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Requirement of Intracellular Calcium Mobilization for Peroxynitrite-Induced Poly(ADP-Ribose) Synthetase Activation and Cytotoxicity

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ABSTRACT

Peroxynitrite is a cytotoxic oxidant produced during shock, ischemia reperfusion, and inflammation. The cellular events mediating the cytotoxic effect of peroxynitrite include activation of poly(ADP-ribose) synthetase, inhibition of mitochondrial respiration, and activation of caspase-3. The aim of the present study was to investigate the role of intracellular calcium mobilization in the necrotic and apoptotic cell death induced by peroxynitrite. Peroxynitrite, in a low, pathophysiologically relevant concentration (20 μM), induces rapid (1 to 3 min) Ca^{2+} mobilization in thymocytes. Inhibition of this early calcium signaling by cell-permeable Ca^{2+} chelators [EGTA-acetoxymethyl ester (AM), 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-AM (BAPTA-AM), 8-amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-*N,N,N′,N′*-tetraacetic acid-tetra-AM] abolished cytotoxicity as measured by propidium iodide uptake. Intracellular Ca^{2+} chelators also inhibited DNA single-strand breakage and activation of poly(ADP-ribose) synthase (PARS), which is a major mediator of cell necrosis in the current model. Intracellular Ca^{2+} chelators also protected PARS-deficient thymocytes from peroxynitrite cytotoxicity, providing evidence for a PARS-independent, Ca^{2+}-dependent cytotoxic pathway. Chelation of intracellular Ca^{2+} blocked the peroxynitrite-induced decrease of mitochondrial membrane potential, secondary superoxide production, and mitochondrial membrane damage. Peroxynitrite-induced internucleosomal DNA cleavage was increased on BAPTA-AM pretreatment in the wild-type cells but decreased in the PARS-deficient cells. Two other apoptotic parameters (phosphatidylserine exposure and caspase 3 activation) were inhibited by BAPTA-AM in both the wild-type and the PARS-deficient thymocytes. Our findings provide evidence for the pivotal role of an early Ca^{2+} signaling in peroxynitrite cytotoxicity.

Peroxynitrite ($ONOO^-$) is a potent oxidant formed in a near diffusion-limited reaction of superoxide (O_2^-) and nitric oxide (NO) (Beckman et al., 1994; Beckman and Koppenol, 1996). Its pathophysiological role has been demonstrated in various forms of shock, inflammation, and ischemia reperfusion (Szabó et al., 1996a,b, 1997a; Zingarelli et al., 1997b). Little is known about the intracellular signals mediating the cytotoxic effect of peroxynitrite. A major cytotoxic pathway triggered by peroxynitrite and other oxidants is the activation of poly(ADP-ribose) synthetase (PARS; Zhang et al., 1994; Szabó et al., 1996a,b, 1997a,b; Eliasson et al., 1997). PARS is a DNA nick sensor enzyme that is activated by DNA single-strand breaks (SSBs); it cleaves NAD^+ to nicotinamide and ADP-ribose and catalyzes the addition of poly(ADP-ribose) adducts to DNA and proteins. Excessive PARS activation leads to depletion of intracellular NAD^+ and ATP and eventually to necrotic-type cell death (Cochrane, 1991; Virág et al., 1998b). Peroxynitrite-induced cytotoxicity has also been linked to direct inhibitory effects of the mitochondrial respiratory chain (Packer and Murphy, 1995; Cassina and Radi, 1996; Xie and Wolin, 1996). The peroxynitrite-mediated apoptosis has been recently linked to the activation of the enzyme caspase-3 (Lin et al., 1998; Virág et al., 1998b).

Mobilization of intracellular calcium is considered an important event in mediating oxidant-induced cellular damage (Ueda and Shah, 1992; Zager and Burkhart, 1997). In the

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ABBREVIATIONS: PARS, poly(ADP-ribose) synthase; SSB, single-strand breaks; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester; Quin-2-AM, 8-amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-*N,N,N′,N′*-tetraacetic acid-tetraacetoxymethyl ester; DiOC6(3), 3,3′-dihexyl-oxacarbocyanine iodide; NAO, nonyl-acridine orange; DEVD, Asp-Glu-Val-Asp; AMC, amino-4-methylcoumarine; FITC, fluorescein isothiocyanate; TMB, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate; BHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; $[Ca^{2+}]_i$, intracellular calcium concentration; Br-A23187, calcimycin; PI, propidium iodide; TCA, trichloroacetic acid.

present study, we investigate the relationship between peroxynitrite-triggered calcium mobilization and PARS activation. Moreover, we determined whether chelation of intracellular calcium modulates the mode of peroxynitrite-induced cell death (apoptosis versus necrosis), and whether this event is proximal or distal to mitochondrial alterations and PARS activation.

Experimental Procedures

Animals. PARS-deficient and wild-type mice (breeding pairs, kind gifts of Dr. Z. Q. Wang, International Agency for Research on Cancer, Lyon, France) (4-week-old male mice) were bred at the animal care facility of the Children's Hospital Medical Center. Animals received food and water ad libitum, and lighting was maintained on a 12-h cycle. Animals were free of diseases and appeared to be healthy and fertile.

Materials. 1,2-bis(2-Aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), EGTA-AM, 8-amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-*N,N,N′,N′*-tetraacetic acid-tetraacetoxymethyl ester (Quin-2-AM), 3,3′-dihexyloxacarbocyanine iodide [DiOC6(3)], dihydroethidium, nonyl-acridine orange (NAO), fluo-3, calcimycin (Br-A23187), and propidium iodide (PI) were obtained from Molecular Probes (Eugene, OR). The tetrapeptide substrate [Asp-Glu-Val-Asp (DEVD)-amino-4-methylcoumarine (AMC)] and inhibitor (DEVD-CHO) of caspase 3 and the purified PARS enzyme were purchased from Biomol (Plymouth Meeting, PA). Proteinase K was obtained from Life Technologies (Grand Island, NY). Annexin V-fluorescein isothiocyanate (FITC) was obtained from Pharmingen. Tris, magnesium chloride, analytical test filter funnels, and Scintisafe scintillation cocktail were purchased from Fisher Scientific (Pittsburgh, PA). [^{3}H]NAD was purchased from DuPont-NEN (Boston, MA). 8-(Diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB) and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ) were obtained from ICN (Costa Mesa, CA). Peroxynitrite (a kind gift of Dr. Harry Ischiropoulos, University of Pennsylvania, Philadelphia, PA) was synthesized, and excess hydrogen peroxide was removed via a zinc oxide column as described previously (Beckman et al., 1994). All the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Thymocyte Preparation and Peroxynitrite Treatment. Thymi from sex-matched PARS-deficient and wild-type mice (3 to 5 weeks old) were aseptically removed and placed into ice-cold RPMI (10% fetal calf serum, 10 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin) medium. Single-cell suspensions were prepared as described (Virág et al., 1998b) by sieving the organs through a stainless wire mesh. Cells isolated this way were routinely 95% viable, as assessed by Trypan blue exclusion assay. Thymocytes (10^6/well in 0.5 ml of medium) were seeded in 24-well plates (0.5 ml/well). Peroxynitrite was diluted in PBS, pH 8.9, and added to the cells in a bolus of 50 μl. Thymocytes were then incubated for various times (20 min for DNA strand break assay and PARS assay, 3 h for the measurement of mitochondrial parameters and for PI/Annexin V staining, or 6 h for DNA fragmentation and caspase activation). Decomposed peroxynitrite (incubated for 30 min at pH 7.0) served as a control. In all figures in *Results*, control (untreated) values represent values obtained in the presence of decomposed peroxynitrite (i.e., reverse-order control).

