Immunology 1998 **94** 345–355

Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly (ADP-ribose) synthetase (PARS) activation

L. VIRÁG, G. S. SCOTT, S. CUZZOCREA, D. MARMER, A. L. SALZMAN & C. SZABÓ *Division of Critical Care, Children's Hospital Medical Center, Cincinnati, OH, USA*

SUMMARY

The mechanisms by which immature thymocyte apoptosis is induced during negative selection are poorly defined. Reports demonstrated that cross-linking of T-cell receptor leads to stromal cell activation, expression of inducible nitric oxide synthase (iNOS) and, subsequently, to thymocyte apoptosis. Therefore we examined, whether NO directly or indirectly, through peroxynitrite formation, causes thymocyte apoptosis. Immuno-histochemical detection of nitrotyrosine revealed *in vivo* peroxynitrite formation in the thymi of naive mice. Nitrotyrosine, the footprint of peroxynitrite, was predominantly found in the corticomedullary junction and the medulla of naive mice. In the thymi of mice deficient in the inducible isoform of nitric oxide synthase, considerably less nitrotyrosine was found. Exposure of thymocytes *in vitro* to low concentrations (10 μM) of peroxynitrite led to apoptosis, whereas higher concentrations (50 μM) resulted in intense cell death with the characteristics of necrosis. We also investigated the effect of poly (ADP-ribose) synthetase (PARS) inhibition on thymocyte apoptosis. Using the PARS inhibitor 3-aminobenzamide (3-AB), or thymocytes from PARS-deficient animals, we established that PARS determines the fate of thymocyte death. Suppression of cellular ATP levels, and the cellular necrosis in response to peroxynitrite were prevented by PARS inhibition. Therefore, in the absence of PARS, cells are diverted towards the pathway of apoptotic cell death. Similar results were obtained with H_2O_2 treatment, while apoptosis induced by non-oxidative stimuli such as dexamethasone or anti-FAS antibody was unaffected by PARS inhibition. In conclusion, we propose that peroxynitrite-induced apoptosis may play a role in the process of thymocyte negative selection. Furthermore, we propose that the physiological role of PARS cleavage by apopain during apoptosis may serve as an energy-conserving step, enabling the cell to complete the process of apoptosis.

INTRODUCTION

Elimination of self-reactive premature T cells occurs during negative selection in the thymus via signals transduced from the T-cell receptor (TCR) following high affinity binding of major histocompatibility complex (MHC)-bound self-peptides presented by bone marrow-derived stromal cells.[1] However, signalling through the TCR alone does not necessarily lead to apoptosis.[2–4] For instance, co-signalling via the cell surface FAS molecule, a member of the tumour necrosis factor (TNF) receptor family, may also contribute to apoptosis induction, as indicated by the high density of FAS on $CD4^+$ $CD8^+$ double-positive thymocytes and the high sensitivity of these cells to FAS-ligand-mediated apoptosis.[5] Moreover co-stimulatory signals are transduced via CD28 and T-helper 1 (Thy-1) surface receptors.[4,6]

Received 22 January 1998; revised 27 March 1998; accepted 27 March 1998.

Correspondence: Dr C. Szabó, Division of Critical Care, Children's Hospital Medical Center, 333 Burnet Avenue, Cincinnati, OH 45229, USA.

The interaction of thymocytes and stromal cells is far from being unidirectional. Cross-linking of TCR on $CD4^+$ $CD8^+$ thymocytes was reported to induce stromal cell activation, characterized by the upregulation of numerous transcripts including cytokine mRNA-s. This cross-talk between TCR-stimulated thymocytes and stromal cells was found to contribute to thymocyte apoptosis.[7]

Of the numerous cell types that together form the thymic stroma, dendritic cells and macrophages are known to play a crucial role in the negative selection. Both cell types are able to produce reactive oxygen and nitrogen intermediates which mediates many of their cytotoxic effects.[8] Reactive oxygen intermediates and nitric oxide (NO) have been shown to induce apoptosis in thymocytes *in vitro*.[9] Recently, it has been demonstrated that inducible NOS (iNOS) is upregulated in the thymus in response to *in vivo* TCR stimulation, with a subsequent decrease in the number of $CD4^+$ $CD8^+$ thymocytes.[10] Based on this study, a role for NO has been proposed in the process of negative selection. However, it is unknown whether this effect is mediated by NO directly or through the formation of more reactive oxidative species such as peroxynitrite.

Peroxynitrite, a potent oxidant formed in the near diffusion limited reaction of NO and superoxide[11] is a feasible candidate as a mediator of the negative selection. Peroxynitrite is a major mediator of tissue injury in inflammation, ischaemia-reperfusion and shock.[12] Recent studies have demonstrated the induction of apoptosis in the HL-60 cells, U936 cells,[13] and human umbilical vein endothelial cells[14] in response to peroxynitrite. Peroxynitrite has also been shown to induce DNA fragmentation in thymocytes.[15]

The cellular response to peroxynitrite exposure can be influenced by the activation of the poly ADP-ribosyl-synthetase (PARS) (also called PARP), a nuclear DNA nick sensor enzyme.[16] Our group has recently reported that treatment of various cell types with peroxynitrite results in the formation of DNA single-strand breaks and consequently to PARS activation.[16–18] Once activated, PARS cleaves NAD^+ to nicotinamide and ADP-ribose and catalyses the addition of poly (ADP-ribose) adducts to nuclear proteins. This process can lead to the depletion of cellular energy pools and to rapid cell death.[19] Considering the fact that apoptosis is an active process that requires energy, activation of PARS may interfere with the execution of the apoptotic program. Thus, the cleavage of PARS by interleukin-1 converting enzyme -like proteases, an event recognized as a hallmark of apoptosis,[20–27] can be interpreted as a preventive measure aimed at the conservation of the cellular energetics to secure the undisturbed execution of the cell death program. Another possible hypothesis for the role of PARS cleavage comes from discovery that one of the substrates of PARS is an endonuclease and poly-ADP-ribosylation is inhibitory to its activity.[28] Therefore, in addition to energy conservation, inactivation of PARS may be necessary for DNA fragmentation. Yet other investigations concluded that PARS cleavage is an epiphenomenon during apoptosis.[29,30]

In the present study we examined the potential role of peroxynitrite in the negative selection of thymocytes by determining: **1.** whether peroxynitrite is produced *in vivo* in the thymus, **2.** defining the sources of peroxynitrite with regard to the multiple NOS isoforms that are active in the thymus. In addition, we investigated: **3.** whether peroxynitrite activates PARS in thymocytes, and **4.** whether this latter process plays a role in the regulation of peroxynitrite-induced thymocyte cell death. Furthermore, **5.** the role of caspase activation in the course of peroxynitrite induced apoptosis as well as **6.** the dependence of the peroxynitrite-induced apoptotic process on protein and RNA synthesis has also been investigated.

