

Poly(ADP-Ribose) Synthetase Activation Mediates Mitochondrial Injury During Oxidant-Induced Cell Death¹

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Reactive oxidant species are important mediators of tissue injury in shock, inflammation, and reperfusion injury. The actions of a number of these oxidants (e.g., hydroxyl radical and peroxynitrite, a reactive oxidant produced by the reaction of nitric oxide and superoxide) are mediated in part by the activation of the nuclear nick sensor enzyme, poly(ADP)-ribose synthetase (PARS), with consequent cellular energy depletion. Here we investigated whether PARS activation contributes to the mitochondrial alterations in cells exposed to oxidants. Authentic peroxynitrite (20 μ M), the peroxynitrite-generating compound 3-morpholinosisidnonimine, the combination of pyrogallol and *S*-nitroso-*N*-acetyl-D,L-penicillamine, as well as hydrogen peroxide induced a time- and dose-dependent decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) in thymocytes, as determined by flow cytometry using the mitochondrial potential sensitive dyes DiOC6(3) and JC-1. A time- and dose-dependent increase in secondary reactive oxygen intermediate production and loss of cardiolipin, an indicator of mitochondrial membrane damage, were also observed, as measured by flow cytometry using the fluorescent dyes dihydroethidine and nonyl-acridine orange, respectively. Inhibition of PARS by 3-aminobenzamide or 5-iodo-6-amino-1,2-benzopyrone attenuated peroxynitrite-induced $\Delta\Psi_m$ reduction, secondary reactive oxygen intermediate generation, cardiolipin degradation, and intracellular calcium mobilization. Furthermore, thymocytes from PARS-deficient animals were protected against the peroxynitrite- and hydrogen peroxide-induced functional and ultrastructural mitochondrial alterations. In conclusion, mitochondrial perturbations during oxidant-mediated cytotoxicity are, to a significant degree, related to PARS activation rather than to direct effects of the oxidants on the mitochondria. *The Journal of Immunology*, 1998, 161: 3753–3759.

Poly(ADP-ribose) synthetase (PARS),³ also known as poly(ADP)-ribose polymerase, is a nuclear nick sensor enzyme that becomes activated by recognizing DNA single-strand breaks. Upon activation, PARS cleaves NAD⁺ to nicotinamide and ADP-ribose and catalyzes the transfer of poly(ADP)-ribose adducts to various proteins. Excessive PARS activation has been shown to lead to cellular NAD⁺ and ATP depletion and cell necrosis (1–3). The PARS-mediated cellular suicide pathway contributes to tissue injury in shock, reperfusion, and inflammation (4–7). The exact mechanisms by which PARS activation contributes to cell death are not understood.

Recently, mitochondria emerged as key regulators of cell death (8–14). Intact mitochondria maintain a large (up to 180 mV) negative membrane potential across the mitochondrial inner membrane. A decrease in mitochondrial transmembrane potential followed by an intense reactive oxygen intermediate (ROI) production and a reduction of mitochondrial mass have been

shown to occur in various models of cell death (10–13). Although most work in this area focuses on the role of mitochondrial alterations during programmed cell death (apoptosis), mitochondrial alterations also play a role in the process of necrotic death (13–15). Oxidants, such as peroxynitrite and hydrogen peroxide, induce mitochondrial permeability transition and inhibit the mitochondrial respiratory chain (16–18).

Here we report that PARS activation mediates the mitochondrial injury in cells exposed to peroxynitrite or hydrogen peroxide. The data presented in the current study demonstrate that the changes in mitochondrial membrane potential, the mitochondrial permeability transition, the increase in ROI production, the increased calcium mobilization, and the destruction of mitochondrial structure are attenuated by inhibition of PARS.

Materials and Methods

Materials

Fluorescent dyes were purchased from Molecular Probes (Eugene, OR). INH2BP was a gift from Dr. E. Kun (State University of San Francisco, Tiburon, CA), and bongkreic acid was generously donated by Dr. J. A. Duine (Technical University of Delft, Delft, The Netherlands). Annexin V-FITC was obtained from PharMingen (San Diego, CA). Peroxynitrite was a kind gift of Dr. H. Ischiropoulos (University of Pennsylvania, Philadelphia, PA). 3-Morpholinosisidnonimine (SIN-1) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) were purchased from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma (St. Louis, MO).

Thymocyte preparation and treatment with oxidants

Thymi from wild-type (WT) and PARS-deficient mice (gift from Dr. Z. Q. Wang, Institute of Molecular Pathology, Vienna, Austria) were aseptically removed and placed into ice-cold RPMI (10% FCS, 10 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 g/ml streptomycin) medium. Single-cell suspensions were prepared by sieving the organs through a stainless wire mesh. Cells isolated this way were routinely 95% viable, as

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Received for publication March 5, 1998. Accepted for publication June 3, 1998.

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¹ This work was supported by grants from the National Institutes of Health (R29GM54773 and R01HL59266).

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³ Abbreviations used in this paper: PARS, poly(ADP-ribose) synthetase; ROI, reactive oxygen intermediates; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; SIN-1, 3-morpholinosisidnonimine; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; PG, pyrogallol; DiOC6(3), 3,3'-dihexyloxycarbocyanine iodide; HE, hydroethidine; NAO, 10-nonyl-acridine orange; 3-AB, 3-aminobenzamide; WT, wild-type.

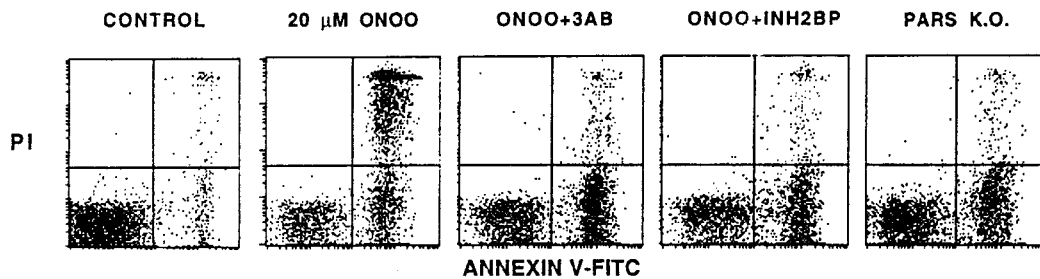


FIGURE 1. Antinecrotic effect of PARS inhibition. WT thymocytes were treated with 20 μ M peroxynitrite in the absence or the presence of 3-AB (1 mM) and INH2BP (100 μ M) for 4 h. PARS-deficient (PARS K.O.) cells were also treated with the same amount of peroxynitrite. Cells were then stained with annexin V-FITC to detect phosphatidylserine exposure and with propidium iodide to determine membrane integrity and were analyzed by flow cytometry.

assessed by trypan blue exclusion assay. Thymocytes were seeded in 24-well plates (0.5 ml/well) for cytofluorometry. Unless stated differently, inhibitors were applied as a pretreatment (15 min before the oxidants, except for bongkrekic acid, where a 30-min pretreatment was used).

