mitochondria also triggers the release of an activity that causes DNA fragmentation in isolated nuclei. The release of this activity into the supernatant is inhibited by the PT inhibitors BA, CsA, and *N*-methyl-4-valine-CsA (Fig. 2B), indicating that it is coupled to PT. In conclusion, NO induces both mitochondrial PT and PT-associated release of apoptogenic factors.

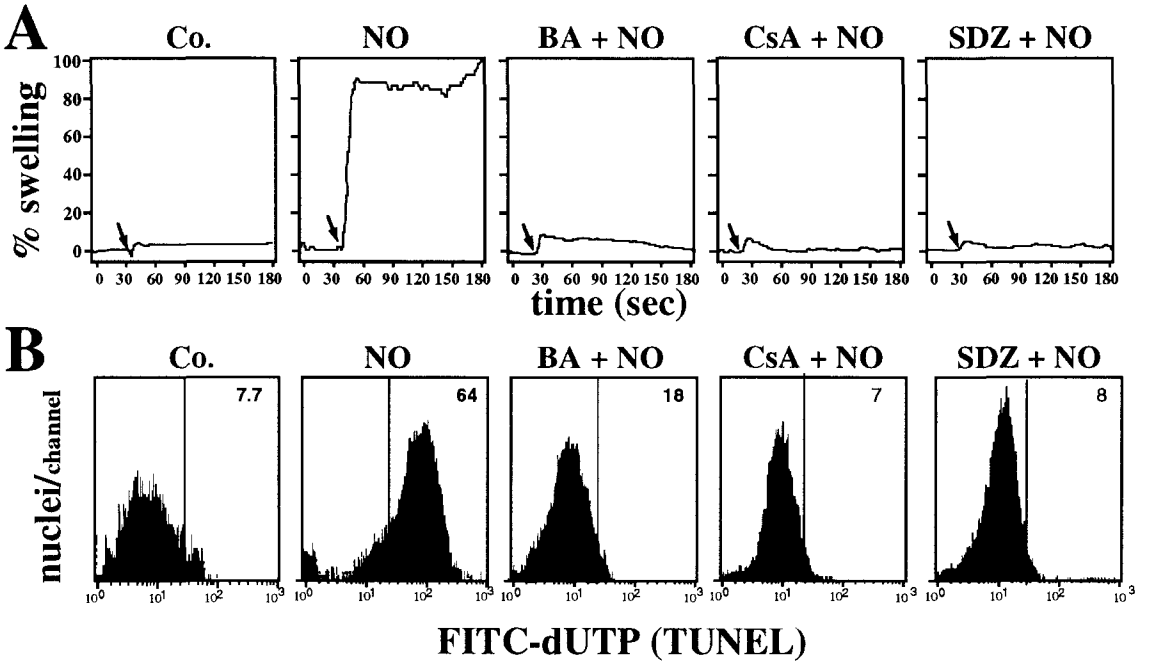

Fig. 2. NO causes permeability transition and liberation of apoptogenic factors from mitochondria. A: large amplitude swelling of mitochondria. Liver mitochondria were incubated with NO donors (data shown for 100 nM GSNO) and/or the PT inhibitors bongkrekic acid (BA; 50 μM), cyclosporin A (CsA; 10 μM), *N*-methyl-Val-4-cyclosporin A (SDZ 220–384, SDZ; 10 μM). Arrows indicate the time points at which the NO donor was added to mitochondria. Similar results were obtained when instead of GSNO, another NO donor, SNAP (100 nM), was used (not shown). B: NO-treated mitochondria release apoptogenic factors. Liver mitochondria were incubated in the presence or absence of the same substances as in A, followed by ultracentrifugation ($15000 \times 10^5 \times g$, 1 h) and recovery of supernatants. Isolated liver nuclei were incubated in the presence of these supernatants (30 min, 37°C), followed by TUNEL staining to determine DNA strand breaks, as described in Section 2.