MATERIALS AND METHODS

Materials

Tetrapeptide caspase substrates (DEVD–AFC and YVAD–AFC), inhibitors (DEVD–fmk and YVAD–fmk) and free AFC (7-amino-4-methylcoumarin) were purchased from Enzyme Systems (Dublin, CA). annexin V–FITC (fluorescein isothyocyanate) was obtained from Pharmingen (San Diego, CA), propidium iodide (PI) from Molecular Probes (Eugene, OR) and proteinase K from Gibco (Grand Island, NY). Rabbit polyclonal antinitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). All other reagents were from Sigma (St Louis, MO). Authentic peroxynitrite was kindly provided by Dr Harry Ischiropoulos (University of Pennsylvania, Philadelphia, PA). The concentration of peroxynitrite was determined spectrophotometrically by measuring absorbance at 302 nm using an extinction coefficient of 1670 M^{-1} cm^1.

Thymocyte preparation and peroxynitrite treatment

Thymi from sex-matched PARS-deficient and wild-type mice (3–5 weeks) were aseptically removed and placed into ice cold RPMI-1640 (10% fetal calf serum, 10 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin) media. Single-cell suspensions were prepared by sieving the organs through a stainless wire mesh. Cells isolated this way were routinely 98% viable, as assessed by Trypan blue exclusion assay. Thymocytes were seeded in 24-well plates (0·5 ml/well) for cytofluorimetry and agarose gel electrophoresis, 96-well plates (0·1 ml/well) for cytotoxic assay and deoxyribonucleic acid (DNA) fragmentation enzyme-linked immunosorbent assay (ELISA) or in Eppendorf tubes (1 ml) for adenosine triphosphate (ATP) and PARS-assays. PARS inhibitors were applied as a pretreatment (15 min prior to peroxynitrite). Authentic peroxynitrite was diluted in phosphate-buffered saline (PBS) pH 8·3 and was added to the cells in 1/10 of the volume of the cell suspension. Control samples were treated with PBS pH 8·3 only. The effect of decomposed peroxynitrite (kept in PBS pH 7·2 at 37° for 30 min) has also been tested in all of the assays and was found to have no effect on any parameters measured. Cells were incubated with peroxynitrite for various time intervals until assayed for DNA fragmentation (6 hr), ATP content (1 hr), viability (MTT) (1 hr), and apoptosis by flow cytometric analysis (4 hr).

Immunohistochemistry

Cryostat sections (10 μm) were treated with 0·3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then rinsed briefly in PBS. Non-specific binding was blocked by incubating the slides for 1 hr in PBS containing 2% goat serum. To detect nitrotyrosine, an indicator of the presence of peroxynitrite,[11,12] rabbit polyclonal antinitrotyrosine antibody was applied in a dilution of 1 : 500 at 4° overnight. (Control sections were incubated in PBS with the primary antibody in the presence of 10 mM nitrotyrosine.) Following extensive washing (5 × 5 min) with PBS, immunoreactivity was detected with a biotinylated goat anti-rabbit secondary antibody and the avidin–biotin–peroxidase complex (ABC) both supplied in the Vector Elite kit (Vector Laboratories, Burlingame, CA). Colour was developed using Ni-DAB substrate (95 mg diaminobenzidine, 1·6 g NaCl, 2 g nickel sulfate in 200 ml 0·1 M acetate buffer). Sections were then counterstained with nuclear fast red, dehydrated and mounted in Permount. Photomicrographs were taken with an Olympus BX-40 microscope.

Flow cytometry

Flow cytometric determination of apoptosis was carried out as previously described.[31] The method relies on the binding of annexin V to phosphatidylserine that is translocated from the inner membrane leaflet to the outer layer in cells undergoing apoptosis.[32] Following treatment of 10^6 thymocytes with peroxynitrite, hydrogen peroxide, anti-FAS antibody or dexamethasone, cells were harvested, washed once in ice-cold PBS and resuspended in 1 ml annexin-binding buffer (10 mM Hepes

pH 7·4, 140 mM NaCl, 2·5 mM $CaCl_2$). Cell suspensions (100 µl) were transferred into new tubes and 5 µl annexin V–FITC and 10 µl propidium iodide stock solution (50 µg/ml) were added to the cells. After 20 min, 400 µl binding buffer was added to each tube and samples were analysed within 1 hr on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) using the standard optics for detecting FL1 (FITC) and FL2 (propidium iodide). Data were analysed with CellQuest software (Becton-Dickinson).

Detection of internucleosomal DNA fragmentation with agarose electrophoresis

Internucleosomal DNA fragmentation was measured as described.[33] 10^6 cells were loaded in 15 µl sample buffer (5% glycerol, 10 mM Tris, pH 8·0, 0·05% bromophenol blue, 5 mg/ml RNase) into the wells of a 2% horizontal agarose gel the top portion of which (above to comb) was replaced after solidification with 1% agarose containing 2% sodium dodecyl sulfate (SDS) and 64 µg/ml proteinase K. Electrophoresis was carried out at 60 V for 12 hr and the gel was subsequently stained with 2 µg/ml ethidium bromide for 1 hr. Excess ethidium bromide was removed by intensive washing with distilled water.

Measurement of DNA fragmentation with ELISA

DNA fragmentation was measured with a commercially available ELISA kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions as previously described.[14] The assay is based on the detection of histone-associated DNA fragments from the cytoplasmic fraction of cell lysates. 2×10^5 thymocytes were seeded into round-bottom 96-well plates in 200 µl culture medium and treated with different concentration of peroxynitrite. After a 5 hr incubation (37°, 5% CO_2) plates were centrifuged (240 ***g***, 10 min), media was aspirated and the cells resuspended in 200 µl incubation buffer (supplied with the kit) and incubated at 4° for 60 min. Cell lysates were then transferred to Eppendorf tubes and centrifuged (10 000 ***g***, 10 min). Supernatants were diluted ×10 and measured in an ELISA (antihistone capturing antibody, peroxidase-conjugated anti-DNA secondary antibody, ABTS substrate). Absorbance was then measured (405 nm–490 nm) versus substrate solution as a blank using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Apoptosis index was calculated by comparing the optical density (OD) values of quadruplicate samples to the values of untreated controls.