Cells were treated with peroxynitrite (20 μ M) diluted in PBS, pH 8.9, and incubated for 3 h at 37°C. Cells treated with decomposed peroxynitrite (incubated for 20 min in PBS, pH 7.2) were used as the vehicle control. Decomposed peroxynitrite had no effect on any parameter measured. Hydrogen peroxide was diluted in PBS, pH 7.4, and added to the cells at various concentrations. In addition to authentic peroxynitrite, we tested the effect of a 3-h exposure of the cells to SIN-1 (200 μ M) and the combination of pyrogallol and SNAP (PG+SNAP; 100–100 μ M) to investigate the effect of continuous peroxynitrite generation.

Flow cytometry

After 3 h of incubation, thymocytes were stained with 40 nM DiOC6(3), 1 μ M JC-1, 2 μ M hydroethidine (HE), and 100 nM 10-N nonyl-acridine orange (NAO) for 15 min or with fura red (5 μ M) and Oregon green BAPTA-AM (3 μ M) for 30 min at 37°C, washed once with PBS, and analyzed with a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA). Forward and side scatters were gated on the major population of normal size cells. In control experiments cells were pretreated (1 h, 37°C) with 50 μ M carbonyl cyanide *m*-chlorophenyl hydrazone, a protonophore that completely de-energizes mitochondria by dissipating $\Delta\Psi_m$.

Samples processed for annexin V-FITC/propidium iodide staining (19) were washed in PBS, and 10^5 cells (in 100 μ l) were stained with 5 μ l annexin V-FITC and 5 μ g/ml propidium iodide in annexin binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂) at room temperature. After 15 min, 400 μ l annexin binding buffer was added to the samples, which were then immediately analyzed with flow cytometry.

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was quantitated by the flow cytometric analysis of DiOC6(3)-stained cells (12). Lipophilic cations such as the fluorescent dyes DiOC6(3), JC-1, or rhodamine are transported into the mitochondria by the negative mitochondrial membrane potential and thus concentrated within the mitochondrial matrix. Since under certain conditions, DiOC6(3) fluorescence may be influenced by the cell membrane potential, we confirmed our results obtained with DiOC6(3) by using JC-1, a fluorescent dye that forms fluorescent aggregates when $\Delta\Psi_m$ decreases (20), a process unaffected by the changes in cell membrane potential.

Determination of secondary ROI generation and mitochondrial membrane damage

Intramitochondrial generation of ROI was determined using a previously established flow cytometry technique based on the superoxide-induced conversion of the oxidant-sensitive dye, hydroethidine (HE), to ethidium (21). Mitochondrial membrane damage was determined by measuring the concentration of cardiolipin, the cellular distribution of which is restricted to mitochondria. The assay uses the fluorochrome NAO, which stoichiometrically interacts with cardiolipin (1:2); this interaction is not influenced by the mitochondrial state (12).

Electron microscopy

Thymocytes were treated with 20 μ M peroxynitrite and then incubated for 3 h at 37°C. Cells were then washed with PBS and fixed with 2% glutar-

aldehyde in 0.1 M cacodylate buffer, pH 7.3, for 2 h at 4°C. Fixed cells were washed in 0.1 M cacodylate buffer (twice, 10 min each time) and postfixed in 1% OsO₄ in 0.1 M cacodylate for 1 h. After osmication and washings, samples were incubated in 2% uranyl acetate (1 h) and then dehydrated with graded ethanols and embedded in epoxy resin. Sections (0.8 nm) were stained with lead citrate and uranyl acetate and examined with a Hitachi transmission electron microscope (Hialeah, FL).

Statistical analysis

All values in the figures and text are expressed as the mean \pm SEM of n observations ($n \geq 4$). Datasets were examined by analysis of variance, and individual group means were then compared with Bonferroni's post-hoc test; $p < 0.05$ was considered statistically significant. When the results are presented as representative flow cytometric analyses, results identical with the ones shown were obtained in at least three different experiments.

Results

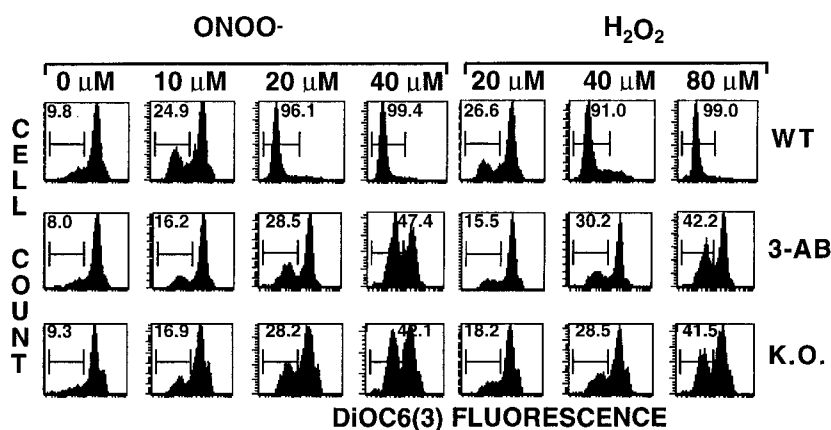
PARS inhibition protects against peroxynitrite-induced thymocyte necrosis

A characteristic feature of necrotic cell death is the loss of plasma membrane integrity, whereas early during apoptosis phosphatidylserine translocates from the inner to the outer plasma membrane layer (22). Exposure of WT thymocytes to 20 μ M peroxynitrite results in phosphatidylserine exposure, as determined by annexin V-FITC binding, and in the loss of plasma membrane integrity, as indicated by propidium iodide uptake (Fig. 1). Inhibition of PARS by 3-AB and INH2BP or the absence of PARS protected cells from the increased membrane permeability, as shown by the shift of the annexin V-FITC/PI double-positive population toward an annexin V-FITC single-positive population (Fig. 1). This finding coupled with our previous observation demonstrating that PARS inhibition causes an increased DNA fragmentation of peroxynitrite-treated thymocytes (23) indicate that PARS inhibition diverts a subpopulation of the cells from the necrotic toward the apoptotic pathway. Furthermore, in peroxynitrite-treated cells in the absence of functional PARS, the proportion of cells exhibiting neither increased annexin V-FITC binding nor increased propidium iodide also increases consistently with complete rescue of a smaller subpopulation of the cells from oxidant-induced death (Fig. 1).