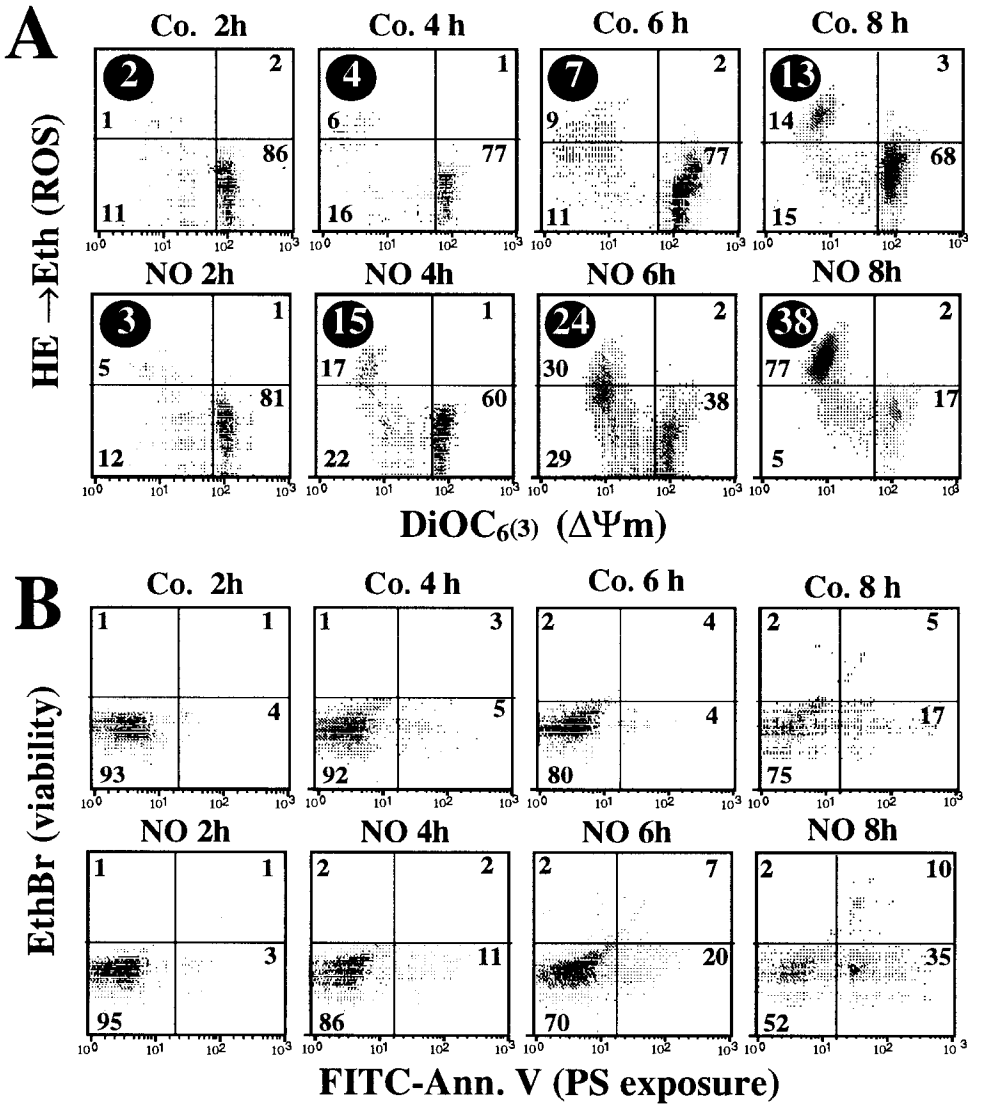

Fig. 3. Kinetics of mitochondrial alterations, nuclear apoptosis and plasma membrane changes induced by NO. A: changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$; measured with $DiOC_6(3)$), superoxide anion generation (measured by means of the conversion of HE→Eth), and nuclear apoptosis (hypoploid cells; black circles). Murine thymocytes were cultured in the presence or absence of the NO donor GSNO (1 mM). At the indicated time intervals, cells were stained with a combination of $DiOC_6(3)$ plus HE, during 15 min at 37°C. The percentage of hypoploid cells (numbers in black circles) was measured in ethanol-permeabilized cells stained with the DNA-intercalating dye propidium iodide. B: exposure of phosphatidyl serine (PS) residues on the cell surface. Thymocytes cultured as in A were simultaneously stained with the vital dye EthBr and the phosphatidylserine-reactive FITC-annexin V. Comparable results were obtained when the NO donor SIN-1 was used (not shown).