Measurement of PARS activity

To 10^7 cells (in 1 ml culture medium), 20 µl peroxynitrite was added to give final concentrations of 10 µM and 50 µM. After 20 min cells were spun, medium was aspirated and cells were 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% digitonin and 0·125 µM ^{3}H-NAD (0·5 µCi/ml)]. PARS activity was then measured as previously described.[17] Briefly, following incubation (20 min at 37°), 200 µl ice-cold 50% TCA was added and samples incubated for 4 hr at 4°. Samples were then spun (10 000 ***g***, 10 min) and pellets washed ×2 in ice-cold 5% TCA and solubilized overnight in 250 µl 2% SDS/0·1 N NaOH at 37°. Contents of the tubes were added to 7 ml ScintiSafe Plus scintillation liquid (Fisher Scientific, Pittsburgh, PA) and radioactivity was determined in a Wallac liquid scintillation counter (Gaithersburg, MD).

Measurement of ATP

Following treatment with peroxynitrite 2×10^7 cells were washed ×1 in PBS and resuspended in ice-cold 0·4 ml 0·6N perchloric acid. Lysates were then sonicated using a Virtis membrane disruptor (Virtis, Gardiner, NY; maximal power, 10 seconds) and left on ice for 1 hr. After adding 180 µl cold K_2HPO_4 (1·0 M, pH 12) samples were vortexed and centrifuged (10 000 ***g***, 10 min, 4°). Supernatants were filtered through 0·2-µm syringe filters and analysed for ATP on high-performance liquid chromatography (HPLC) (Pharmacia, Piscataway, NJ) in parallel to ATP standards. Results are expressed as nmol ATP/mg protein.

Determination of peroxynitrite-induced cytotoxicity

Cytotoxic effect of peroxynitrite was measured with the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay as described.[17] Cells were treated the same way as for the assay for DNA fragmentation. One hour after peroxynitrite treatment, MTT was added to the cells (0·5 mg/ml) and incubated for an additional hour. Plates were then centrifuged (240 ***g***, 10 min), media was aspirated and the formazan crystals were dissolved by the addition of 100 µl dimethyl sulphoxide (DMSO). Optical density was determined in the Spectramax plate reader (Spectramax Molecular Devices, Sunnyvale, CA) at 550-nm test wavelength and 690 nm as reference wavelength.

Measurement of apopain and ICE-activity

Caspase activity was measured by the cleavage of the fluorogenic tetrapeptide-amino-4-methylcoumarine conjugate (DEVD–AFC for apopain assay or YVAD–AFC for ICE) as described.[34] Thymocytes ($3 \times 10^{6)}$ were treated with 30 µM peroxynitrite. In parallel, some samples were pretreated with 10 µM of the specific cell permeable tetrapeptide inhibitors (DEVD–fmk and YVAD–fmk) 1 hr before the peroxynitrite treatment. Cells were harvested 0, 2, 4 and 6 hr after peroxynitrite treatment, washed ×1 in PBS and then lysed in a lysis buffer (10 mM HEPES, 0·1% CHAPS, 5 mM dithiothreitol, 2 mM EDTA, 10 µg/ml aprotinin, 20 µg/ml leupeptin, 10 µg/ml pepstatin A, and 1 mM PMSF, pH 7·25) by sonication. 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% CHAPS, pH 7·25). AFC liberation was monitored over time with a Perkin-Elmer fluorimeter (Norwalk, CT) using 400-nm excitation and 505-nm emission wavelength. Fluorescence units were converted to picomoles of AFC using a calibration curve generated with free AFC. Data are given as DEVD–fmk inhibitable AFC generation (mean ± SD).

Statistical analysis

All values in the figures and text are expressed as the mean + standard error of the mean (SEM) of n observations; $n > 4$. Data sets were examined by analysis of variance and individual group means were then compared with Bonferroni's *post hoc* test. A P-value less than 0·05 was considered statistically significant. When the results are presented as representative gels, immunohistochemical pictures or representative flow

cytometry analyses, results identical to the ones shown were obtained in at least three different experiments.

RESULTS

Detection of peroxynitrite in the thymus

For the detection of peroxynitrite production *in vivo* we have taken advantage of the fact that peroxynitrite, unlike NO, reacts with tyrosine residues of proteins. Therefore the presence of nitrated protein in tissues is regarded as a specific footprint of peroxynitrite.[11,12] We have detected cells containing nitrated proteins in the thymi of untreated mice (Fig. 1), indicating the *in vivo*, 'basal' production of peroxynitrite in this organ. The nitrotyrosine positive cells were predominantly found in the medulla (Fig. 1a,c). Nitrotyrosine positive cells have also been found in the thymi of iNOS-deficient mice (Fig. 1b,d), although they were less abundant than in the wild-type mice and displayed a predominantly perivascular distribution.

Effect of peroxynitrite on thymocyte apoptosis

Apoptotic cell death is characterized by numerous morphological and biochemical changes. One of the features of apoptotic cells is the loss of cell membrane asymmetry during the early phases of programmed cell death. Phosphatidylserine (PS), a phospholipid that can only be found in the inner layer of the intact cell membrane, becomes exposed to the outer leaflet where it can be detected by the selective binding of annexin V to PS under defined salt and calcium concentrations. In the early phase of apoptosis, cells bind annexin V but exclude propidium iodide. On the other hand, double-positive cells (stained by both annexin V–FITC and propidium iodide) may