Peroxyntite-induced $\Delta\Psi_m$ reduction is mediated by PARS activation

Since mitochondrial dysfunction has been proposed to represent a point of no return during cell death (12), we hypothesized that PARS activation-related changes may act proximal to the disruption of mitochondrial membrane potential. Thymocytes exposed to peroxynitrite or hydrogen peroxide displayed a dose- and time-dependent decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$; Figs. 2 and 3). Using 3-AB (1 mM) and INH2BP (100 μ M), cellular inhibitors of PARS (24, 25), we found that both

FIGURE 2. PARS activation causes $\Delta\Psi_m$ reduction. WT thymocytes were treated with the indicated amounts of peroxynitrite (ONOO⁻) or H₂O₂ in the presence or the absence of the PARS inhibitor 3-AB. In addition, PARS-deficient thymocytes (K.O.) were treated with the same amount of oxidants and were incubated for 3 h. Cells were then stained with DiOC6(3) and analyzed for flow cytometry. Numbers indicate the percentage of gated cells displaying decreased $\Delta\Psi_m$.



pharmacologic inhibitors of PARS abrogated the peroxynitrite-induced $\Delta\Psi_m$ reduction (Figs. 2 and 3). Similarly, cells from PARS knockout mice were resistant to changes in $\Delta\Psi_m$ in response to hydrogen peroxide or peroxynitrite (Figs. 2 and 3). However, when using agents that do not induce DNA single-strand breakage (which is the obligatory trigger of PARS activation), such as dexamethasone, $\Delta\Psi_m$ reduction was unaffected by inhibition of PARS (data not shown), indicating the specific role of DNA single-strand break-induced PARS activation in aggravating cell death.

Bongkrekcic acid, an inhibitory ligand of the mitochondrial adenine nucleotide translocator, inhibits the formation of mitochondrial channels, an event found to occur in various models of apoptotic (11–13) and in some models of necrotic (13–15) death. The peroxynitrite-induced (15 μM) $\Delta\Psi_m$ reduction was significantly ($p < 0.01$) reduced by 50 μM bongkrekcic acid ($64.68 \pm 4.9\%$ in the absence of bongkrekcic acid vs $45.9 \pm 2.1\%$ in the presence of the inhibitor). This finding indicates that PARS activation-induced $\Delta\Psi_m$ reduction is mediated by pore formation to only a minor extent.

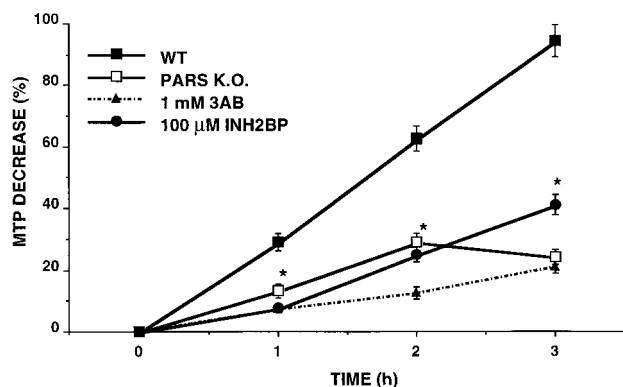


FIGURE 3. Time course of peroxynitrite-induced $\Delta\Psi_m$ disruption. WT and PARS-deficient thymocytes were treated with peroxynitrite (20 μM) in the absence or the presence of 3-AB (1 mM) and INH2BP (100 μM) for 1 to 3 h. Cells were then stained with DiOC6(3) and analyzed by flow cytometry. Peroxynitrite induced $\Delta\Psi_m$ disruption was calculated as follows: $100 \times (T - C) / (100 - C)$, where T is the percentage of peroxynitrite-treated cells displaying decreased mitochondrial transmembrane potential (MTP), and C is the corresponding value in control samples. Data represent the mean \pm SEM of four observations. *, Significant ($p < 0.05$) suppression of the effect of peroxynitrite in the groups where PARS was inhibited compared with the response in WT cells.

Secondary ROI production and mitochondrial membrane damage are attenuated by PARS inhibition

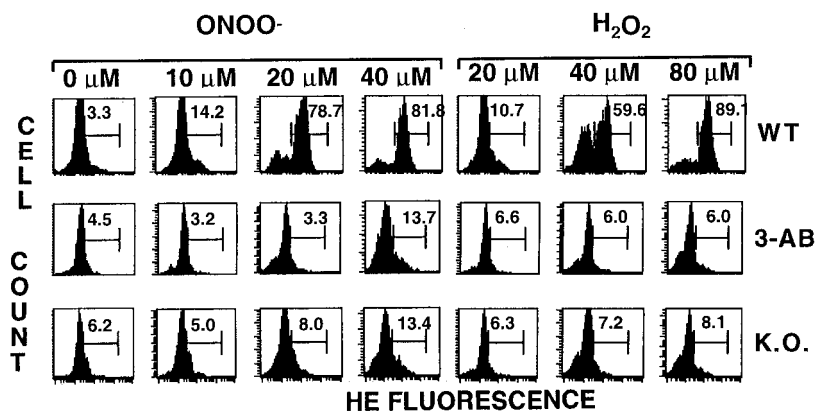
Disruption of the mitochondrial transmembrane potential is usually followed by increased mitochondrial ROI production and mitochondrial membrane damage (10). We have found that peroxynitrite and hydrogen peroxide induced a dose- and time-dependent increase in ROI production (Figs. 4 and 5) and triggered a loss of mitochondrial cardiolipin (Fig. 6). Both effects were blocked by the PARS inhibitors 3-AB and INH2BP or by the PARS-negative phenotype (Figs. 5–7). The loss of cardiolipin may result from the secondary ROI production and is unlikely to be related to a direct effect of peroxynitrite, since treatment of the cells (1 h after peroxynitrite exposure) with the antioxidants glutathione (10 mM) and *N*-acetyl-cysteine (10 mM) significantly reduced secondary ROI production and the loss of cardiolipin content (Fig. 7). Since at physiologic pH, the half-life of peroxynitrite is < 1 s, this inhibition cannot be attributed to the scavenging of peroxynitrite itself by the antioxidants. Moreover, antioxidant treatment also inhibited $\Delta\Psi_m$ reduction to a small extent (Fig. 7C). Although ROI production is considered to be the consequence of $\Delta\Psi_m$ reduction, our finding is in line with the concept proposed by Kroemer and colleagues that mitochondrial perturbations trigger self-amplifying vicious circles. *L*-N^G-methyl arginine (1 mM), an inhibitor of nitric oxide synthase, did not inhibit the peroxynitrite-induced progressive mitochondrial membrane damage (Fig. 7), indicating that endogenous formation of nitric oxide or peroxynitrite does not play a role in the peroxynitrite-induced progressive mitochondrial alterations.

