

PARTIAL PROTECTION BY POLY(ADP-RIBOSE) POLYMERASE INHIBITORS FROM NITROXYL-INDUCED CYTOTOXICITY IN THYMOCYTES

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Abstract—Nitroxyl (NO⁻/HNO), has been proposed to be one of the NO[•]-derived cytotoxic species. Although the biological effect of nitroxyl is largely unknown, it has been reported to cause DNA breakage and cytotoxicity. We have therefore investigated whether NO⁻/HNO-induced DNA single-strand breakage activates the nuclear nick sensor enzyme poly(ADP-ribose) polymerase (PARP) and whether PARP activation affects the mode of NO⁻/HNO-induced cell death. NO⁻/HNO generated from Angeli's salt (AS, sodium trioxodinitrate) (0–300 μM) induced DNA single-strand breakage, PARP activation, and a concentration-dependent cytotoxicity in murine thymocytes. AS-induced cell death was also accompanied by decreased mitochondrial membrane potential and increased secondary superoxide production. The cytotoxicity of AS, as measured by propidium iodide uptake, was abolished by electron acceptors potassium ferricyanide, TEMPOL, the intracellular calcium chelator BAPTA-AM, and by PARP inhibitors 3-aminobenzamide (3-AB) and PJ-34. The cytoprotective effect of 3-AB was paralleled by increased output of AS-induced apoptotic parameters such as phosphatidylserine exposure, caspase activation, and DNA fragmentation. No significant increase in tyrosine nitration could be observed in AS-treated thymocytes as opposed to peroxynitrite-treated cells, indicating that tyrosine nitration is not likely to contribute to NO⁻/HNO-induced cytotoxicity. Our results demonstrate that NO⁻/HNO-induced PARP activation shifts the default apoptotic cell death toward necrosis in thymocytes. However, as total PARP inhibition resulted only in 30% cytoprotection, PARP-independent mechanisms dominate NO⁻/HNO-induced cytotoxicity in thymocytes. © 2001 Elsevier Science Inc.

Keywords—Nitroxyl, Poly(ADP-ribose) polymerase, Apoptosis, Necrosis, Nitric oxide, Free radicals

INTRODUCTION

The Janus face of nitric oxide (NO[•]) has generated a considerable controversy over the beneficial vs. detrimental effect of NO[•] under various pathophysiological conditions. Nitric oxide has been shown to damage cells and contribute to tissue injury, e.g., in shock, ischemia-reperfusion, and inflammatory diseases on the one hand [1], and to protect cells from cytotoxicity and to inhibit tissue injury on the other hand [2–4]. A plausible explanation to resolve this controversy was that NO[•] per se serves beneficial, regulatory roles, whereas NO[•]-derived reactive species, such as peroxynitrite, are responsible for the cytotoxicity and tissue injury associated with

increased NO[•] production [5]. Peroxynitrite is a potent oxidant and cytotoxic agent formed in the near diffusion-limited reaction of NO[•] and superoxide. The cytotoxic effect of peroxynitrite has been attributed to protein oxidation and nitration, lipid peroxidation, inhibition of mitochondrial respiration, inactivation of ion channels, DNA breakage, and the overactivation of poly(ADP-ribose) polymerase (PARP) [6]. PARP is a nuclear nick sensor enzyme that becomes activated in response to DNA breakage [7]. Activated PARP cleaves NAD⁺ into nicotinamide and polymerizes the latter on nuclear acceptor proteins. Overactivation of PARP depletes cellular stores of its substrate NAD⁺, and consequently ATP, leading to necrotic cell death [8–10]. Peroxynitrite-induced PARP activation is now recognized as an important pathway of tissue injury in various forms of inflammation, reperfusion injury, and shock [11].

Recently, another candidate has emerged as a poten-

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tial cytotoxic intermediate responsible for nitric oxide-related cytotoxicity. Several reports have put forward the hypothesis that nitroxyl (NO^-), the one-electron reduction product of NO^* , is the cytotoxic reactive nitrogen species that may also contribute to tissue injury under pathophysiological conditions [12,13]. Nitroxyl can be formed in vivo in a variety of reactions [14]: (i) nitric oxide synthase (NOS) generates NO^- either directly or indirectly via metabolism of the decoupled NOS product N^G -hydroxyl-L-arginine [15–17]; (ii) the quinone/hydroquinone redox system or ferricytochrome *c* can also carry out the one-electron reduction of NO^* [18,19]; and (iii) NO^- can also be produced from nitrosothiols or nitrosylhemoglobin [20,21].