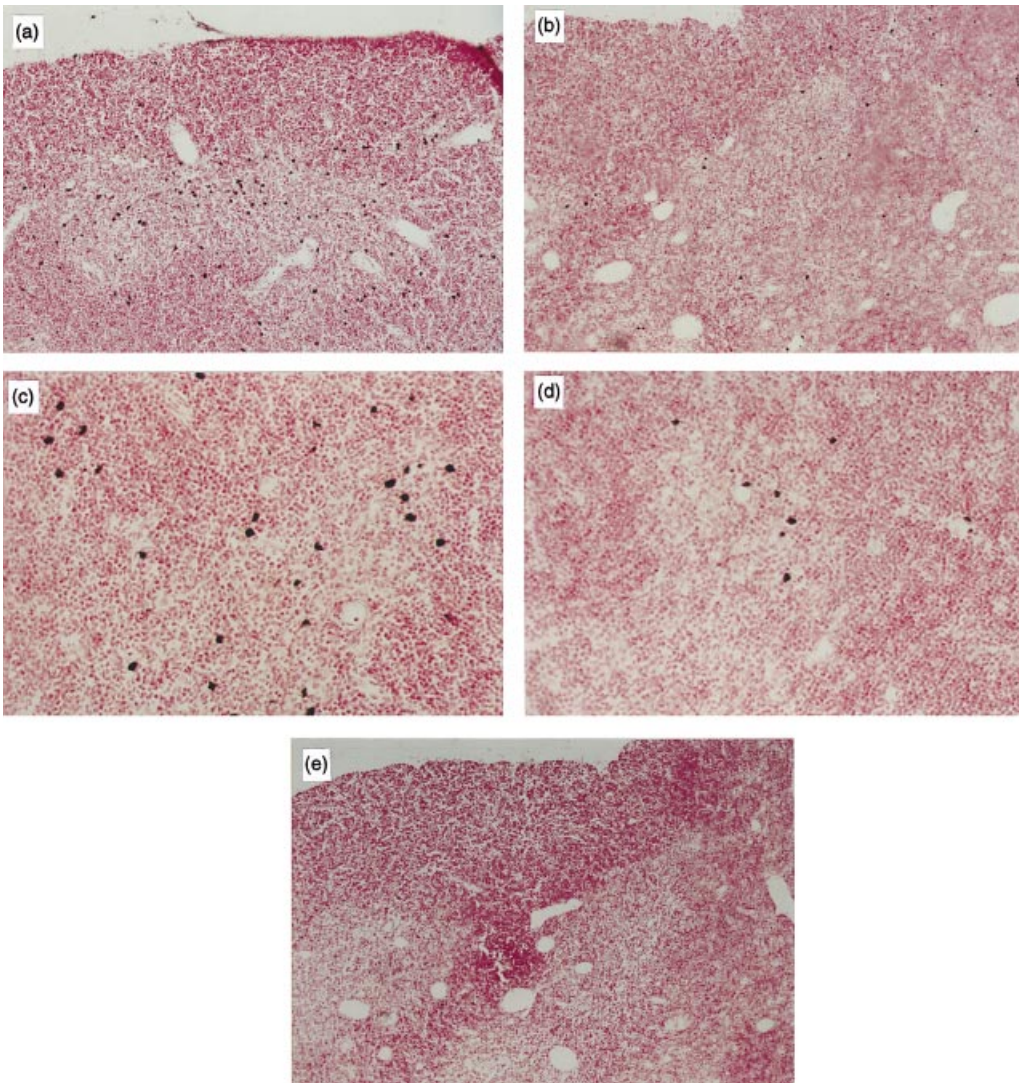

Figure 1. Immunochistochemical detection of nitrotyrosine in the thymus. *Cryostat sections of naive wild-type* (a, c) and iNOS knockout (b, d) mice were stained for nitrotyrosine. Immunoreactive cells were predominantly found in the medulla. A few nitrotyrosine positive cells of predominantly perivascular localization could also be detected in the thymi of iNOS-deficient mice (b,d). Control sections (e) were stained with the primary antibody in the presence of 10 mM nitrotyrosine. Histological sections shown are representative of three experiments. (Panels a, b, e: ×80, 1 cm = 140 μm; panels c, d: ×320, 1 cm = 35 μm.)

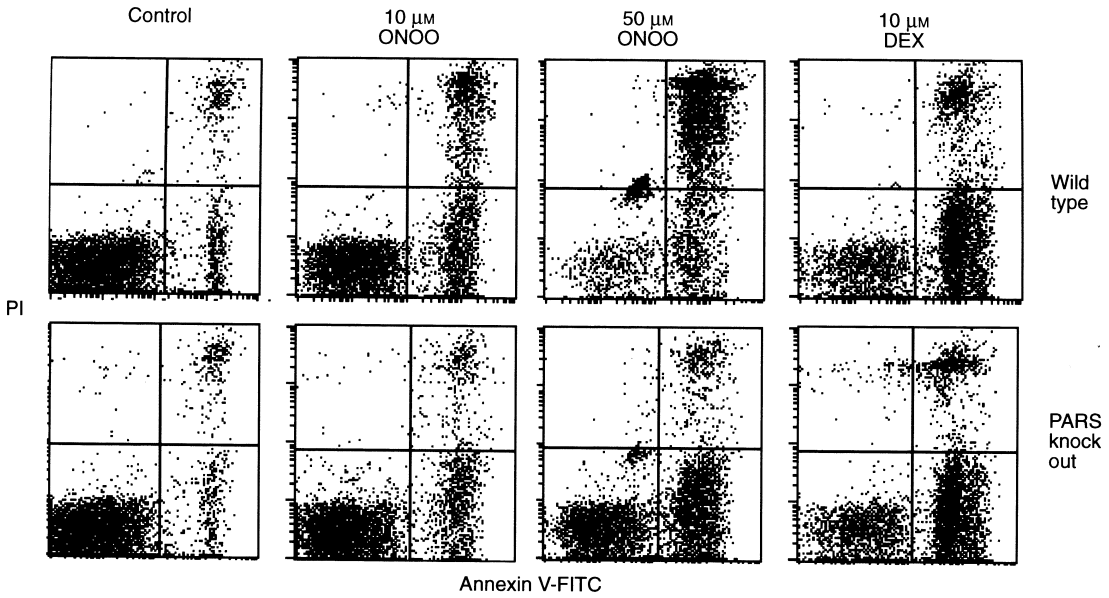

Figure 2. Cytofluorimetric analysis of thymocyte apoptosis induced by peroxynitrite or dexamethasone. Thymocytes were treated for 4 hr with 10 μm and 50 μm peroxynitrite or 10 μm dexamethasone. Cells were then stained with annexin V–FITC and propidium iodide (PI) and two-colour analysis was performed by flow cytometry. An increase in the number of apoptotic (annexin V–FITC single positive) cells was observed in response to 10 μM peroxynitrite treatment, whereas necrotic (stained by both annexin V–FITC and PI) cells dominate in response to 50 μM peroxynitrite. PARS-deficient cells were protected against the loss of membrane integrity, as indicated by decreased PI uptake. No differences were found between the wild-type and PARS-deficient thymocytes in response to dexamethasone. Data shown are representative of three to four experiments performed on different experimental days.