Continuous generation of peroxynitrite with either SIN-1 or PG+SNAP exerted similar effects as authentic peroxynitrite (Table I). Similar to the findings with authentic peroxynitrite, mitochondrial $\Delta\Psi_m$ reduction, ROI production, and cardiolipin loss were reduced in thymocytes from PARS^{-/-} animals compared with the response in WT cells (Table I).

PARS activation leads to calcium mobilization

Since disruption of mitochondrial function and subsequent oxidative stress are often followed by an elevated intracellular Ca²⁺ level, we have investigated whether Ca²⁺ is mobilized in the cells exposed to the oxidants. Peroxynitrite caused a dose-dependent increase in cytosolic free Ca²⁺ as indicated by the decreased fluorescence of the Ca²⁺-sensitive dye fura red (Fig. 8). (Similar results were obtained using another Ca²⁺ sensitive dye, Oregon green BAPTA-AM, which shows an increased fluorescence upon binding to Ca²⁺.) PARS-deficient thymocytes and cells treated

FIGURE 4. PARS activation leads to secondary ROI production. WT thymocytes were treated with various concentrations of peroxynitrite (ONOO⁻) or H₂O₂ in the presence or the absence of the PARS inhibitor 3-AB. In addition, PARS-deficient thymocytes (K.O.) were treated with the same concentration of oxidants and were incubated for 3 h. Cells were then stained with the superoxide-sensitive dye HE and analyzed for flow cytometry. Numbers indicate the percentage of gated cells displaying increased ROI production.



with the PARS inhibitors mobilized significantly less Ca²⁺, indicating the crucial role of PARS activation-induced changes in the induction of Ca²⁺ efflux (Fig. 8).

PARS activation leads to mitochondrial destruction

To provide morphologic evidence of mitochondrial destruction indicated by NAO staining, we conducted electron microscopic examinations on peroxynitrite-treated (15 μM) cells. WT thymocytes challenged with the oxidants displayed a typical necrotic morphology (swollen cytoplasm and organelles and decreased electron density), with mitochondrial damage signs ranging from broken cristae to high amplitude mitochondrial swelling, total disruption of ultrastructure, and appearance of flocculent matrix densities. In comparison, mitochondria of the PARS-deficient cells showed no or minor changes (Fig. 9). Gross morphologic changes in the mitochondria have been thought to be characteristic of necrosis. However, recently, severe mitochondrial damage has also been found to occur during apoptosis (26).

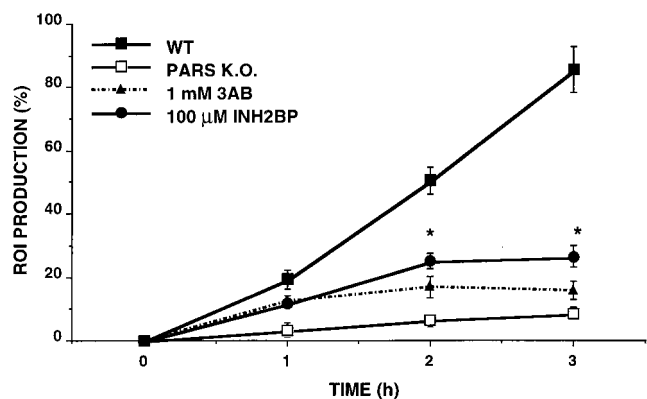


FIGURE 5. Time course of peroxynitrite-induced secondary ROI production. WT and PARS-deficient thymocytes were treated with peroxynitrite (20 μM) in the absence or the presence of 3-AB (1 mM) and INH2BP (100 μM) for 1 to 3 h. Cells were then stained with HE and analyzed by flow cytometry. Peroxynitrite-induced secondary ROI production was calculated as follows: $100 \times (T - C) / (100 - C)$, where T is the percentage of peroxynitrite-treated cells displaying increased ROI production, and C is the corresponding value in control samples. Data represent the mean \pm SEM of four observations. *, Significant ($p < 0.05$) suppression of the effect of peroxynitrite in the groups where PARS was inhibited compared with the response in WT cells.

Discussion

ROIs and reactive nitrogen intermediates are now considered major mediators of tissue injury in various pathophysiologic conditions (27–30). Peroxynitrite, hydrogen peroxide, and hydroxyl radical can cause DNA single-strand breaks and induce PARS activation (31, 32). Since PARS activation rapidly depletes cellular NAD⁺ and ATP (1–6), both of which are important regulators of mitochondrial functions (33–38), in the present study we hypothesized that besides having direct inhibitory effect on mitochondrial function, ROIs and reactive nitrogen intermediates may also exert PARS-mediated effect on mitochondria. Our current findings provide evidence that the mitochondrial perturbations in cells exposed to relatively low, pathophysiologically relevant concentrations of oxidants are related to a PARS-related indirect route, rather than to a direct damaging effect of the oxidants toward the mitochondria.