The chemistry of NO^- has been intensively investigated [22,23] and was found to significantly differ from that of NO^* and peroxynitrite [24]. However, the biological effects of NO^- have not been investigated in detail. At physiological pH nitroxyl exists both in the anion form (NO^-) and the protonated form [23], and will hereafter be referred to as NO^-/HNO . Using the nitroxyl donor compound Angeli's salt, it has been reported that nitroxyl (2–4 mM) is cytotoxic to Chinese Hamster V79 lung fibroblasts [12] and MCF-7 human breast cancer cells [25]. However, the mechanism of NO^- -induced cytotoxicity is largely unknown. As NO^-/HNO has been shown to cause DNA breakage [19,25], we hypothesized that NO^-/HNO , similar to peroxynitrite, can also lead to PARP activation and trigger the PARP-mediated suicidal pathway.

MATERIALS AND METHODS

Materials

AS was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Fluorescent dyes (propidium iodide, hydroethidium) were purchased from Molecular Probes (Eugene, OR, USA). Annexin V-FITC was from BD Pharmingen (San Diego, CA, USA). The PARP inhibitor PJ34 was synthesized as previously described [26]. All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), except when stated otherwise.

Preparation and treatment of thymocytes

Thymocytes were isolated from 3–4 week old male C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME, USA) as described [9]. Single-cell suspension was made by pressing the thymus through a wire mesh. Cells were seeded in 24-well tissue culture plates (0.450 ml/well) in RPMI medium complemented with 10% fetal bovine serum. PARP inhibitors and electron acceptors were added in 50 μl medium and incubated

with the cells for 30 min. AS was dissolved in PBS (pH 11.0) and was added to the cells in a volume of 25 μl . Addition of AS to the medium did not result in pH change. Decomposed AS (kept for 1 week at room temperature in PBS pH 7.0) was also used as control, and was found to have no effect on any parameters measured.

Cytotoxicity assay

AS-induced cytotoxicity was determined by propidium iodide uptake, as described previously [27]. AS-treated cells were stained with 2.5 $\mu\text{g}/\text{ml}$ propidium iodide for 15 min, washed once in PBS, and analyzed by flow cytometry. Cytotoxicity was calculated as $100 \times (\text{T}-\text{C}/100-\text{C})$ where T = % number of PI positive AS-treated cells and "C" means the corresponding value of control samples.

Double staining of thymocytes with Annexin V-FITC and propidium iodide

Annexin V-FITC and propidium iodide was carried out as described [9]. Three hours after AS treatment, thymocytes were washed in PBS and 10^5 cells were stained with 5 μl Annexin V-FITC and 5 $\mu\text{g}/\text{ml}$ propidium iodide (PI) in 100 μl annexin-binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) at room temperature. After 15 min, 400 μl annexin-binding buffer was added to the samples, which were then immediately analyzed with flow cytometry.

Detection of mitochondrial depolarization and superoxide production

The mitochondrial membrane potential was quantitated by the flow cytometric analysis of 3,3'-dihexyloxycarbocyanine iodide [DiOC6(3)]-stained cells as described [28]. The assay utilizes a lipophilic cationic fluorescent dye DiOC6(3) that is transported into the mitochondria by the negative mitochondrial membrane potential and thus concentrated within the mitochondrial matrix. Decreased mitochondrial membrane potential results in reduced cellular fluorescence. Intramitochondrial generation of superoxide was determined using a previously established flow cytometry technique, which is based on the superoxide-induced conversion of the superoxide-sensitive dye, hydroethidium, to the highly fluorescent ethidium. Thymocytes were stained with 40 nM DiOC6(3) or 2 μM hydroethidium (HE) for 15 min at 37°C, washed once with PBS, and analyzed with a FACS-Calibur flow cytometer. Forward and side scatters were gated on the major population of normal-sized cells.