Table 1. Cytofluorimetric analysis of thymocyte apoptosis induced by peroxynitrite, hydrogen peroxide, anti-FAS antibody or dexamethasone. Thymocytes were treated for 4 hr with 10 μm, 25 μm and 50 μm peroxynitrite, 50 μm and 300 μm H_2O_2, 10 μg/ml anti-FAS antibody or 10 μm dexamethasone. Cells were then stained with annexin V–FITC and propidium iodide (PI) and two-colour analysis was performed by flow cytometry. Values indicate percentage number of annexin V/PI double-negative (normal), annexin V single-positive (apoptotic) and annexin V/PI double-positive (necrotic) cells. Data represent the mean of three experiments performed on different experimental days. Variations between individual determinations did not exceed 10% of the mean

	Wild type			3-AB			PARS knock out		
	Normal	APO	NECR	Normal	APO	NECR	Normal	APO	NECR
CTL	84	10	4	88	8	3	88	7	3
10 μM ONOO	59	24	14	62	28	8	75	18	5
25 μM ONOO	30	21	47	46	43	9	55	35	7
50 μM ONOO	13	17	64	48	43	7	49	42	7
50 μM H2O2	34	21	43	47	42	10	56	36	6
300 μM H2O2	9	11	74	41	47	10	39	50	8
10 μg/ml FAS Ab	57	29	13	60	28	9	54	35	9
10 μm DEX	26	59	13	29	58	11	31	57	10

represent a necrotic population, since necrotic cells lose membrane integrity and can not exclude propidium iodide. Cytofluorimetric analysis of cells stained with annexin V–FITC and propidium iodide allows the identification of intact (unstained), apoptotic (annexin V–FITC positive) and necrotic (annexin V–FITC and propidium iodide double positive) populations.[31,35]

Peroxynitrite treatment (10 μM) resulted in an increase of the number of apoptotic cells (Fig. 2, Table 1). However, higher concentrations (50 μM) caused a marked necrosis. Decomposed peroxynitrite had no effect (data not shown). By comparison, treatment with 3-aminobenzamide (3-AB), an inhibitor of PARS, produced a dramatic decrease in the number of double-positive cells, with an increase of apoptotic (i.e. annexin V single positive) cells. PARS-deficient thymocytes also displayed a typical apoptotic staining pattern in response to peroxynitrite, with a dose-dependent increase in the number of annexin V-binding, PI-excluding cells. The patterns of the cellular responses were very similar in the PARS-deficient cells and in the wild-type thymocytes pretreated with the PARS inhibitor (Fig. 2, Table 1). 3-AB had no effect on the apoptosis of PARS-deficient thymocytes (data not shown).

The effect of H_2O_2 on thymocytes was similar to that of peroxynitrite (Table 1). Low concentration (50 μM) of H_2O_2 caused apoptosis whereas higher doses (300 μM) caused

necrosis, which was prevented by 3-AB pretreatment. No differences were found in the anti-FAS antibody or dexamethasone-induced apoptosis between the $PARS^{+/+}$ and $PARS^{-/-}$ cells or the $PARS^{+/+}$ cells in the presence of 3-AB treatment (Table 1).

Peroxynitrite-induced DNA fragmentation

During apoptosis DNA is cleaved by endonucleases into oligonucleosomal fragments, the detection of which in the cytoplasmic fraction of cell lysates is commonly used to verify apoptotic cell death.[33] In line with the flow cytometric data, agarose gel electrophoresis of cellular DNA showed that 10 μM peroxynitrite, but not higher concentrations of the oxidant, induced DNA fragmentation. In fact, at higher concentrations of the oxidant, a marked inhibition of the apoptotic process was found, which was reversed by pretreatment with 3-aminobenzamide (Fig. 3a). PARS-deficient thymocytes exposed to peroxynitrite also demonstrated a dose-dependent increase of DNA fragmentation (Fig. 3b) (similar to the results of the wild-type cells treated with 3-aminobenzamide). The PARS inhibitor did not affect DNA fragmentation in the thymocytes from the PARS-deficient animals, demonstrating that the agent, at the concentration used, did not have additional pharmacological effects. Results similar to that obtained with peroxynitrite were found with H_2O_2 treatment (Fig. 3c,d), whereas anti-FAS- or dexamethasone-induced apoptosis was unaffected by inhibition of PARS (Fig. 3c,d).

Peroxynitrite activates PARS leading to energy depletion and inhibition of mitochondrial respiration

As previously demonstrated, the acute cellular metabolic effects of peroxynitrite are mediated, at least in part, by PARS activation.[18] Therefore, we investigated herein whether PARS is activated in thymocytes in response to peroxynitrite treatment and whether PARS activation leads to energy depletion. Treatment of thymocytes with 20 μM peroxynitrite led to a