Measurement of Intracellular Calcium Mobilization. Measurement of intracellular Ca^{2+} flux was performed by flow cytometry, as described (Gergely et al., 1997). Briefly, cells were loaded with 1 μM fluo-3-AM at 37°C for 30 min and washed twice with PBS. Half of the cell suspension was then treated with 5 μM BAPTA-AM for 30 min at 37°C, washed twice with PBS, and resuspended in Hanks' balanced salt solution flux buffer (without phenol red, calcium chloride, and magnesium sulfate; supplemented with 5% calf serum, 10 mM HEPES, 1.5 mM calcium chloride, pH 7.2) at a cell density of 10^6/ml. Aliquots of 5×10^4 cells were added to 1 ml of Hanks' balanced salt solution flux buffer and samples were run on a Facscalibur flow cytometer for 20 s. Acquisition was then paused, and peroxynitrite or Br-A23187 was added to the samples. Tubes were mixed and acquisition continued for a total of 600 to 1000 s. Linear fluorescence data were collected over time and analyzed with the CellQuest software (Becton-Dickinson, San Jose, CA). The fluorescence of fluo-3-stained cells has been converted into absolute intracellular calcium concentration ($[Ca^{2+}]_i$) by a calibration procedure, as described previously (Vandenberghe and Ceuppens, 1990). Briefly, the 300-s recordings have been divided into 21 segments of approximately 14 s each. Mean fluorescent intensity in Fl1 has been used for the calculations of $[Ca^{2+}]_i$. The equation used for the conversion of fluorescence intensity into $[Ca^{2+}]_i$ was:

$$[Ca^{2+}]_i = K_d*(F - F_{min}) * (F_{max} - F)^{-1}$$

where K_d represents the dissociation constant for Ca^{2+}-bound fluo-3 and is 400 nM. F_{max} represents the maximum fluorescence of cells rendered permeable to Ca^{2+} by 10 μM Br-A23187 in a 1 mM Ca^{2+}-containing medium. F_{min} was obtained by adding 2 mM $MnCl_2$ to Ca^{2+} ionophore-treated cells. Because Mn^{2+} displaces Ca^{2+} from fluo-3 and because the Mn^{2+}-fluo-3 complex is eight times as fluorescent as the metal-free dye but only one-fifth as fluorescent as the Ca^{2+}-fluo-3 complexes, F_{min} can be calculated as follows:

$$F_{min} = F_{max} - (F_{max} - F_{MnCl_2}) \times 1.25 = 1.25 \times F_{MnCl_2} - 0.25 \times F_{max}.$$

Measurement of Mitochondrial Membrane Potential, Superoxide Production, and Cardiolipin Content. The mitochondrial membrane potential was quantitated by the flow cytometric analysis of DiOC6(3)-stained cells (Zamzami et al., 1995). 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 are thus concentrated within the mitochondrial matrix. Intramitochondrial generation of the reactive oxygen intermediate was determined by analyzing with flow cytometry the superoxide-induced conversion of the oxidant-sensitive dye dihydroethidium to ethidium (Zamzami et al., 1995). Mitochondrial membrane damage was determined by measuring the concentration of cardiolipin, the cellular distribution of which is restricted to mitochondria. The fluorochrome NAO stoichiometrically interacts with cardiolipin (1:2); this interaction is not influenced by the mitochondrial state (Zamzami et al., 1995).

Flow Cytometry. Thymocytes were stained with 40 nM DiOC6(3), 2 μM dihydroethidine, 100 nM NAO for 15 min at 37°C, washed once with PBS, and analyzed with a FacsCalibur flow cytometer as described previously (Zamzami et al., 1995). Forward and side scatters were gated on the major population of normal-sized cells. In control experiments cells were pretreated (1 h, 37°C) with 50 μM carbonyl cyanide *m*-chlorophenyl hydrazone, a protonophore that completely de-energizes mitochondria by dissipating mitochondrial membrane potential ($\Delta\psi_m$).

Samples processed for Annexin V-FITC/PI staining (Vermes et al., 1995) were washed in PBS and 10^5 cells (in 100 μl) were stained with 5 μl of Annexin V-FITC and 5 μg/ml PI in 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 of annexin binding buffer was added to the samples, which were then immediately analyzed with a FacsCalibur flow cytometer (Becton-Dickinson, San Jose, CA).

Measurement of Cellular PARS Activity. Thymocytes (10^7 cells in 1 ml of culture medium) were treated with peroxynitrite. After 20 min, cells were spun, medium was aspirated, and cells were resuspended in 0.5 ml of assay buffer [56 mM HEPES pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 0.125 μM [^{3}H]NAD (0.5 μCi/ml)]. PARS activity was then measured as described previously (Virág et al., 1998b). Briefly, after incubation (20 min at 37°C), 200 μl of ice-cold 50% trichloroacetic acid (TCA) was added and samples were incubated for 4 h at 4°C. Samples were then

spun (10,000*g*, 10 min) and pellets washed twice in ice-cold 5% TCA and solubilized overnight in 250 μl of 2% SDS/0.1 N NaOH at 37°C. Contents of the tubes were added to 7 ml of ScintiSafe Plus scintillation liquid (Fisher Scientific) and radioactivity was determined in a liquid scintillation counter (Wallac, Gaithersburg, MD).

Cell-Free PARS Assay. An in vitro cell-free assay for the measurement of PARS activity was adapted from the method of Banasik et al. (1992). Briefly, 1 μl of PARS enzyme (286 μg/ml) was added to 200 μl of buffer containing 100 mM TRIS**,** 5 mM MgCl, 2.5 mM dithiothreitol, pH 7.5. BAPTA-AM and other test agents were added at various concentrations and allowed to preincubate at room temperature for 30 min. After preincubation, 10 μl of DNA**/**Histone (20 μg/ml final concentration each) in analogous buffer was added along with 10 μl of [^{3}H]NAD$^+$ (25 μCi/ml). Tubes were vortexed then centrifuged at 14,000*g* for 15 s and the reaction was allowed to proceed at 37°C for 15 min. The reaction was stopped by addition of 100 μl of 50% TCA (ice-cold) and was precipitated at 4°C for 30 min. The TCA-insoluble precipitate was collected by filtration through 0.2 μm nitrocellulose analytical test filter funnels under vacuum and washed 5 times with 4 ml of 5% TCA (ice-cold). The membranes were counted in 7-ml scintillation vials with ScintiSafe cocktail for a 2-min β-spectrum in a Wallac 1409 scintillation counter.