Detection of DNA single-strand breakage

DNA breakage was detected by a commercially available single-cell gel electrophoresis (also known as comet assay) kit (Trevigen, Gaithersburg, MD, USA) following the manufacturer's instructions. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates more slowly and remains within the confines of the nucleus. Cells treated with AS or decomposed AS (control) were incubated at 37°C for 20 min, washed once in ice-cold PBS, and resuspended in PBS at a density of 10^5 cells/ml. Cell suspensions were mixed with low melting point agarose (at 42°C) at a ratio of 1:10 (v/v) and 75 μ l was immediately pipetted onto CometSlides (supplied with the kit). Gel was allowed to solidify at 4°C for 20 min and were then submerged in lysis solution (2.5M NaCl, 100 mM EDTA pH 10, 10 mM TRIS base, 1% sodium lauryl sarcosinate, 1% Triton X) for 60 min. Slides were then transferred into alkaline unwinding solution pH > 13 (0.6g NaOH, 250 μ l of 200mM EDTA solution in 50 ml deionized water) and were incubated for 60 min at room temperature in the dark. Slides were then placed into a horizontal electrophoresis apparatus filled with alkaline electrophoresis solution (12 g NaOH, 2 ml of 500 mM EDTA solution pH 8, in 1 l deionized water) and were electrophoresed for 30 min at 300 mA. Slides were then silver stained and examined with a Zeiss Axiolab microscope.

Immunodot blot detection of poly(ADP-ribose)

PARP activation was evidenced by the detection of poly-ADP-ribosylated proteins, as previously described by Affar *et al.* [29]. The thymocytes were preincubated with 3-aminobenzamide (3-AB, 3 mM) or medium for 30 min. Then the cells were treated with AS for 3 h. The cells were lysed in RIPA buffer supplemented with NaOH and EDTA (Tris 50 mM, NP-40 1%, Na-deoxycholate 0.25%, NaCl 150 mM, PMSF 1 mM, aprotinin, leupeptin, pepstatin 1 μ g/ml each, Na_3VO_4 1 mM, NaF 1 mM, NaOH 0.4 M, EDTA 10 mM). Twenty micrograms protein was loaded to a Hybond N+ membrane (Amersham Biosciences, Freiburg, Germany) at each AS concentration. The blot was washed with 0.4 M NaOH for 20 min then rinsed in PBST. The membrane was blocked with 5% milk powder dissolved in PBST for 1 h. Poly(ADP-ribose) was detected with anti-poly(ADP-ribose) antibody (1:400 dilution, Alexis). The membrane was washed with PBST and was incubated with peroxidase-conjugated secondary antibody (Boehringer Mannheim, Mannheim, Germany). After three washes with PBST and one wash with PBS, signal was detected by ECL.

Measurement of PARP activity

PARP activity of cell lysates has been determined with the classical PARP activity assay based on the incorporation of isotope from $^3\text{H-NAD}$ into trichloroacetic acid (TCA) precipitable proteins, as described [9]. Briefly, thymocytes (10^7 cells in 0.5 ml culture medium) were treated with Angeli's salt (0–300 μ M). After 3 h, cells were collected into eppendorf tubes, spun, and resuspended in 0.5 ml assay buffer (56 mM HEPES pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM MgCl_2 , 0.01% w v^{-1} digitonin and 0.125 μ M NAD^+ and 0.5 $\mu\text{Ci ml}^{-1}$ $^3\text{H-NAD}^+$). Following incubation (10 min at 37°C), 200 μ l ice-cold 50% w v^{-1} TCA was added and samples incubated for 4 h at 4°C. Samples were then spun ($10,000 \times g$, 10 min) and pellets washed twice with ice-cold 5% w v^{-1} TCA and solubilized overnight in 250 μ l 2% w v^{-1} SDS/0.1 N NaOH at 37°C. Contents of the tubes were added to 6.5 ml ScintiSafe Plus scintillation liquid (Fisher Scientific, Pittsburgh, PA, USA) and radioactivity was determined using a liquid scintillation counter (Wallac, Gaithersburg, MD, USA).