Disrupted mitochondrial membrane potential followed by increased ROI generation, loss of mitochondrial cardiolipin, and increased intracellular Ca²⁺ level have recently been described as common features of the apoptotic process. The current results demonstrate that these changes can also occur during oxidant-induced necrotic cell death. Although peroxynitrite can cause delayed apoptosis (39, 40), peroxynitrite-induced apoptosis is not attenuated by PARS inhibitors (5, 23, 41, 42). On the contrary, PARS inhibition of peroxynitrite-treated thymocytes can shift the necrotic cell death toward apoptosis, as indicated by an increased output of apoptotic parameters (DNA fragmentation and phosphatidylserine exposure) (23). In hydrogen peroxide-treated human myeloid leukemia U937 cells, inhibition of PARS has been shown to reduce necrosis, but increase apoptosis (43), whereas in a human epithelial cell line, the suppression of hydrogen peroxide-induced necrosis by 3-AB was not associated with increased apoptosis (44).

The data presented in the present study put the mode of oxidant-induced cell death into a new perspective (Fig. 10). 1) The current data, contrary to the previously held view, demonstrate that oxidant-induced mitochondrial alterations are not due to a direct effect of the oxidants on the mitochondria, but are related to an indirect mechanism governed by PARS. 2) It is a widely held view that that necrosis is a process that cannot be influenced by pharmacologic means, and apoptosis is the process that is under the control of a sophisticated cellular machinery. The present data, demonstrating protection against mitochondrial injury by inhibition of PARS, support the view that cellular necrosis and related mitochondrial injury indeed are governed by an endogenous regulatory mechanism, i.e., PARS. 3) The role of PARS in the process of apoptotic cell death is generally viewed as a terminal effector step, whereby

FIGURE 6. PARS activation leads to mitochondrial membrane damage. WT thymocytes were treated with the indicated amounts of peroxynitrite (ONOO⁻) or H₂O₂ in the presence or the absence of the PARS inhibitor 3-AB. In addition, PARS-deficient thymocytes (K.O.) were treated with the same amount of oxidants and were incubated for 3 h. Cells were then stained with the cardiolipin-binding dye NAO and analyzed for flow cytometry. Numbers indicate the percentage of gated cells displaying decreased cardiolipin content.

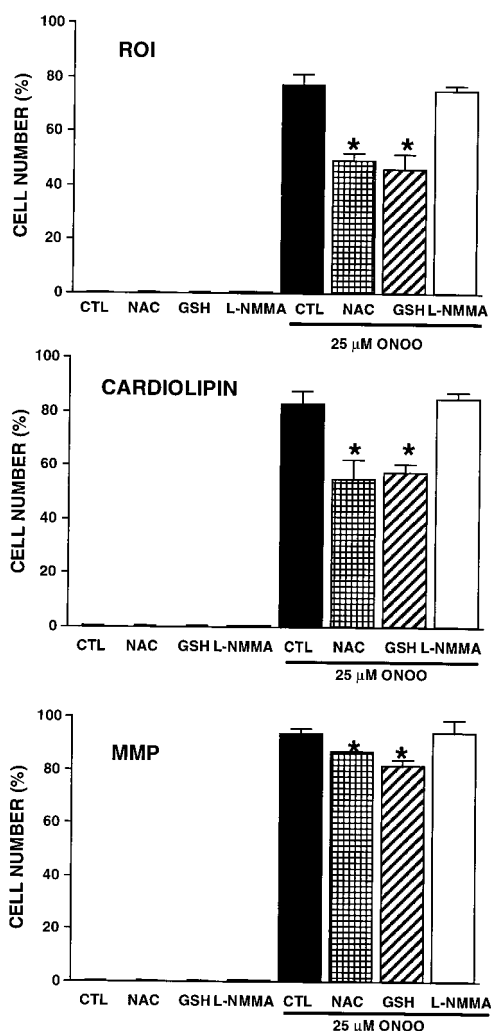
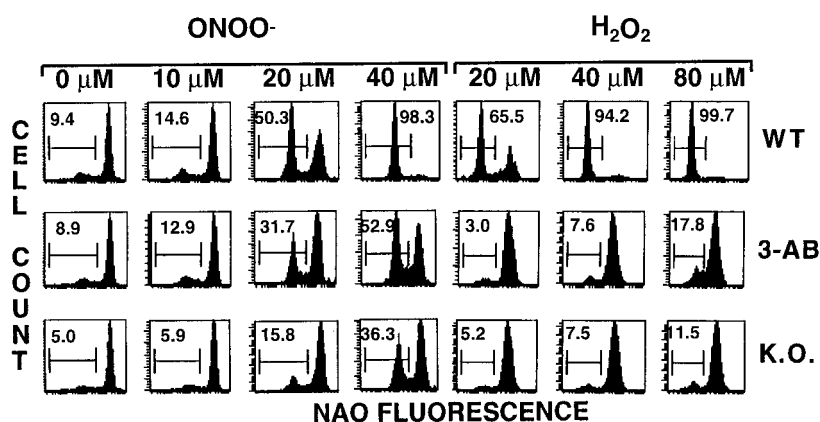


FIGURE 7. Pharmacologic modulation of peroxynitrite-induced mitochondrial alterations. Thymocytes were treated with 25 μM peroxynitrite. Cells were either pretreated with L-N^G-methyl arginine (1 mM) 15 min before peroxynitrite treatment or post-treated with glutathione (GSH; 10 mM) or N-acetyl cysteine (NAC; 10 mM). After 3 h of incubation cells were stained with HE, NAO, and DiOC6(3) and analyzed for flow cytometry. Values were calculated as 100 × (T - C/100 - C), where T is the percentage of peroxynitrite-treated cells displaying increased HE, decreased NAO, or DiOC6(3) staining, and C is the corresponding value in control samples. Data represent the mean ± SEM of five observations. *, Significant (p < 0.05) suppression of the mitochondrial alterations by the antioxidants.

PARS acts as a “death substrate” for caspases (45–48). The present data demonstrate that during oxidant-induced cell necrosis, PARS mediates an early, rather than a delayed, effector mechanism, at a level proximal to mitochondrial alterations.