Measurement of DNA SSB. The formation of strand breaks in double-stranded DNA in thymocytes exposed to peroxynitrite was determined by the alkaline unwinding method as described previously (Zingarelli et al., 1996). Cells were homogenized in 0.2 ml of solution A buffer (250 mM *myo*-inositol, 10 mM NaH_2PO_3, 1 mM $MgCl_2$, pH 7.2). The cell lysate was then transferred into plastic tubes designated T (maximum fluorescence), P (fluorescence in sample used to estimate extent of DNA unwinding), or B (background fluorescence). To each tube, 0.2 ml of solution B (alkaline lysis solution: 10 mM NaOH, 9 M urea, 2.5 mM EDTA, and 0.1% SDS) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption. Solutions C (0.45 volume solution B in 0.2 N NaOH) and D (0.4 volume solution B in 0.2 N NaOH) (0.1 ml each) were then added to the P and B tubes. Solution E (0.1 ml; neutralizing solution: 1 M glucose, 14 mM mercaptoethanol) was added to the T tubes before solutions C and D were added. From this point, incubations were carried out in the dark. A 30-min incubation period at 0°C was then allowed during which the alkali diffused into the viscous lysate. Because the neutralizing solution, solution E, was added to the T tubes before addition of the alkaline solutions C and D, the DNA in the T tubes was never exposed to a denaturing pH. At the end of the 30-min incubation, the contents of the B tubes were sonicated for 30 s to ensure rapid denaturation of DNA in the alkaline solution. All tubes were then incubated at 15°C for 10 min. Denaturation was stopped by chilling to 0°C and adding 0.4 ml of solution E to the P and B tubes. Solution F (1.5 ml; 6.7 μg/ml ethidium bromide in 13.3 mM NaOH) was added to all the tubes and fluorescence (excitation, 520 nm; emission, 590 nm) was measured by a Perkin-Elmer fluorometer. Under the conditions used, in which ethidium bromide binds preferentially to double-stranded DNA, the percentage of double stranded DNA (D) may be determined using the equation: $\%D = 100 \times [F(P) - F(B)]/[F(T) - F(B)]$; where F(P) is the fluorescence of the sample, F(B) the background fluorescence (i.e., fluorescence caused by all cell components other than double-stranded DNA), and F(T) is the maximum fluorescence.

Cell-Free DNA Breakage Assay. To investigate the potential direct effect of BAPTA on peroxynitrite-induced DNA SSB, a cell-free assay was used as described previously (Yermilov et al., 1996). Peroxynitrite (2.5 mM) was added to a reaction mixture (final volume, 10 μl) containing 100 mM sodium phosphate buffer, pH 7.4, 100 ng of plasmid pBR322 DNA, BAPTA, or BAPTA-AM, and an appropriate amount of HCl to neutralize the NaOH present in the peroxynitrite solution. The conversion of the covalently closed circular double-stranded supercoiled DNA (form I) to a relaxed open circle (form II) was used to investigate DNA strand breakage. Percentages of supercoiled and relaxed forms were calculated by an imaging densitometer (model GS-670; Bio-Rad, Hercules, CA). From these values, the average number of SSBs per pBR322 DNA molecule was calculated according to the method of Epe and Hegler (1994), taking into account that when stained with ethidium bromide, the relaxed form gives fluorescence intensity 1.4-fold higher than the supercoiled form and that relaxation is caused by one SSB per DNA molecule. Results (mean ± S.D.) are expressed as numbers of SSB per 1000 base pairs (pBR322 consists of 4363 base pairs) after correcting for the numbers of SSB in untreated plasmid, which contained 5 to 15% form II (corresponding to 0.08–0.27 sites/104bp). All experiments were carried out in triplicate.

Detection of Internucleosomal DNA Fragmentation of Thymocytes. Thymocytes were pretreated with the calcium chelators for 20 min and then treated with peroxynitrite. After 6 h, cells were washed once in cold PBS and pellets resuspended in loading buffer. DNA fragmentation was detected as described previously (Eastman, 1995). Agarose (2%) was poured on a horizontal gel support. After solidification of the gel, the top part (above the comb) was replaced with 1% agarose containing 2% SDS and 64 μg/ml proteinase K. Cells (2×10^6) were loaded in 20 μl of sample buffer (5% glycerol, 10 mM Tris, pH 8.), 0.05% bromophenol blue, 5 mg/ml RNase. Electrophoresis was carried out at 60 V for 12 h and the gel was stained with 2 μg/ml ethidium bromide for 1 h.

Measurement of Caspase 3 Activity. Caspase activity was measured by the cleavage of the fluorogenic tetrapeptide-amino-4-methylcoumarine conjugate (DEVD-AMC) as described (Virág et al., 1998b). Cells were harvested at 6 h after peroxynitrite treatment, washed once in PBS, and then lysed in a lysis buffer (10 mM HEPES, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 5 mM dithiothreitol, 2 mM EDTA, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, pH 7.25) for 10 min on ice. Cell lysates and substrates (50 μM) were combined in triplicates in the caspase reaction buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, pH 7.25) in the presence or absence of 10 μM caspase 3 inhibitor DEVD-CHO. AMC liberation was monitored over time with a Perkin-Elmer fluorometer using 380-nm excitation and 460-nm emission wavelengths. Data are given as DEVD-CHO inhibitable increase of absolute fluorescence units (mean ± S.D.).

Statistical Analysis. All values in the figures and text are expressed as mean ± S.E.M. of n observations ($n > 4$). Data sets were examined by ANOVA and individual group means were then compared with Bonferroni's post hoc test. A p value less than .05 was considered statistically significant. When the results are presented as representative gels, or flow cytometry analysis, results identical with the ones shown were obtained in at least three different experiments.

Results

Peroxynitrite Induces Calcium Mobilization in Thymocytes. Peroxynitrite treatment of thymocytes resulted in a rapid calcium flux indicated by the increased fluo-3 fluorescence (Fig. 1A). The intracellular calcium level started to increase 3 to 5 min after the addition of peroxynitrite. Pretreatment of cells with 5 μM BAPTA-AM abolished or markedly inhibited the peroxynitrite-induced increase in $[Ca^{2+}]_i$. The calcium ionophore Br-A23187 was used as a positive control. Pretreatment of thymocytes with 100 μM TMB, an inhibitor of the mobilization of Ca^{2+} from intracellular pools, inhibited peroxynitrite-induced calcium mobilization (Fig. 1B). Chelation of extracellular Ca^{2+} by mM EGTA did not inhibit the first phase of Ca^{2+} mobilization. However, in the presence of EGTA, there was an accelerated decline of the Ca^{2+} levels during the latter phase of the response (Fig. 1B).