Measurement of caspase activation

Caspase-3-like activity was determined as described previously [30], with modifications as follows. Six hours after AS exposure, thymocytes were lysed in lysis buffer (10 mM HEPES, 0.1% w/v CHAPS, 5 mM dithiothreitol, 2 mM EDTA, 10 μ g/ml aprotinin, 20 μ g/ml leupeptin, 10 μ g/ml pepstatin A, and 1 mM PMSF, pH 7.25). Lysates were combined with assay buffer (100 mM HEPES, 10% w/v sucrose, 5 mM dithiothreitol, 0.1% w/v CHAPS, pH 7.25) containing para-nitroanilin-conjugated tetrapeptide caspase substrate DEVD-pNA (300 μ M) and incubated for 1 h. Absorbance of pNA was measured spectrophotometrically and compared to pNA standard curve.

Detection of DNA fragmentation

Internucleosomal DNA fragmentation was visualized by agarose electrophoresis exactly as described previously [9]. Agarose (2% w/v) was poured on a horizontal gel support. After solidification of the gel, the top part (above the comb) was replaced with 1% w/v agarose containing 2% w/v SDS and 64 μ g/ml proteinase K. Cells (2×10^6) were loaded in 20 μ l sample buffer (5% v/v glycerol, 10 mM Tris, pH 8.0, 0.05% w/v bromophenol blue, 5 mg/ml RNase). Electrophoresis was carried out at 60 V for 12 h and the gel was stained with 2 μ g/ml ethidium bromide for 1 h. Excess dye was removed by intensive washing in distilled water.

Detection of tyrosine nitration by western blotting

Cells exposed for 30 min to different concentrations of AS and peroxynitrite were lysed in RIPA buffer. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Tyrosine nitration was detected by a rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA). The secondary antibody was a mouse monoclonal antirabbit IgG-peroxidase conjugate (Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Reaction was developed by enhanced chemiluminescence.

RESULTS

Nitroxyl-induced cytotoxicity in thymocytes

Treatment of thymocytes with AS caused a concentration-dependent necrosis, as measured by propidium iodide uptake (Fig. 1A). AS-induced cytotoxicity was abolished by TEMPOL (1 mM) and potassium ferricyanide (1 mM), indicating that the cytotoxic effect of AS was due to nitroxyl release (Fig. 1B). Inhibition of PARP by 3-aminobenzamide (2 mM) provided significant protection against AS-induced necrosis (Fig. 1B). Similar results were obtained by using the novel phenanthridine-based PARP inhibitor PJ34 (2 μ M) (not shown). Chelation of intracellular calcium by BAPTA-AM (5 μ M), which has previously been shown by us to block PARP activation [27], also protected thymocytes from the cytotoxic effect of AS (Fig. 1B).

We have also carried out Annexin V-FITC/propidium iodide (PI) double staining on AS-treated thymocytes (Fig. 1C). As Annexin V detects phosphatidylserine exposure in the plasma membrane of apoptotic cells, this technique can differentiate between normal (double negative), early apoptotic (Annexin V-FITC single positive), and necrotic (or late apoptotic) (Annexin V-FITC + PI double positive) cells. However, as at 3 h after AS treatment when late apoptotic cells are not likely to be present, the double positive population most likely represents necrotic cells. AS (150 μ M) induced both apoptotic and necrotic cell death in thymocytes. Pretreatment of cells with 3-AB resulted in a shift from necrotic toward the apoptotic and normal phenotype (Fig. 1C).

Nitroxyl-induced mitochondrial alterations

We have reported previously that in peroxynitrite- and hydrogen peroxide-treated thymocytes, PARP activation leads to decreased mitochondrial membrane potential and secondary superoxide production [28]. Here we show that AS-induced necrotic cell death is also accompanied by these mitochondrial alterations (Fig. 2). Treat-