Previous studies on peroxynitrite-induced apoptosis or necrosis (in neurons, epithelial cells, endothelial cells, macrophages, smooth muscle cells, and other cell types) routinely used exposure of cells to peroxynitrite concentrations ranging from 50 μM to 1.5 mM to elicit cytotoxic effects (3, 4, 6, 17, 39–41, 49, 50). In the current study, a lower concentration of peroxynitrite (20 μM) was found to induce significant cytotoxicity in WT thymocytes. Nevertheless, the question arises as to whether the concentrations of peroxynitrite used in the current study are pathophysiologically relevant. Ischiropoulos and colleagues reported rates of peroxynitrite formation in the range of 0.1 nmol/10⁶ cells/min from stimulated macrophages (51). This translates into rates of peroxynitrite formation in the range of 0.8 μM/min for an inflamed organ (lung) or 7 μM/min within a blood vessel (18, 51). Cells that are in direct contact with immunostimulated cells (e.g., macrophages) are likely to be exposed to even higher rates (51). In comparison, in the current study the use of a single bolus of 20 μM peroxynitrite is equivalent to 0.52 μM peroxynitrite maintained for 1 min, calculated as previously described (52). Therefore, the concentration of peroxynitrite used in the current study is likely to be in the pathophysiologically relevant range. The concomitant generation of superoxide and nitric oxide from SIN-1 for 1 h, which corresponds to a rate of peroxynitrite generation of 2.5 μM/min (18), also resulted in significant mitochondrial injury, which was attenuated in the absence of functional PARS. Again, this rate of peroxynitrite generation is in the pathophysiologically relevant range (see

Table I. Effect of continuous generation of peroxynitrite with SIN-1 or PG+SNAP on mitochondrial parameters of cell death

	WT		PARS Knockout	
	PG+SNAP	SIN-1	PG+SNAP	SIN-1
MMP ↓	62.9 ± 1.2	43.5 ± 3.0	9.6 ± 0.8	6.0 ± 0.2
ROS ↑	43.7 ± 0.8	38.0 ± 3.1	1.7 ± 0.3	4.8 ± 0.4
Cardiolipin ↓	63.3 ± 0.6	43.0 ± 0.5	14.9 ± 0.5	7.6 ± 0.5

^a Thymocytes from WT and PARS-deficient mice were treated with 200 μM SIN-1 or PG+SNAP (100–100 μM) for 3 h. Cells were then washed free of the drugs and incubated for an additional 3 h at 37°C. Percent number of cells (± SEM of n = 4 measurements) displaying decreased ΔΨ_m, increased ROI production, and decreased NAO staining are shown. In the PARS knockout thymocytes, the oxidants induced a significantly lower (p < 0.01) degree of mitochondrial injury than in the WT control cells.

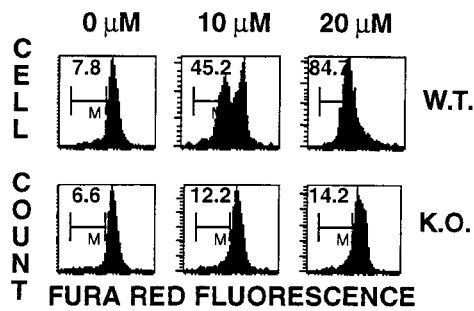


FIGURE 8. PARS activation leads to calcium mobilization. Thymocytes from WT and PARS knockout (K.O.) mice were loaded with the calcium-sensitive dye fura red and treated with 15 and 30 μM peroxynitrite. Calcium mobilization is indicated by decreased red fluorescence. Numbers indicate the percentage of gated cells with increased cytosolic free calcium.

above). Similarly, in pulmonary type II cells, fluxes of peroxynitrite in the range of 0.5 to 2.5 $\mu\text{M}/\text{min}$ have been shown to induce significant cytotoxicity (18).

Necrotic cell death is an important pathway of cell death, which has direct relevance for various forms of reperfusion injury and for various forms of inflammation. Under such conditions, overwhelming oxidant production can occur, and cells die via the necrotic route. In recent *in vivo* experiments, pharmacologic inhibition or inactivation of PARS protected against stroke, myocardial reperfusion injury, shock, and inflammation (4–7, 31). Based on the current work, we propose that prevention of mitochondrial injury and consequent cell necrosis is one of the mechanisms by which PARS inhibitors exert beneficial effects in various pathophysiological conditions.

Acknowledgments

We thank Dr. J. A. Duine (Technical University of Delft, Delft, The Netherlands) for the gift of bongkreic acid, Mr. R. Montione and Ms. L. Engelman (University of Cincinnati, Cincinnati, OH) for their help with electron microscopy, Dr. Z. Q. Wang (Institute of Molecular Pathology,

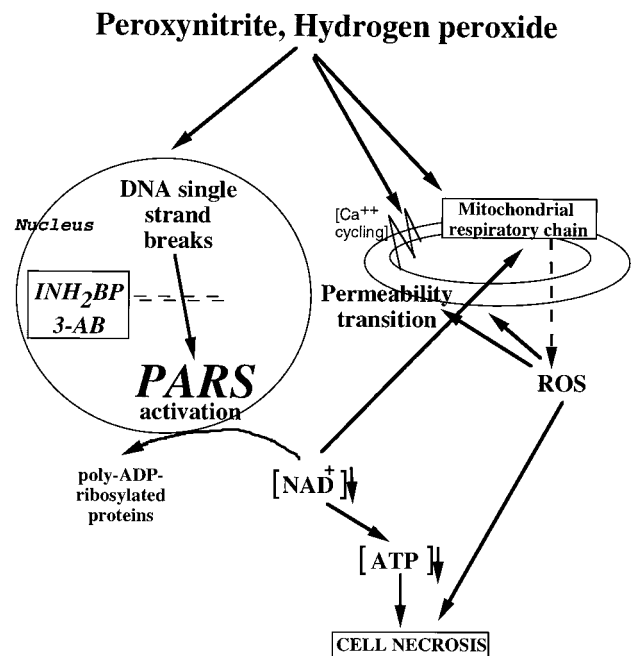


FIGURE 10. Proposed scheme of PARS-dependent and PARS-independent mitochondrial alterations in cells exposed to hydrogen peroxide or peroxynitrite. Hydrogen peroxide and peroxynitrite trigger the development of DNA single-strand breakage, with consequent activation of PARS. Massive poly(ADP) ribosylation leads to NAD^+ depletion, which potentiates the oxidant-induced mitochondrial dysfunction and mitochondrial free radical generation, resulting in cell necrosis.