Intracellular Calcium Chelators Protect from Peroxynitrite-Induced Cytotoxicity. Treatment of wild-type thymocytes with peroxynitrite (20 μM) resulted in cell death, as indicated by the uptake of the membrane-impermeable fluorescent dye PI (Fig. 2a). Pretreatment of the cells with BAPTA-AM (5 μM), Quin-2-AM (2.5 μM), or EGTA-AM (10 μM) abolished peroxynitrite-induced cytotoxicity. Chelation of extracellular calcium with EDTA or EGTA had no protective effect (data not shown). Because PARS-deficient thymocytes were resistant to peroxynitrite-induced cytotoxicity (Vi-

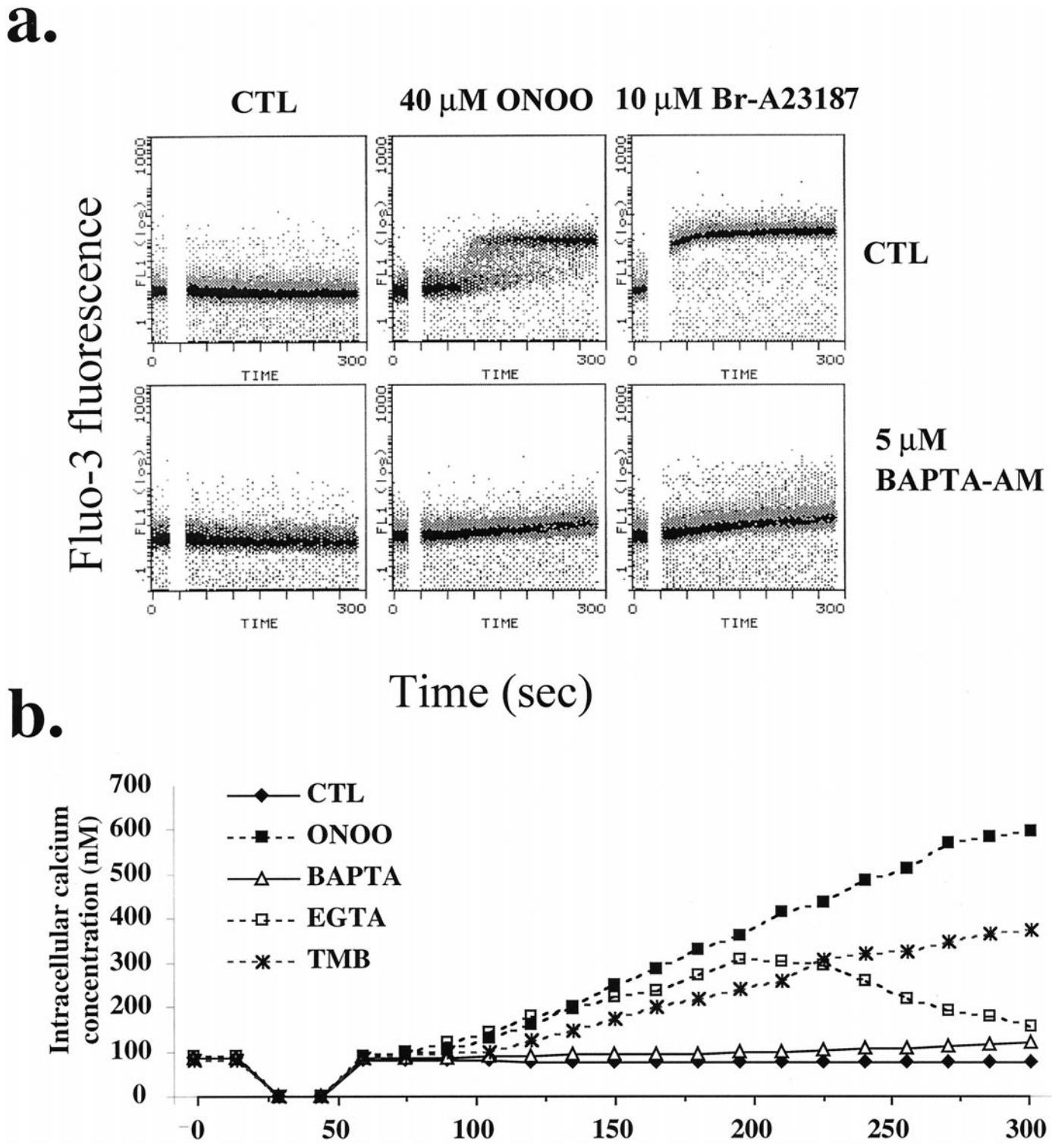

Fig. 1. Peroxynitrite induces calcium mobilization in thymocytes. Thymocytes loaded with the calcium-sensitive dye fluo-3 were treated with 40 μM peroxynitrite and fluo-3 fluorescence was recorded over time for 300 s. Some fluo-3-loaded samples were also treated with 5 μM BAPTA-AM before peroxynitrite treatment. The calcium ionophore Br-A23187 (10 μM) was used as a positive control. Dot plots shown are representative of three independent experiments. b, actual $[Ca^{2+}]_i$ (nM), calculated from fluorescence values. The effect of pretreatment with 100 μM TMB, 5 mM EGTA, and 5 μM BAPTA-AM on peroxynitrite-induced Ca^{2+} mobilization are also presented. Data represent mean of three independent experiments.

rág et al., 1998b), to achieve a similar degree of cell death in PARS-deficient thymocytes, doses of peroxynitrite four times higher (80 μM) needed to be used. Intracellular calcium chelators also provided significant protection in the PARS-deficient thymocytes (Fig. 2b), which indicates that the mechanism of the cytoprotective action of the calcium chelators is, at least in part, PARS-independent.

Inhibition of Intracellular Ca^{2+} Mobilization, but Not Chelation of Extracellular Calcium, Protects from Peroxynitrite-Induced Cytotoxicity. To identify the contribution of the mobilization of Ca^{2+} from intracellular and extracellular pools, we have pretreated the cells with 30 μM BHQ, an intracellular Ca^{2+} depletor, and 5 mM EGTA before peroxynitrite exposure. We have found that BHQ, but not EGTA, provided significant protection against peroxynitrite-induced thymocyte necrosis (Fig. 3).

Action of Intracellular Calcium Chelators is Proximal to Mitochondrial Alterations. Our recent work has demonstrated that peroxynitrite-induced cell death is characterized by the collapse of mitochondrial membrane potential followed by the production of superoxide anion and the loss of cardiolipin (Virág et al., 1998a). These perturbations were followed by a late elevation of intracellular calcium level (Virág et al., 1998a). We have now investigated whether the protective effect of the calcium chelators is proximal or distal to mitochondrial alterations. Pretreatment of the cells with the calcium chelators reduced the changes in mitochondrial function (Fig. 4). Although BAPTA-AM treatment reduced the cytotoxicity of peroxynitrite (20 μM) by approximately 88% (Fig. 2), there was a lesser degree of protection by BAPTA-AM against the peroxynitrite-related changes in membrane potential (approximately 71% protection; from 81 $\pm$ 7 to 23 $\pm$ 4%, $n = 3$). Mitochondrial membrane damage was reduced in the presence of BAPTA-AM to a degree comparable with the protection against overall cell death (from 82 $\pm$ 5 to 11 $\pm$ 4%, $n = 3$). In the presence of BAPTA-AM, however, superoxide production was nearly completely abolished (Fig. 4). These findings support the crucial role of the early, rapid Ca^{2+} mobilization in the peroxynitrite-induced cytotoxic process and support the view that part of the protection provided by BAPTA-AM against peroxynitrite-mediated cytotoxicity is related to its protective effect against the mitochondrial dysfunction.