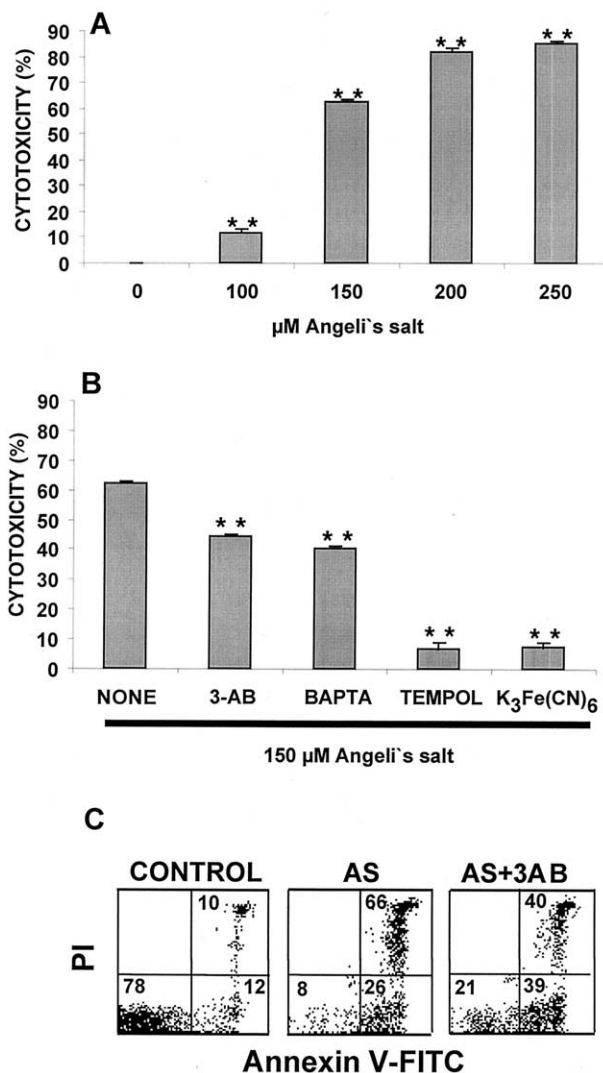


Fig. 1. Nitroxyl-induced cytotoxicity in thymocytes. (A) Thymocytes were treated with the indicated concentrations of Angeli's salt for 3 h. Cytotoxicity was measured by propidium iodide uptake. (B) Thymocytes were pretreated with 2 mM 3-AB, 5 μ M BAPTA-AM, 1 mM TEMPOL, or 1 mM $K_3Fe(CN)_6$ for 30 min and then exposed to 150 μ M Angeli's salt for 3 h. Cytotoxicity was determined by propidium iodide uptake. Stars indicate significant ($p < .01$) cytotoxicity (A) or significantly ($p < .01$) reduced cytotoxicity by the pharmacological agents (B). In (C), Annexin V-FITC/propidium iodide double staining of thymocytes 3 h after treatment with 150 μ M AS in the absence or presence of 2 mM 3-aminobenzamide (3-AB). Numbers indicate percent ratio of normal (lower left quadrant), apoptotic (lower right quadrant), or necrotic (upper right quadrant) populations.

ment of thymocytes for 3 h with 150 μ M AS resulted in decreased DiOC6(3) fluorescence (i.e., decreased mitochondrial membrane potential) and increased HE fluorescence (secondary superoxide production) in thymocytes. Furthermore, inhibition of PARP by 3-AB significantly reduced the AS-induced decrease of mitochondrial membrane potential and also inhibited secondary superoxide production (Fig. 2).

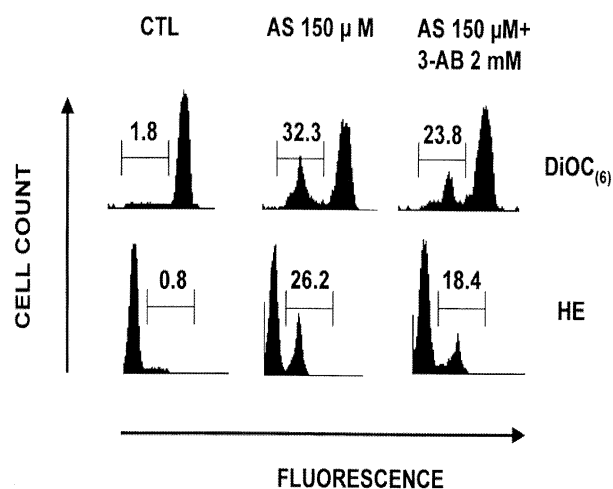


Fig. 2. Nitroxyl-induced mitochondrial alterations. Thymocytes were treated with 150 μM Angeli's salt in the presence or absence of 2 mM 3-aminobenzamide. Mitochondrial membrane potential and overproduction of superoxide were determined by flow cytometry using the mitochondrial membrane potential-selective dye DiOC₆(3) and the superoxide-sensitive dye hydroethidine (HE). Numbers represent mean percentage values of cells displaying decreased mitochondrial membrane potential or increased superoxide production. ($n = 4$).