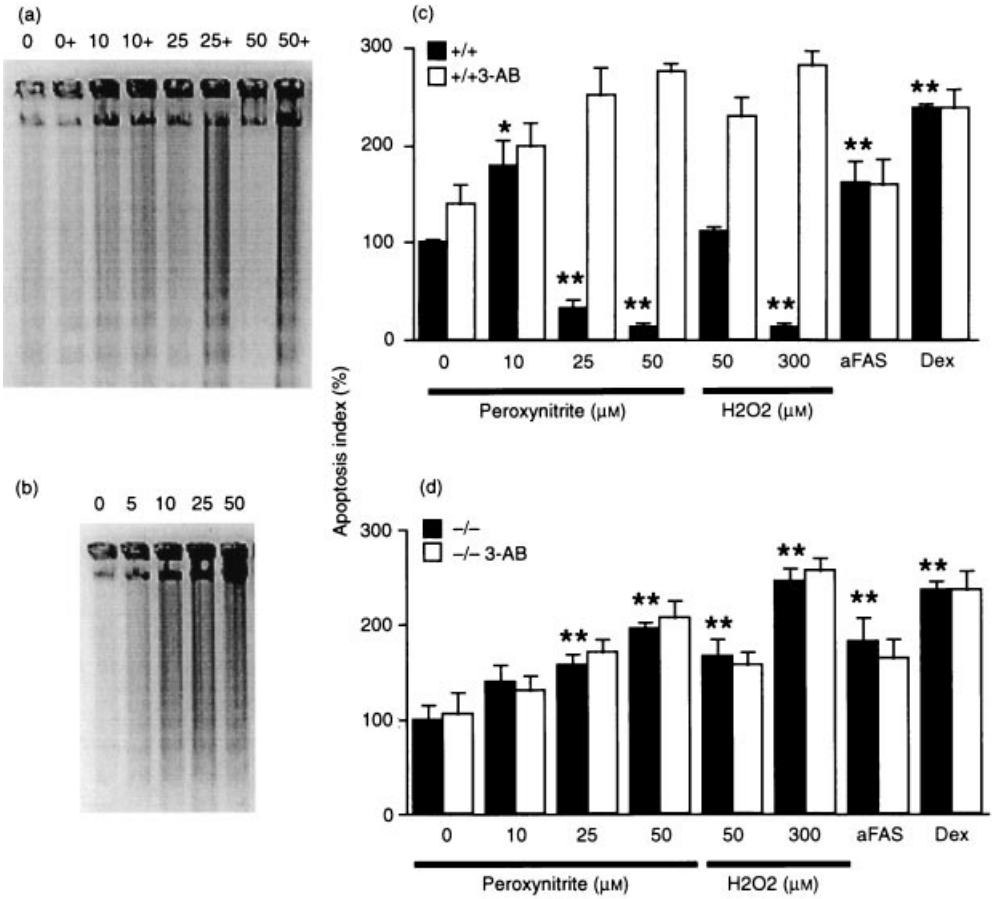

Figure 3. Detection of internucleosomal DNA fragmentation in thymocytes treated with different concentrations of peroxynitrite, H_2O_2, dexamethasone and anti-FAS-Ab. Following a 6-hr treatment with different concentrations of peroxynitrite, H_2O_2, anti-FAS-Ab and dexamethasone, cells were analysed for internucleosomal DNA cleavage with agarose gel electrophoresis (a, b) and cytosolic DNA fragments were also quantified by DNA fragmentation ELISA (c, d). $PARS^{+/+}$ thymocytes were treated with 0 μM, 10 μM, 25 μM or 50 μM peroxynitrite in the absence or presence (+) of 1 mM 3-AB and were then analysed for DNA laddering (a). DNA laddering of $PARS^{-/-}$ cells treated with the indicated concentration of peroxynitrite has also been determined (b). Gels shown are representative of three to four experiments performed on different experimental days. In addition to agarose gel electrophoresis, DNA fragmentation of $PARS^{+/+}$ (c) and $PARS^{-/-}$ (d) thymocytes treated with peroxynitrite, H_2O_2, dexamethasone and anti-FAS-Ab has been quantified with DNA fragmentation ELISA. Data shown on parts (c) and (d) represent means ± SEM of $n = 9$ observations.

marked elevation in PARS activity as shown in Fig. 4(a). Higher concentrations of peroxynitrite (50 μM) resulted in maximal PARS activation. Pretreatment of cells with 3-AB significantly decreased PARS activation.

We have previously demonstrated that activation of PARS is followed by consumption of NAD^+, the substrate of PARS and leads in turn to a dramatic decrease in the intracellular ATP level, inhibition of mitochondrial respiration and eventual cell death.[18] In line with these data, a significant decrease in the intracellular ATP level was found 1 hr after peroxynitrite pretreatment of thymocytes, with a protection provided by inhibition of PARS (Fig. 4b). Similarly, we have found a PARS-related, concentration-dependent decrease in mitochondrial respiration in thymocytes exposed to peroxynitrite (Fig. 4c).

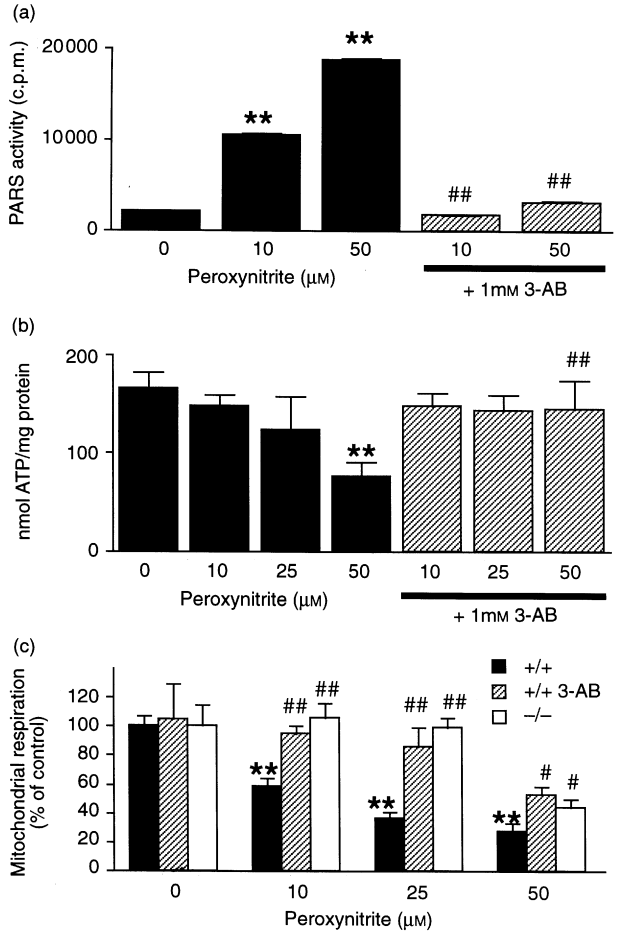

Figure 4. Peroxynitrite-induced PARS activation (a), ATP consumption (b) and inhibition of mitochondrial respiration (c). Thymocytes were treated in the presence or absence of 1 mM 3-AB with peroxynitrite and following 20 min PARS activity was determined. Peroxynitrite induced a significant ($^{**}P<0{\cdot}01$) increase in PARS activity when compared with the control, an effect that was inhibitable by 3-AB ($^{\#\#}P<0{\cdot}01$). A 3-aminobenzamide-inhibitable decrease in the cellular ATP content (as measured by HPLC) and mitochondrial respiration (as determined by MTT reduction) were also observed. Thymocytes from PARS-deficient mice were less sensitive to the peroxynitrite-induced inhibition of mitochondrial respiration. In parts (b) and (c), $^{**}P<0{\cdot}01$ represents significant inhibition of cellular ATP levels or mitochondrial respiration in response to peroxynitrite, whereas $^{\#}P<0{\cdot}05$ and $^{\#\#}P<0{\cdot}01$ represent significant protection against these effects by inhibition of PARS. Data shown represent means ± SEM of $n=9$–12 observations.