Intracellular Calcium Chelators Inhibit Cellular DNA Strand Breaks. Pretreatment of thymocytes with BAPTA-AM, EGTA-AM, and Quin-2-AM, significantly reduced peroxynitrite-induced DNA SSBs in thymocytes (Fig. 5a), as measured with the alkaline unwinding assay. In the cell-free system, however, BAPTA had no effect on the peroxynitrite-induced DNA breakage (Fig. 5b). These findings

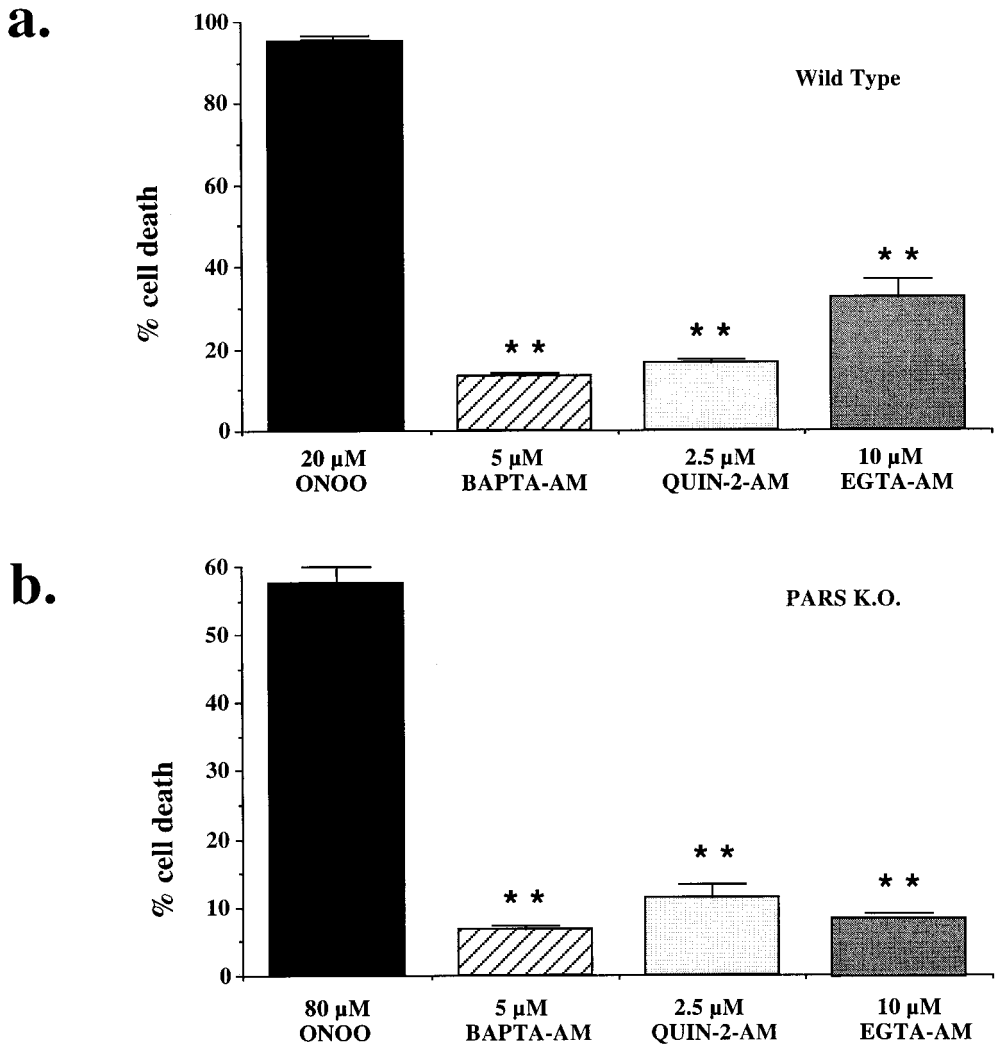

Fig. 2. Intracellular calcium chelators protect from peroxynitrite-induced cytotoxicity. Wild-type (a) and PARS-deficient (b) thymocytes were pretreated with the indicated amount of calcium chelators and then treated with 20 or 80 μM peroxynitrite, respectively. After 4 h, cytotoxicity was determined by the flow cytometric measurement of PI uptake. Percentage number of PI positive cells $\pm$ S.D. of triplicate samples are shown. **, Indicates a significant ($p < .01$) protection from cytotoxicity.

suggest that an indirect mechanism mediates the DNA SSBs induced by peroxynitrite. In studies investigating the cytotoxic effects of *tert*-butyl-hydroperoxide, for example, the agent has been shown to cause DNA breakage via mitochondria-derived H_2O_2.

Effect of Intracellular Calcium Chelation on PARS Activation. Because PARS activation has been identified previously as a major cytotoxic pathway mediating peroxynitrite and hydrogen peroxide-induced cytotoxicity (Virág et al., 1998a,b), we have investigated the effect of calcium mobilization on PARS activation. Intracellular calcium chelators abolished peroxynitrite-induced PARS activation (Fig. 6). This is not likely to be a direct inhibitory effect of the drugs on the enzyme, because a high dose (1 mM) of BAPTA had no effect on the activity of the enzyme in a cell-free PARS assay (data not shown). Therefore, BAPTA does not act as a direct scavenger of peroxynitrite, and failed to affect the oxidation of cytochrome *c* by peroxynitrite in a cell-free assay (data not shown).

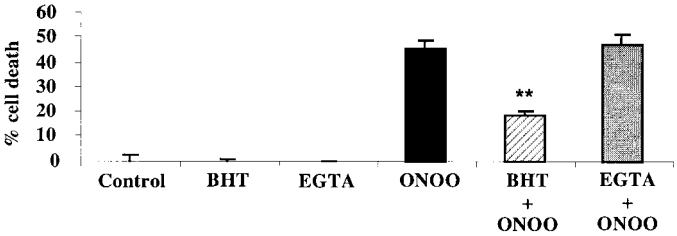

Fig. 3. An intracellular Ca^{2+} depletor (BHQ), but not an extracellular Ca^{2+} chelator protects from peroxynitrite cytotoxicity. Thymocytes were pretreated with 30 μM BHQ or 5 mM EGTA for 30 min at 37°C. Cells were then treated with 15 μM peroxynitrite. After 2 h, cytotoxicity was determined by the flow cytometric measurement of PI uptake. Percentage of PI-positive cells ± S.D. of triplicate samples are shown. ** Indicates a significant ($p < .01$) protection from cytotoxicity.