Nitroxyl-induced caspase activation and DNA fragmentation

AS treatment caused a slight increase in caspase-3-like activity in thymocytes as indicated by DEVD-ase activity (Fig. 3A). However, in the presence of 3-AB, AS-induced caspase activation was significantly elevated but declined at higher concentrations (400–600 μM). Essentially similar changes could be observed in DNA fragmentation, another characteristic marker of apoptotic

cell death (Fig. 3B). At low concentrations (100–200 μM), AS induced DNA fragmentation. However, at higher concentrations, DNA fragmentation was blocked by AS (Fig. 3B). Inhibition of DNA fragmentation observed at higher concentrations of AS could be reversed by the PARP inhibitor 3-AB.

Nitroxyl-induced DNA breakage and PARP activation

Single-cell electrophoresis of thymocytes exposed to AS (200 μM) showed intense DNA single-strand breakage as indicated by the migration of damaged DNA from the nuclei resulting in a "comet" formation (Fig. 4A). On the contrary, cells treated with decomposed AS (control) showed no signs of DNA damage. Furthermore, AS induced PARP activation in a concentration-dependent manner as shown by the immunodot blot detection of poly-ADP-ribosylated proteins (Fig. 4B). PARP activation was inhibited by pretreatment of the cells with 2 mM 3-AB (Fig. 4B). AS-induced PARP activation was also confirmed by the measurement of tritium incorporation from 3H-NAD into trichloroacetic acid precipitable proteins (Fig. 4C).

Lack of effective tyrosine nitration by nitroxyl

We have also investigated whether AS, similar to peroxynitrite, causes protein tyrosine nitration. We have found that exposure of thymocytes to AS (0–600 μM) resulted only in a slightly increased tyrosine nitration. Peroxynitrite treatment, however, induced intense protein tyrosine nitration (Fig. 5).

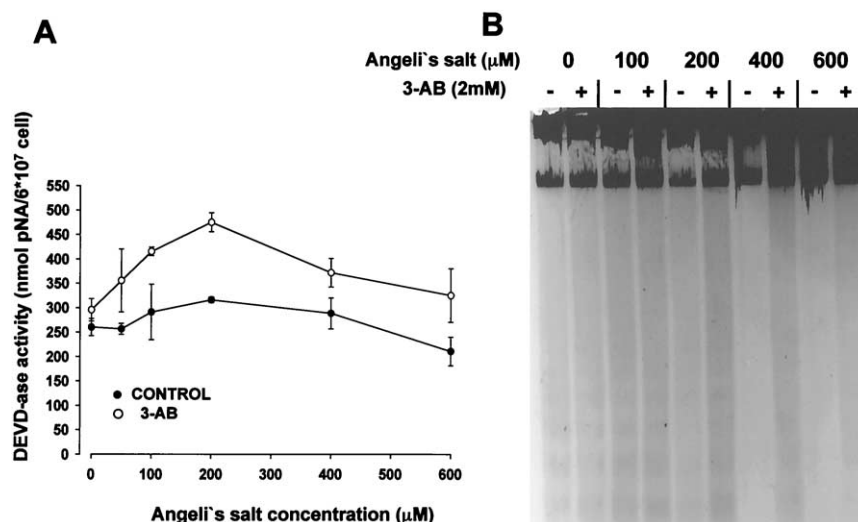


Fig. 3. Nitroxyl-induced apoptosis in thymocytes. Thymocytes were treated with the indicated concentrations of Angeli's salt in the presence or absence of 3-aminobenzamide for 6 h. Apoptosis was assessed by measuring caspase activity (A) and detecting DNA fragmentation (B).