Vienna, Austria) for providing the PARS knockout animals, Dr. H. Ischiropoulos (Institute of Environmental Medicine, University of Pennsylvania, Philadelphia, PA) for providing peroxynitrite and for helpful discussions, Dr. Rafael Radi (Universidad de la Republica, Montevideo, Uruguay) for helpful discussions, and Dr. E. Kun (San Francisco State University, Tiburon, CA) for the kind gift of INH2BP.

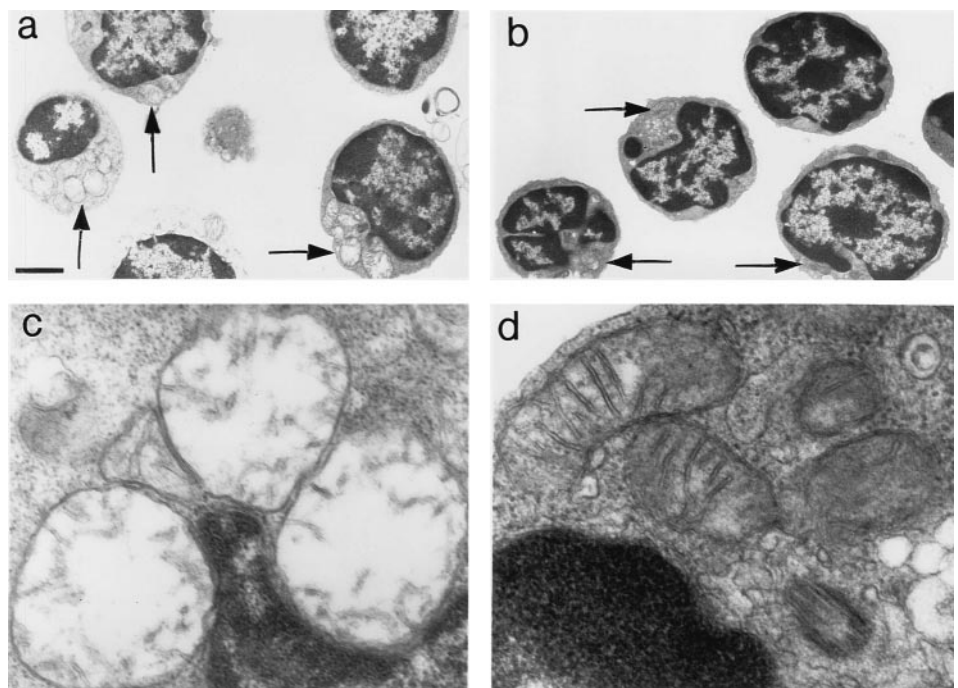


FIGURE 9. PARS activation results in mitochondrial destruction. Electron microscopic picture of WT thymocytes (*a* and *c*) treated with 20 μM peroxynitrite revealed typical necrotic morphology with decreased electron density, swollen mitochondria (arrows), disrupted cristae, and flocculant matrix densities. PARS-deficient thymocytes (*b*, and *d*) exposed to the same concentration of peroxynitrite exhibited markedly reduced mitochondrial injury. Magnification for *a*, *b*, *c*, and *d*, $\times 6640$, $\times 6640$, $\times 33200$, and $\times 58100$, respectively. Scale bar = 2.5 μm for *a* and *b*, 0.5 μm for *c*, and 0.26 μm for *d*.