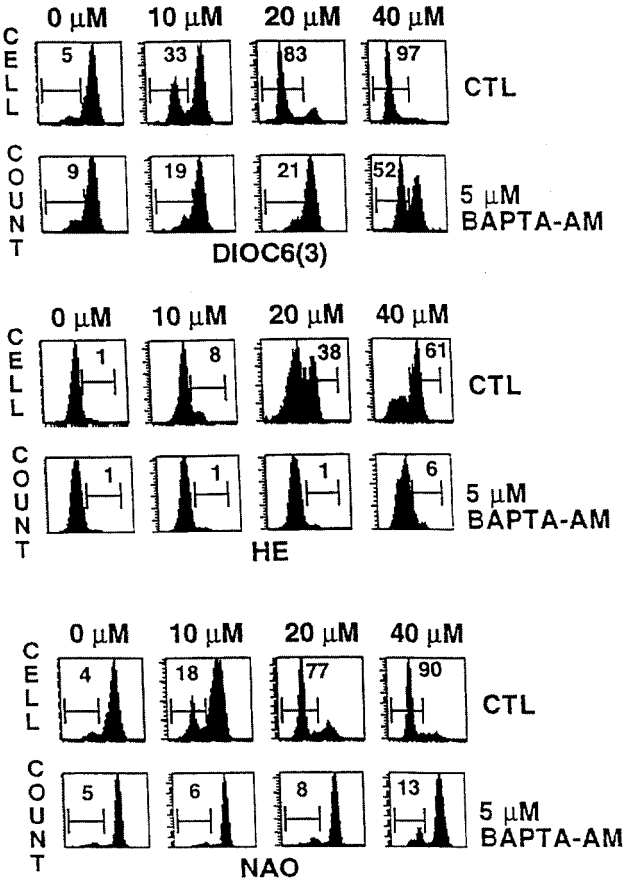

Fig. 4. Calcium buffering prevents peroxynitrite-induced mitochondrial alterations. Thymocytes were either left untreated or were pretreated with 5 μM BAPTA-AM. Cells were then exposed to various concentrations of peroxynitrite, incubated for 3 h, and stained with DiOC6(3), hydroethidine, or NAO for the measurement of mitochondrial membrane potential, superoxide production, and mitochondrial membrane damage, respectively. Values indicate percentages of cells displaying decreased mitochondrial membrane potential, increased superoxide production, or decreased cardiolipin content. Histograms presented are representatives of three different experiments.

Effect of BAPTA-AM on Peroxynitrite-Induced Caspase 3 Activation and DNA Fragmentation. Our recent work has shown that the cytoprotection provided by PARS inhibition results in a shift from necrosis toward apoptotic cell death (Virág et al., 1998b). In the absence of PARS, peroxynitrite induced a dose-dependent increase in caspase 3 activity and DNA fragmentation whereas in wild-type cells, PARS activation leads to necrosis in the absence of DNA fragmentation (Virág et al., 1998b). Here we have investigated the effect of calcium chelation on two apoptotic parameters: caspase activation and DNA fragmentation. In line with the predominantly necrotic and apoptotic nature of cell death in the wild-type and PARS deficient cells, respectively, here we show an increased caspase 3 activation in the PARS deficient cells, compared with the wild-type thymocytes (Fig. 7). BAPTA-AM inhibited caspase activation in both the wild-type and the PARS-deficient cells (Fig. 7).

In the PARS deficient cells, peroxynitrite induced a dose-dependent increase in DNA fragmentation that was inhibited by BAPTA-AM. In the wild-type cells, however, a low dose of peroxynitrite increased fragmentation, whereas high doses of the oxidant blocked DNA fragmentation. Pretreatment with BAPTA-AM prevented the blockade of DNA fragmentation caused by the high doses of peroxynitrite (Fig. 8). Results similar to those obtained with BAPTA-AM have also been found with EGTA-AM.

Effect of BAPTA-AM on Phosphatidylserine Exposure. Peroxynitrite-induced necrotic and apoptotic cell death was accompanied by the appearance of phosphatidylserine in the outer membrane leaflet, as indicated by Annexin V-FITC binding. PI/Annexin V-FITC double-staining revealed that BAPTA-AM blocked both the breakdown of membrane integrity (PI uptake) and phosphatidylserine exposure 3 h after peroxynitrite treatment (Fig. 9). Similar effects were obtained with Quin-2-AM and EGTA-AM (data not shown).

Discussion

The aim of the current study was to elucidate the role of calcium mobilization in the peroxynitrite-induced cytotoxicity, with special emphasis on the PARS pathway. Peroxynitrite caused a rapid increase in $[Ca^{2+}]_i$. The primary source of elevated $[Ca^{2+}]_i$ is most likely intracellular, because TMB (an intracellular Ca^{2+}-release inhibitor), but not EGTA (a nonmembrane-permeable chelator), inhibited the rise of $[Ca^{2+}]_i$. Ca^{2+} may be liberated from the endoplasmic reticulum and/or mitochondria. The efflux of Ca^{2+} from the mitochondria can be triggered by the opening of the mitochondrial megachannel (permeability pore). Peroxynitrite can directly induce permeability transition in mitochondria (Packer and Murphy, 1995); however, we have shown previously that in

the currently used experimental system, the reduction of mitochondrial membrane potential is a consequence of PARS activation (Virág et al., 1998a) and is most likely caused by PARS-mediated NAD/ATP depletion. ATP depletion can also disable the function of the ATP-dependent Ca^{2+} pump of the endoplasmic reticulum, leading to Ca^{2+} mobilization from the endoplasmic reticulum. The decline of $[Ca^{2+}]_i$ in the presence of EGTA may indicate that mobilization of Ca^{2+} from intracellular stores is followed by a capacitative Ca^{2+} entry.

Peroxynitrite-induced cytotoxicity was abolished by BAPTA-AM, EGTA-AM, or QUIN2-AM, indicating a pivotal role of calcium mobilization for peroxynitrite cytotoxicity. Thus, the present data support the view that the rapid early calcium mobilization is essential for the peroxynitrite-induced cytotoxicity. The calcium signal is proximal to the mitochondrial perturbations, because the calcium chelators efficiently blocked [similarly to PARS inhibition (Virág et al., 1998a)] the collapse of mitochondrial membrane potential, the secondary reactive oxygen species generation, and mitochondrial membrane damage. The calcium signal proved to be essential to trigger the cascade of cellular events leading to DNA SSBs and PARS activation. Using other oxidants, it has previously been reported that in intact cells, the DNA SSBs are mediated by mitochondria-derived radicals, rather than being a direct effect of the oxidant on DNA (Guidarelli et al., 1997). A similar mechanism may operate in the peroxynitrite-induced DNA strand breakage, because BAPTA had no effect on peroxynitrite-induced DNA breakage in the cell-free system. (Notably, in the cell-free system, substantially higher concentrations of peroxynitrite were required to induce DNA SSBs compared with intact cells. This observation is likely to support the view that different mechanisms underlie the DNA SSBs in cells and in cell-free conditions.) BAPTA did not scavenge peroxynitrite and was found not to act as a direct inhibitor of PARS, as demonstrated in a cell-free PARS assay. These findings indicate that early calcium signaling is required to initiate the "DNA single strand breakage to PARS activation to cell death" route. It is also likely that self-amplifying feedback processes operate between mitochondrial injury, calcium mobilization, and PARS-dependent cell death. Nevertheless, calcium chelators also provided significant protection to PARS-deficient cells, which required higher concentrations of peroxynitrite to induce comparable cytotoxicity. These observations support the existence of a second, calcium-dependent but PARS-independent pathway of peroxynitrite cytotoxicity. As higher concentrations of oxidants might directly damage organelles (e.g., mitochondria), we hypothesize that this damage may result