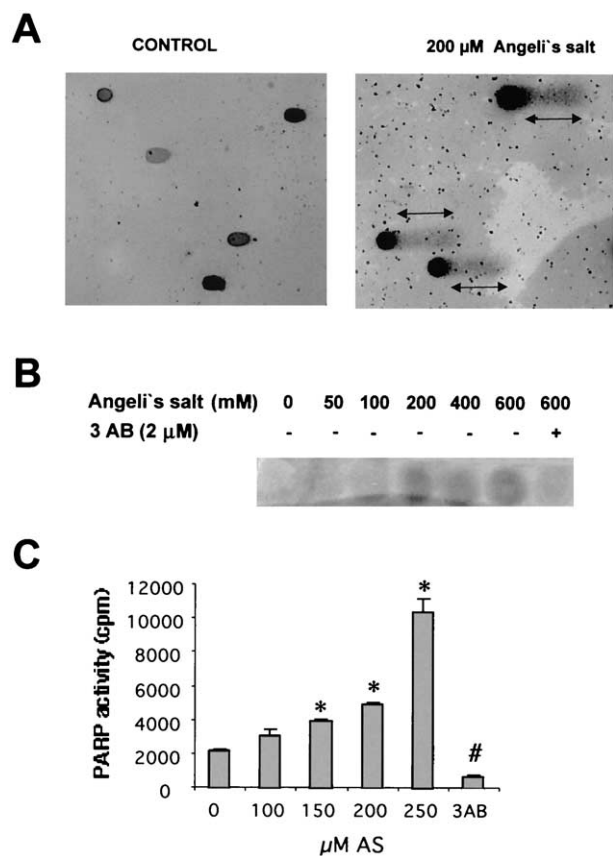


Fig. 4. Nitroxyl induces DNA breakage and PARP activation. (A) Thymocytes were exposed to Angeli's salt for 30 min and DNA breakage was detected by single-cell gel electrophoresis (top panel). Angeli's salt-induced DNA damage is indicated by comet formation (arrows). (B) Angeli's salt-induced PARP activation was evidenced by the immunodot blot detection of the enzyme's product poly(ADP-ribose). (C) Determination of AS-induced PARP activation with 3 H-NAD incorporation assay. Stars indicate significantly ($p < .01$) elevated PARP activity as compared to control. 3-AB significantly ($\#p < .01$) inhibited PARP activation induced by 250 μ M AS.

DISCUSSION

The chemistry of nitroxyl has attracted considerable interest in the last couple of years and it has been revealed that the reactivity of NO^-/HNO significantly differs from that of NO^\bullet and peroxynitrite (ONOO^-). The biological effects of nitroxyl, however, are mainly unexplored. In neuronal cells, nitroxyl has been shown to protect from NMDA receptor-mediated toxicity by modifying critical cystein residues in the NMDA receptor [31]. On the other hand, nitroxyl anion has been shown to contribute to tissue injury in myocardial ischemia [13] and, in millimolar concentrations, also proved cytotoxic in fibroblasts and breast cancer cells [12,25]. In our present work, thymocytes were about an order of magnitude more sensitive targets for NO^- -induced cytotoxicity. This observation is in line with our previous observations that significantly smaller concentrations of

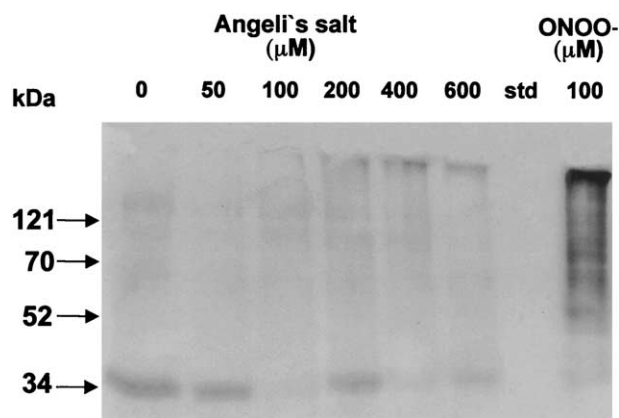


Fig. 5. Lack of tyrosine nitration in AS-treated thymocytes. Thymocytes were treated with the indicated concentrations of Angeli's salt or peroxynitrite for 1 h. Proteins in cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed for the presence of tyrosine-nitrated proteins.

oxidants (hydrogen peroxide, peroxynitrite) were required to kill thymocytes [9] than fibroblasts [32]. The explanation for cell type-specific differences in oxidant sensitivity of cells may lie in differences in metabolism, cell cycle, and antioxidant defense systems.