3.2. *Disruption of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) is an early event in NO-induced apoptosis*

Thymocytes undergoing glucocorticoid-induced death exhibit a reduction in mitochondrial transmembrane potential ($\Delta\Psi_m$) preceding exposure of phosphatidylserine residues on the plasma membrane, enhanced generation of superoxide anions, and nuclear DNA degradation [8,10]. Here, we show that NO-induced apoptosis follows a similar chronology. Incubation of mouse thymocytes with HE and $DiOC_6(3)$ allows for the simultaneous determination of $\Delta\Psi_m$ and mitochondrial ROS generation [8]. As demonstrated in Fig. 3A, thymocytes cultured in the presence of NO donors manifest a reduction in $DiOC_6(3)$ incorporation followed by an increased superoxide anion-driven HE→Eth conversion, as well as nuclear DNA loss. In addition to mitochondrial signs of apoptosis, we determined plasma membrane alterations associated with the apoptotic process. Loss of membrane integrity was measured using ethidium bromide (EthBr), and the aberrant surface exposure of phosphatidylserine was monitored by means of an FITC-annexin V conjugate. As shown in Fig. 3B, NO also triggers the "flipping out" of phosphatidyl serine residues on yet viable ($EthBr^-$) cells. As it has been observed for apoptosis triggered by the glucocorticoid receptor agonist DEX [10], the kinetic data suggest that $\Delta\Psi_m$ disruption precedes phosphatidyl serine exposure and nuclear apoptosis (Fig. 3).

3.3. *Inhibition of PT prevents NO-induced apoptosis*

To determine whether PT is a rate-limiting event of NO-induced apoptosis, we have taken advantage of three inhibitors of PT, namely BA (a ligand of the adenine nucleotide translocator of the inner mitochondrial membrane), CsA and *N*-methyl-L-Val-CsA (a ligand of mitochondrial matrix cyclo-