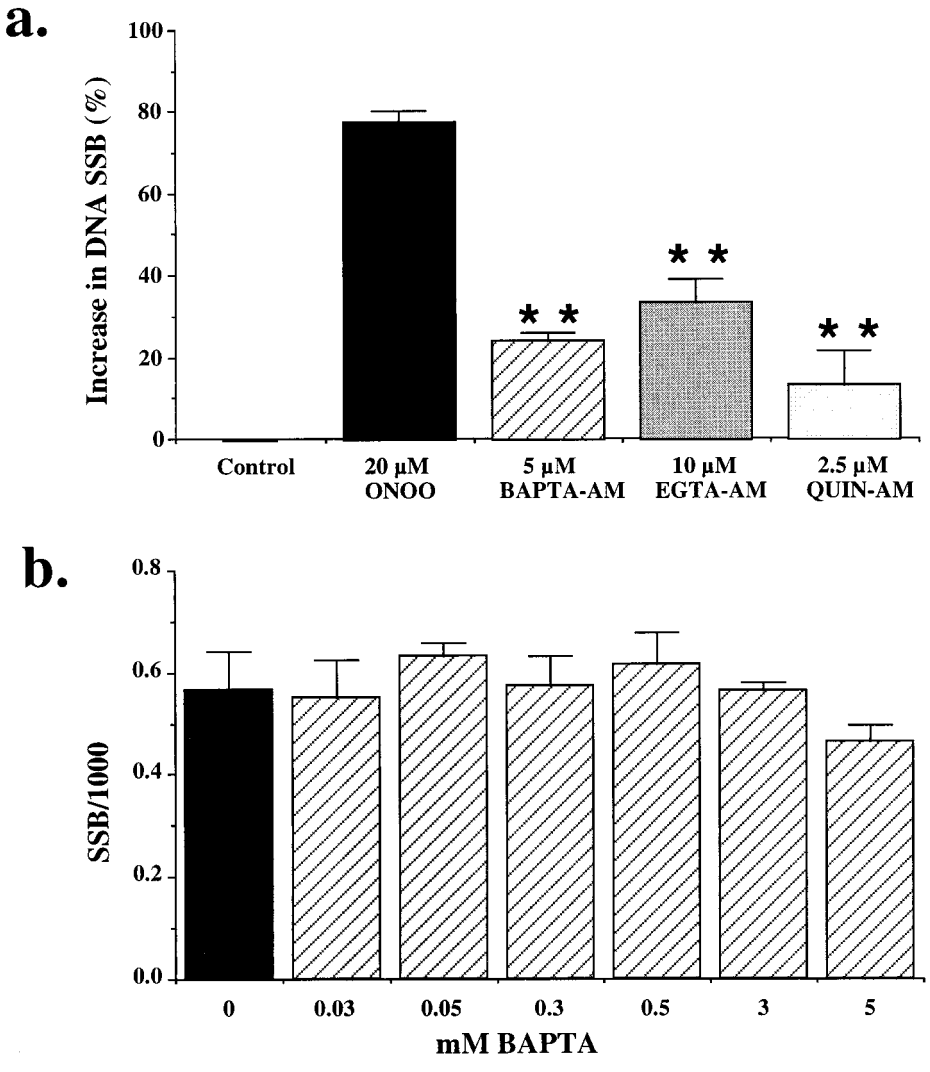

Fig. 5. Calcium chelators inhibit cellular DNA SSBs but do not affect the direct DNA breaking effect of peroxynitrite. Thymocytes were pretreated with the indicated concentrations of chelators and were then exposed to 20 μM peroxynitrite (a). After 20 min, cellular DNA breakage was determined with the alkaline unwinding assay. b, lack of effect of BAPTA on the peroxynitrite-induced DNA breakage in the cell free system. Data are given as mean ± S.E.M. of triplicate experiments. ** Indicates a significant ($p < .01$) protection from cytotoxicity.

in leakage of Ca^{2+} from these pools, which in turn may contribute to cell death.

It is now established that lower fluxes of oxidants induce apoptosis, whereas higher fluxes of the oxidants can induce necrosis. Our previous work demonstrated that inhibition of PARS effectively reduces necrotic death without suppressing the apoptotic machinery (Virág et al., 1998b). The cytoprotection provided by PARS inhibitors in oxidant-induced cytotoxic models resulted in decreased necrosis, accompanied by a higher output of such apoptotic markers as DNA fragmentation and phosphatidylserine exposure (Virág et al., 1998b). Here we have investigated the effect of PARS activation on yet another biochemical marker of apoptosis, the activation of caspase 3, and carried out experiments to establish the relationship between calcium chelation and the manner of cell death (i.e., apoptosis versus necrosis).

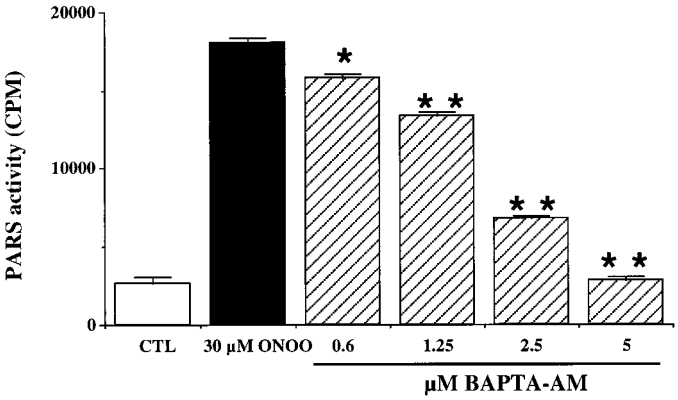

Fig. 6. Calcium signaling is required for PARS activation. Thymocytes were pretreated for 30 min with BAPTA-AM. Cells were then stimulated with 30 μM peroxynitrite for 20 min and PARS activity was determined with the [^{3}H]NAD assay. Data are given as mean ± S.E.M. of triplicate experiments. Asterisks indicate a significant (*$p < .05$; **$p < .01$) inhibition of PARS activity.

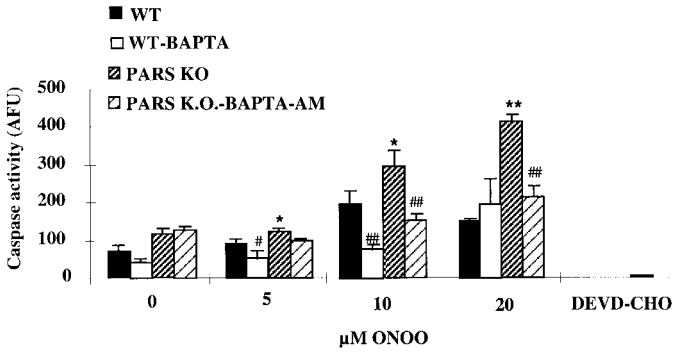

Fig. 7. Calcium chelation inhibits caspase activation. Wild-type (WT) and PARS knock-out (PARS K.O.) cells were pretreated with 5 μM BAPTA-AM and then exposed to different concentrations of peroxynitrite ($ONOO^-$). After 6 h, cells were lysed and caspase activity was measured by DEVD-AMC cleavage. Data are given as mean ± S.E.M. of triplicate measurements. Asterisks indicate significant difference between wild-type and PARS knock-out cells: *$p < .05$ and **$p < .01$; # indicates a significant inhibition of caspase activity by BAPTA-AM: #$p < .05$ and # #$p < .01$.