Based on previous observations reporting the DNA breaking effect of NO^- , we hypothesized that DNA breakage and poly(ADP-ribose) polymerase activation occurs also in cells exposed to NO^-/HNO . Indeed, we found that nitroxyl induced both DNA breakage and PARP activation in thymocytes. PARP activation by peroxynitrite or hydrogen peroxide has been shown by us and others to contribute to cell necrosis in a variety of cell types including thymocytes, fibroblasts, keratinocytes, macrophages, and neurons [9,10,32–35]. Nitroxyl-induced PARP activation also contributes to the AS-induced cytotoxicity in thymocytes as PARP inhibitors provided significant protection in this model. The mechanism by which PARP activation leads to cell death likely involves depletion of cellular NAD^+ and ATP stores as shown previously with other oxidants. Of note, protection provided by PARP inhibitors in nitroxyl-induced cytotoxicity was not as dramatic as previously observed with other PARP-activating stimuli, such as peroxynitrite or hydrogen peroxide [9,28].

This finding may point towards the involvement of PARP-independent mechanisms in nitroxyl-induced cell death. One such mechanism may be the reaction of nitroxyl with thiols. It has been shown that nitroxyl reacts with thiols [24,36]. Previous reports demonstrating that depletion of intracellular glutathion pools by L-buthionine sulfoxide sensitizes cells to NO^-/HNO toxicity indicate that thiols are in the first line of defense in cells exposed to NO^-/HNO [12]. Alternatively, NO^- decomposition could lead to the formation of the potent

cytotoxic oxidant peroxynitrite. In its triplet state, NO^- favorably reacts with molecular oxygen [37], giving rise to peroxynitrite formation [21,22]. Peroxynitrite nitrates tyrosine residues in proteins allowing convenient detection of peroxynitrite production in biological systems. However, under our experimental conditions we were unable to detect significant tyrosine nitration in AS-treated thymocytes, indicating that peroxynitrite is not likely to be responsible for cytotoxicity in nitroxyl-treated thymocytes. It may be worthwhile to note here that the apparent lack of AS-induced tyrosine nitration may also be due to a slow release of nitroxyl from AS as opposed to bolus addition of authentic peroxynitrite. NO^* per se can not be made responsible for cytotoxicity as conversion of NO^- into NO^* by electron acceptors [12] abolished AS-induced cytotoxicity. This finding is consistent with the current understanding of the biological role of nitric oxide viewing NO^* as a radical being more cytoprotective than cytotoxic.

In addition to necrotic cell death, AS also triggered apoptosis as evidenced by caspase activation and DNA fragmentation. At higher concentrations of AS, PARP activation shifted apoptosis toward necrosis as indicated by the reversal of suppression of DNA fragmentation and caspase activation by PARP inhibitors. The PARP-mediated apoptosis to necrosis switch observed in NO^- -treated thymocytes is likely to be caused by NAD^+ and ATP depletion, as previously described in hydrogen peroxide- or peroxynitrite-treated thymocytes and fibroblasts [9,10]. Again, necrosis to apoptosis switch by PARP inhibition was less pronounced than previously reported with peroxynitrite and hydrogen peroxide.

The question arises as to what pathophysiological implications our work may have. First, intrathymic nitric oxide production has been implicated in the elimination of potentially autoreactive thymocytes in a process called negative selection [9,38]. It may be plausible to assume that NO^-/HNO and/or peroxynitrite is the cytotoxic mediator involved in the nitric oxide-dependent negative selection. Furthermore, cytotoxic mechanisms identified in thymocytes may also operate in other cell types and may therefore have implications for a wide variety of pathophysiological conditions (e.g., myocardial ischemia) in which reactive nitrogen species have been implicated. However, as cell type-specific differences may occur, cytotoxic mechanisms of NO^-/HNO in other cell types need to be investigated.

In conclusion, in the present study we provide evidence for the involvement of multiple pathways in nitroxyl-induced cytotoxicity. Nitroxyl is capable of triggering both apoptotic and necrotic cell death with poly(ADP-ribose) polymerase activation serving as a switch between these two basic forms of cell death. Our

work may stimulate further research to identify the cytotoxic pathways triggered by NO^-/HNO .

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