References

1. Schraufstatter, I. U., P. A. Hyslop, D. B. Hinshaw, R. G. Spragg, L. A. Sklar, and C. G. Cochrane. 1986. Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA* 83:4908.
2. Zhang, J., V. L. Dawson, T. M. Dawson, and S. H. Snyder. 1994. Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science* 263:687.
3. Szabó, C., B. Zingarelli, M. O'Connor, and A. L. Salzman. 1996. DNA strand breakage, activation of poly(ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc. Natl. Acad. Sci. USA* 93:1753.
4. Szabó, C., B. Zingarelli, and A. L. Salzman. 1996. Role of poly-ADP ribosyltransferase activation in the vascular contractile and energetic failure elicited by exogenous and endogenous nitric oxide and peroxynitrite. *Circ. Res.* 78:1051.
5. Endres, M., Z. Q. Wang, S. Namura, C. Waerber, and M. A. Moskowitz. 1997. Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J. Cereb. Blood Flow Metab.* 17:1143.
6. Eliasson, M. J., K. Sampei, A. S. Mandir, P. D. Hurn, R. J. Traystman, J. Bao, A. Pieper, Z. Q. Wang, T. M. Dawson, S. H. Snyder, and V. L. Dawson. 1997. Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med.* 3:1089.
7. Szabó, C., L. H. Lim, S. Cuzzocrea, S. J. Getting, B. Zingarelli, R. J. Flower, A. L. Salzman, and M. Perretti. 1997. Inhibition of poly(ADP-ribose) synthetase attenuates neutrophil recruitment and exerts antiinflammatory effects. *J. Exp. Med.* 186:1041.
8. Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275:1129.
9. Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275:1132.
10. Kroemer, G., N. Zamzami, and S. A. Susin. 1997. Mitochondrial control of apoptosis. *Immunol. Today* 18:44.
11. Petit, P. X., N. Zamzami, J. L. Vayssiere, B. Mignotte, G. Kroemer, and M. Castedo. 1997. Implication of mitochondria in apoptosis. *Mol. Cell. Biochem.* 174:185.
12. Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, and G. Kroemer. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med.* 181:1661.
13. Zamzami, N., T. Hirsch, B. Dallaporta, P. X. Petit, and G. Kroemer. 1997. Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. *J. Bioenerg. Biomembr.* 29:185.
14. Ankarcrona, M., J. M. Dypbukt, E. Bonfoco, B. Zhivotovsky, S. Orrenius, S. A. Lipton, and P. Nicotera. 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* 15:961.
15. Aguilar, H. I., R. Botla, A. S. Arora, S. F. Bronk, and G. J. Gores. 1996. Induction of the mitochondrial permeability transition by protease activity in rats: a mechanism of hepatocyte necrosis. *Gastroenterology* 110:558.
16. Lizasoain, I., M. A. Moro, R. G. Knowles, V. Darley-Usmar, and S. Moncada. 1996. Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *Biochem. J.* 314:877.
17. Packer, M. A., and M. P. Murphy. 1995. Peroxynitrite formed by simultaneous nitric oxide and superoxide generation causes cyclosporin-A-sensitive mitochondrial calcium efflux and depolarisation. *Eur. J. Biochem.* 234:231.
18. Gow, A. J., S. R. Thom, and H. Ischiropoulos. 1998. Nitric oxide and peroxynitrite-mediated pulmonary cell death. *Am. J. Physiol.* 274:L112.
19. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* 184:39.
20. Reers, M., T. W. Smith, and L. B. Chen. 1991. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry* 30:4480.
21. Rothe, G., and G. Valet. 1990. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J. Leukocyte Biol.* 47:440.
22. Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, S. R. C. van, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* 182:1545.
23. Virág, L., G. S. Scott, A. L. Salzman, and C. Szabó. 1998. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly-(ADP-ribose) synthetase (PARS) activation. *Immunology* 94:345.
24. Banasik, M., H. Komura, M. Shimoyama, and K. Ueda. 1992. Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. *J. Biol. Chem.* 267:1569.
25. Szabó, C., S. Cuzzocrea, B. Zingarelli, M. O'Connor, and A. L. Salzman. 1997. Endothelial dysfunction in a rat model of endotoxic shock. Importance of the activation of poly(ADP-ribose) synthetase by peroxynitrite. *J. Clin. Invest.* 100:723.
26. Vander, H. M. G., N. S. Chandel, E. K. Williamson, P. T. Schumacker, and C. B. Thompson. 1997. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 91:627.
27. Moncada, S. 1997. Nitric oxide in the vasculature. *Ann. N Y Acad. Sci.* 811:60.
28. McCord, J. M. 1993. Oxygen-derived free radicals. *New Horiz.* 1:70.
29. Beckman, J. S., and W. H. Koppenol. 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* 271:C1424.
30. Szabó, C. 1996. DNA strand breakage and activation of poly-ADP ribosyltransferase: a cytotoxic pathway triggered by peroxynitrite. *Free Radical Biol. Med.* 21:855.
31. Szabó, C., V. L. Dawson. 1998. Role of poly(ADP-ribose) synthetase activation in inflammation and reperfusion injury. *Trends Pharmacol. Sci.* 19:287.
32. Halliwell, B., and O. I. Aruoma. 1991. DNA damage by oxygen-derived species: its mechanism and measurement in mammalian systems. *FEBS Lett.* 281:9.
33. Devin, A., B. Guerin, and M. Rigoulet. 1997. Cytosolic NAD⁺ content strictly depends on ATP concentration in isolated liver cells. *FEBS Lett.* 410:329.
34. Rustin, P., B. Parfait, D. Chretien, T. Bourgeron, F. Djouadi, J. Bastin, A. Rotig, and A. Munnich. 1996. Fluxes of nicotinamide adenine dinucleotides through mitochondrial membranes in human cultured cells. *J. Biol. Chem.* 271:14785.
35. Takeyama, N., N. Matsuo, and T. Tanaka. 1993. Oxidative damage to mitochondria is mediated by the Ca²⁺-dependent inner-membrane permeability transition. *Biochem. J.* 294:719.
36. Costantini, P., B. V. Chernyak, V. Petronilli, and P. Bernardi. 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J. Biol. Chem.* 271:6746.
37. Lee, A. C., X. Xu, and M. Colombini. 1996. The role of pyridine dinucleotides in regulating the permeability of the mitochondrial outer membrane. *J. Biol. Chem.* 271:26724.
38. Zizi, M., M. Forte, E. Blachly-Dyson, and M. Colombini. 1994. NADH regulates the gating of VDAC, the mitochondrial outer membrane channel. *J. Biol. Chem.* 269:1614.
39. Salgo, M. G., G. L. Squadrito, and W. A. Pryor. 1995. Peroxynitrite causes apoptosis in rat thymocytes. *Biochem. Biophys. Res. Commun.* 215:1111.
40. Lin, K. T., J. Y. Xue, M. Nomen, B. Spur, and P. Y. Wong. 1995. Peroxynitrite-induced apoptosis in HI-60 cells. *J. Biol. Chem.* 270:16487.
41. Leist, M., B. Single, G. Kunstle, C. Volbracht, H. Hentze, and P. Nicotera. 1997. Apoptosis in the absence of poly-(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.* 233:518.
42. Wang, Z. Q., L. Stingl, C. Morrison, M. Jantsch, M. Los, K. Schulze-Osthoff, and E. F. Wagner. 1997. PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev.* 11:2347.
43. Palomba, L., P. Sestili, F. Cattabeni, A. Azzi, and O. Cantoni. 1996. Prevention of necrosis and activation of apoptosis in oxidatively injured human myeloid leukemia U937 cells. *FEBS Lett.* 390:91.
44. Watson, A. J., J. N. Askew, and R. S. Benson. 1995. Poly(adenosine diphosphate ribose) polymerase inhibition prevents necrosis induced by H₂O₂ but not apoptosis. *Gastroenterology* 109:472.
45. Kaufmann, S. H., S. Desnoyers, Y. Ottaviano, N. E. Davidson, and G. G. Poirier. 1993. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.* 53:3976.
46. Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, et al. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37.
47. Tewari, M., L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen, and V. M. Dixit. 1995. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81:801.
48. Nicholson, D. W., and N. A. Thornberry. 1997. Caspases: killer proteases. *Trends Biochem. Sci.* 22:299.
49. Sandoval, M., X. Liu, E.E. Mannick, D.A. Clark, and M. J. Miller. 1996. Peroxynitrite-induced apoptosis in human intestinal epithelial cells is attenuated by mesalamine. *Gastroenterology* 113:1480.
50. Cookson, M. R., P. G. Ince, and P. J. Shaw. 1998. Peroxynitrite and hydrogen peroxide induced cell death in the NSC34 neuroblastoma × spinal cord cell line: role of poly(ADP-ribose) polymerase. *J. Neurochem.* 70:501.
51. Ischiropoulos, H., L. Zhu, and J. S. Beckman. 1992. Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* 298:446.
52. Beckman, J. S., J. Chen, H. Ischiropoulos, and J.P. Crow. 1994. Oxidative chemistry of peroxynitrite. *Methods Enzymol.* 233:229.