Our group (Virág et al., 1998b) as well as another group (Lin et al., 1998) have recently shown that caspase 3 but not caspase 1 becomes activated in peroxynitrite-treated thymocytes and HL-60 cells and that caspase 3 inhibition abolishes peroxynitrite-induced DNA fragmentation. Consistent with our previous finding, demonstrating that PARS inhibition diverts cell death from necrosis toward apoptosis, we have found significantly higher caspase 3 activation in the PARS-deficient thymocytes compared with the wild-type cells. The calcium chelators inhibited caspase activation in both wild-type and PARS-deficient cells. Because the activity of caspases is known to be unaffected by calcium concentrations in the range of 0 to 100 mM (Stennicke and Salvesen, 1997), it is most likely that calcium regulates peroxynitrite-induced caspase activation as a second messenger.

Consistent with the caspase inhibitory effect of intracellular calcium chelation, BAPTA-AM and EGTA-AM markedly inhibited DNA fragmentation in the PARS-deficient cells. In the wild-type cells, however, BAPTA-AM reversed the inhibition of DNA fragmentation observed at higher doses (20 and 40 μM) of peroxynitrite, which may be attributable to the PARS inhibitory effect of the chelator. The effect of the chelators on the DNA fragmentation of the peroxynitrite-treated, wild-type thymocytes may reflect a balance between PARS inhibition (which increases DNA fragmentation because it improves cellular energetics, which enhances apoptosis, an energy-dependent process; Virág et al., 1998a), and caspase inhibition (which would decrease DNA fragmentation). A direct inhibitory effect of the calcium chelators on the activity of calcium-dependent nucleases (Peitsch et al., 1993) may also be considered.

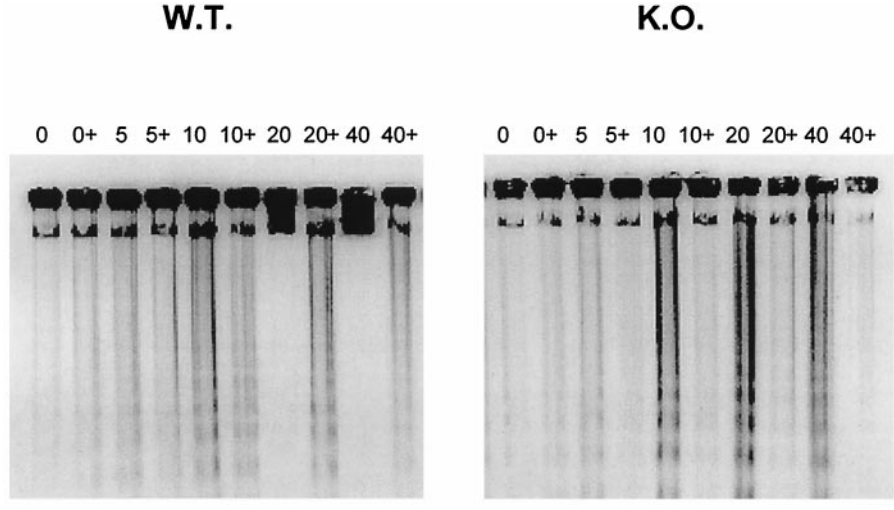

Fig. 8. Effect of calcium chelators on peroxynitrite-induced DNA fragmentation. Wild-type (W.T.) and PARS-deficient (K.O.) thymocytes were either left untreated or were pretreated with 5 μM BAPTA-AM. Thymocytes were then exposed to the indicated concentrations (μM) of peroxynitrite and incubated for 6 h. DNA fragmentation was visualized by agarose gel electrophoresis. BAPTA pretreatment is indicated by +.

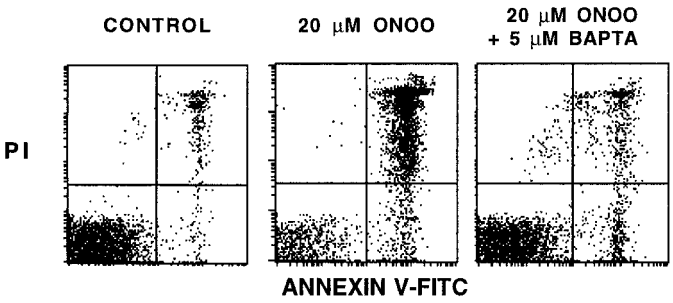

Fig. 9. Effect of BAPTA-AM on phosphatidylserine exposure. Thymocytes were either left untreated or were pretreated with 5 μM BAPTA-AM. Cells were then treated with 20 μM peroxynitrite and incubated for 3 h followed by Annexin V-FITC/PI double-staining. Dot plots shown are representative of three independent experiments.

Clearly, many aspects of peroxynitrite cytotoxicity, such as the intracellular sources of the mobilized calcium, the mechanisms of peroxynitrite-induced DNA SSBs, as well as the signaling cascade leading to caspase activation and DNA fragmentation require further investigation. The relationship between calcium, PARS, mitochondrial dysfunction, and permeability transition and mitochondrial cytochrome C release may be a fruitful area of future investigations, especially in light of the fact that the release of cytochrome C from the mitochondria is known to trigger the activation of caspase-3. Although it is clear that much further work is required to delineate the above processes, our current study provided experimental basis for the following conclusions: 1) peroxynitrite induces a rapid, early mobilization of Ca from intracellular pools; 2) this calcium mobilization plays an important role in peroxynitrite cytotoxicity; 3) rapid Ca^{2+} mobilization is required for peroxynitrite-induced DNA SSBs and PARS activation; 4) the PARS-independent cytotoxic pathways of peroxynitrite also involve early calcium signaling; 5) peroxynitrite-induced phosphatidylserine exposure requires calcium mobilization; 6) calcium signaling promotes caspase 3 activation; and 7) intracellular calcium chelation of peroxynitrite-treated thymocytes results in increased DNA fragmentation, despite decreases in caspase 3 activation.

Inhibition of calcium mobilization by BAPTA-AM has been reported to protect from glutamate-induced neurotoxicity in vitro and from cerebral ischemia-reperfusion in vivo (Frandsen and Schousboe, 1991; Tymianski et al., 1993, 1994a,b; Clementi et al., 1996). The activation of PARS plays a key role in the pathogenesis of excitotoxicity and the development of stroke in vivo (Zhang et al., 1994; Dawson, 1995; Eliasson et al., 1997). Therefore, it is possible that the suppression of calcium mobilization may regulate PARS activation in these models, and, consequently, the protective effect of intracellular calcium chelation may be caused by the inhibition of the PARS pathway. Activation of PARS has been recently reported to play a pathogenic role in a number of other pathophysiological conditions, including myocardial reperfusion injury (Zingarelli et al., 1997a) and various forms of shock and multiple organ failure (Szabó et al., 1996b, 1997b). Further studies are required to elucidate whether 1) the currently identified early calcium-dependent step is also present in oxidant-induced death of cell types other than thymocytes and 2) an initial calcium signaling plays a role in the activation of PARS in various pathophysiological conditions.

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