Peroxynitrite-induced thymocyte apoptosis requires intact protein and RNA synthesis

To further characterize the mechanism of peroxynitrite-induced apoptosis, we have investigated whether it requires protein and RNA synthesis. We tested the effect of cycloheximide, a protein synthesis inhibitor, and actinomycin D, an inhibitor of RNA synthesis, on the apoptosis induced by peroxynitrite. As shown on Fig. 5., peroxynitrite-induced apoptosis depends on both protein and RNA synthesis, as evidenced by a remarkably decreased DNA fragmentation (Fig. 5a,b) and annexin V exposure (Fig. 5c) of cycloheximide or actinomycin D-treated thymocytes. (Since actinomycin D-derived fluorescence interferes with the cytofluorimetric method, the effect of actinomycin D could not be investigated by flow cytometry.)

Caspase-3 but not caspase-1 is an important mediator of the peroxynitrite-induced thymocyte apoptosis

Since a novel group of cysteine proteases called caspases is now recognized as key regulators of apoptosis, we investigated whether caspase-1 (also called interleukin convertase enzyme-1) and caspase-3 (also known as apopain/YAMA/CPP32) are involved in the regulation of peroxynitrite-induced apoptosis. For these experiments we have used PARS-deficient thymocytes for two reasons. **1.** As shown above, PARS activation may compromise cellular energetics and thus suppress apoptosis. **2.** In addition, the use of PARS-deficient cells allowed us to investigate, whether the pro-apoptotic effect of apopain is confined to PARS cleavage or cleavage of other substrates may also be important in this process. We have found that caspase-3 becomes activated in peroxynitrite-treated thymocytes (Fig. 6a) with an onset of 4 hr, whereas caspase-1 activation could not be detected (data not shown). Pretreatment of cells with DEVD–fmk (100 μM), an inhibitor of caspase-3, inhibited DNA fragmentation as evidenced with agarose gel electrophoresis (Fig. 6b). Quantitation of the effect of DEVD–fmk on DNA fragmentation of peroxynitrite-treated thymocytes showed a dose-dependent effect of the inhibitor (Fig. 6c).

DISCUSSION

'Basal' formation of peroxynitrite in the thymus: role of iNOS

The mechanisms through which potentially autoreactive premature T cells are eliminated in the thymus remain undefined. The crucial role of high-avidity binding of self-antigenic peptide to the TCR in this process is well known. Additionally, costimulatory signals appear to be necessary for the thymocytes to undergo apoptosis. Likely candidates for this role are reactive nitrogen intermediates, of which peroxynitrite is one of the most potent oxidant species (see: Introduction).

Here we have shown that nitrotyrosine, the 'footprint' of peroxynitrite, is detectable in the mouse thymus by immunohistochemistry. Therefore, our studies provide evidence for the *in vivo* 'basal' production of peroxynitrite in this organ.

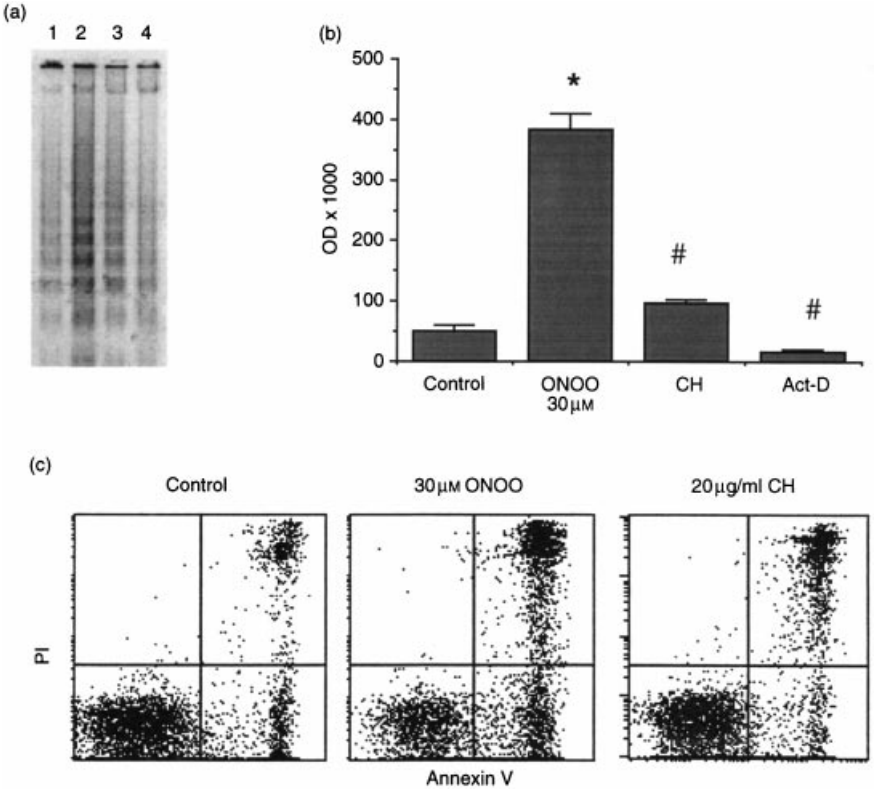

Figure 5. Peroxynitrite-induced apoptosis requires protein and RNA synthesis. The effect of protein and RNA synthesis inhibitors cycloheximide and actinomycin D was investigated on the DNA fragmentation of peroxynitrite treated (30 μM) PARS-deficient thymocytes with agarose electrophoresis (a) and DNA fragmentation ELISA (b). A basal level of DNA fragmentation was detected in untreated cells (lane 1) corresponding to spontaneous thymocyte apoptosis. Peroxynitrite treatment resulted in increased DNA fragmentation (lane 2). Pretreatment of thymocytes with 20 μg/ml cycloheximide (lane 3) or 5 μg/ml actinomycin D (lane 4) prevented peroxynitrite-induced DNA fragmentation. Protective effect of cycloheximide has also been demonstrated with flow cytometric analysis of PS exposure and PI uptake.

Regarded as the 'ugly side of superoxide-nitric oxide-peroxynitrite triangle',[11,12] peroxynitrite has previously been reported to be mainly formed under pathophysiological conditions, contributing to tissue injury in various forms of inflammation, shock and ischaemia reperfusion.[12] Based on the present studies, we propose that the production of peroxynitrite in the lymphoid organs is a normal, 'physiological' process, which probably occurs in response to activation of antigen-presenting cells (dendritic cells, macrophages), and the subsequent oxidative burst and upregulation of iNOS. Our finding, that a less abundant, predominantly perivascular peroxynitrite production has also been detected in the thymi of iNOS-deficient mice, indicates that NO derived from constitutively expressed NOS isoforms (such as the endothelial NOS isoform, eNOS) may also combine with superoxide to form peroxynitrite in the thymus. Since (i) nitrotyrosine reactivity were found in the thymus, and (ii) since peroxynitrite is a potent trigger of thymocyte apoptosis (see below), we propose that peroxynitrite may serve as a physiological mediator of thymocyte negative selection.