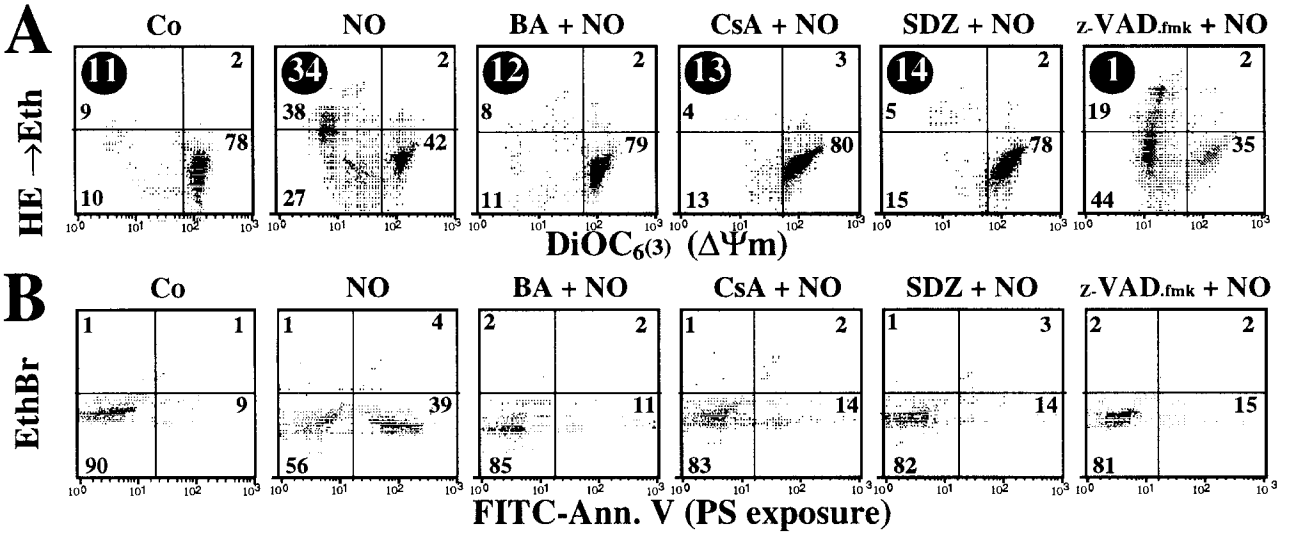

Fig. 4. Effect of different PT inhibitors on apoptosis-associated changes in mitochondria, superoxide anion generation, nuclear DNA content and plasma membranes. Thymocytes were exposed to the indicated substances (GSNO 1 mM), BA (50 μM), CsA (10 μM), *N*-methyl-Val-CsA (SDZ; 10 μM), or Z-VAD.fmk (50 μM) during 7 h. Thereafter, cells were labeled with HE, $DiOC_6(3)$ (A), FITC-annexin V, and EthBr (B). Numbers in circles (A) refer to the percentage of hypoploid cells determined by staining with propidium iodide. Results are representative for three independent experiments.

philin) [12]. As shown in Fig. 4A, these agents inhibit the mitochondrial alterations induced by NO: $\Delta\Psi_m$ disruption and hyperproduction of superoxide anion. In addition, they effectively prevent nuclear DNA loss and phosphatidyl serine exposure on the outer leaflet of the plasma membrane (Fig. 4B). The protease inhibitor Z-VAD.fmk, which neutralizes most of the apoptogenic activity contained in mitochondrial supernatants [25], can dissociate the mitochondrial and post-mitochondrial signs of apoptosis induced by NO. Whereas Z-VAD.fmk fails to stabilize the $\Delta\Psi_m$, it completely prevents nuclear apoptosis and strongly reduces phosphatidyl serine exposure (Fig. 4A,B). This suggests that Z-VAD.fmk-inhibitable proteases participate in the post-mitochondrial rather than in the mitochondrial phase of the NO-triggered apoptotic process.

3.4. Concluding remarks

In this paper, we present data indicating that NO can trigger apoptosis via inducing mitochondrial PT. This suggests that NO is triggering the same cascade of molecular events as do other apoptosis inducers including glucocorticoids, reactive oxygen species, and p53-dependent genotoxic stress [6,8,10,12]: first mitochondrial PT with release of apoptogenic factors, then the nuclear and plasma membrane feature of advanced apoptosis. The idea that NO provokes apoptosis via induction of PT is in accord with the facts that Bcl-2 hyperexpression prevents NO-induced apoptosis [4,5] and that Bcl-2 functions as an endogenous inhibitor of mitochondrial membrane changes associated with early apoptosis [8,10,11,25,30–33].

As a note of caution, however, it has to be stated that NO is not a universal inducer of apoptosis, perhaps due to differences in the expression level of members of the Bcl-2 family and/or differences in the capacity of NO detoxification in cell lines [34]. Moreover, it appears that, according to its pleiotropic nature, NO can trigger alternative apoptotic pathways. Thus, NO has been reported to upregulate the CD95/Fas/Apo-1 death receptor on vascular smooth muscle cells [35], to trigger overexpression of the apoptosis-triggering protein p53 in a macrophage cell line [36], and to prevent NFκB binding to DNA, thus disrupting an apoptosis-inhibitory pathway [37]. Regardless of these additional possibilities, however, the present data suggest that, at least in certain cell types, NO may induce apoptosis via a direct effect on mitochondria. Future studies will have to elucidate which exact molecular targets are modified by NO or peroxynitrate at the level of mitochondrial membranes.

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