Peroxynitrite-induced thymocyte apoptosis: the role of PARS

We have found that peroxynitrite (10 μM) can cause apoptosis in thymocytes, as evidenced by annexin V binding, DNA fragmentation and caspase activation. Our findings that apopain but not ICE becomes activated in response to peroxynitrite are in line with our current understanding of the biological role of the different subgroups of caspases. Members of the ICE subfamily (caspase-1, -4 and -5) are known to be predominantly involved in inflammation, whereas enzymes of the CED-3 subfamily, including caspase 3, have a regulatory role in apoptosis.[25,34,35] Requirement of apopain activation and intact protein and RNA synthesis for the peroxynitrite-induced apoptosis indicate that it is an active process and not merely a consequence of peroxynitrite-induced single-strand breaks.

In response to higher concentrations of peroxynitrite (e.g. 50 μM), we observed that peroxynitrite reduced the spontaneous DNA fragmentation and caused membrane damage as indicated by propidium iodide uptake. This is consistent with previous reports that mild or intense oxidative insults may cause apoptosis or necrosis, respectively.[36] PARS-related changes in cellular ATP concentration may be important determinants regulating this balance. Low concentrations of peroxynitrite, which induced apoptosis, did not affect cellular ATP levels (Fig. 4b). On the other hand, high concentrations of peroxynitrite led to reduced cellular ATP levels, an effect which was prevented by inhibition of PARS. Considering the fact that the execution of the apoptotic program is ATP dependent, we propose that higher levels of oxidant stress

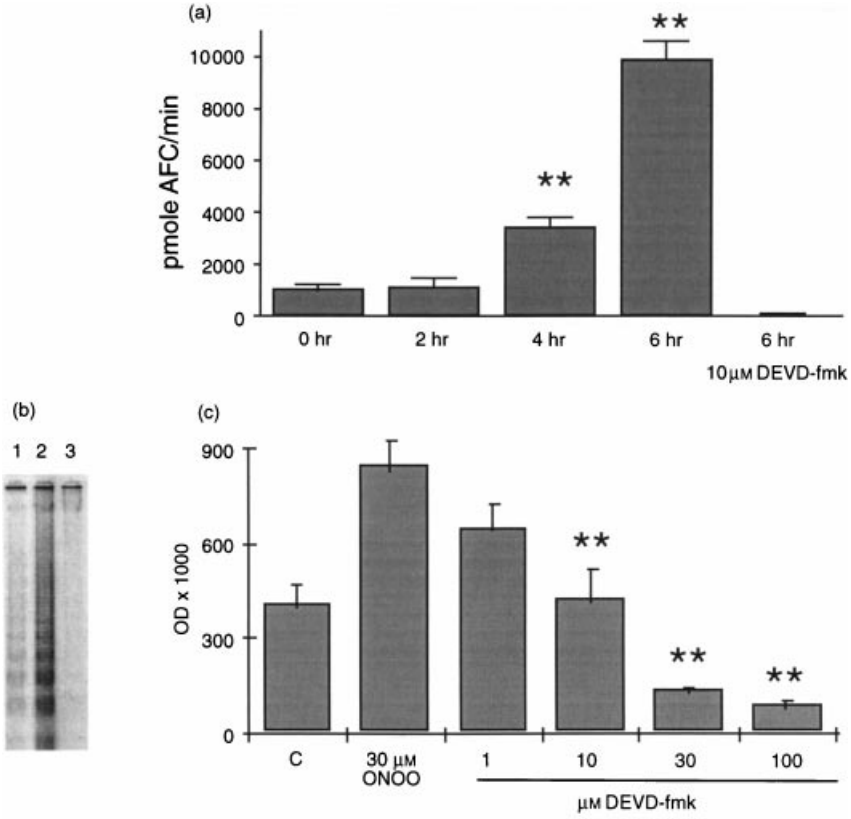

Figure 6. Peroxynitrite-induced apopain activation is indispensible for DNA fragmentation. PARS-deficient thymocytes were treated with 50 μm peroxynitrite and DEVD–AFC-cleaving activity of cell lysates prepared 0 hr, 2 hr, 4 hr, and 6 hr after peroxynitrite treatment has been measured fluorimetrically (a). Control samples pretreated with 10 μM apopain inhibitor, DEVD–fmk were also included. AFC liberation was calculated from a standard curve prepared with free AFC. Results are given as DEVD–fmk inhibitable AFC liberation in pmole AFC/min. Data represent mean ± SD of triplicate samples. Effect of DEVD–fmk on peroxynitrite-induced DNA fragmentation of PARS-deficient thymocytes has been determined with agarose gel electrophoresis (b) (lane 1 = control, lane 2 = 50 μM peroxynitrite, lane 3 = 100 μM DEVD–fmk + peroxynitrite). Data obtained by DNA fragmentation ELISA (c) show a dose-dependent effect of the inhibitor on the peroxynitrite-induced DNA fragmentation. Note that DEVD–fmk suppressed not only peroxynitrite-induced but also spontaneous DNA fragmentation (**$P < 0{\cdot}01$).

inhibit apoptosis because they lower ATP and thus they reduce the activity of enzymes involved in the process of apoptosis. Control of cell death by the cellular energetics is supported by numerous observations.[37–39] Furthermore, recently mitochondria emerged as key organelles determining the fate (apoptosis versus necrosis) of cells exposed to oxidative stress, and cytochrome c released from mitochondria was identified as an activator of caspase-3.[40–42] These observations may provide a direct link between the peroxynitrite-induced suppression of mitochondrial respiration and its caspase-mediated apoptotic effect.

Does PARS-activation interfere with peroxynitrite-induced thymocyte apoptosis *in vivo*?

In vivo, consumption of ATP as a result of PARS activation may be prevented by the inactivation of the enzyme via cleavage by apopain. Thus, the dose-dependent apoptotic effect of peroxynitrite in PARS-deficient thymocytes or cells pretreated with 3-aminobenzamide probably better reflects the *in vivo* physiological situation than the necrotic cell death seen in the wild-type cells. This suggestion is based on the following consideration: by the time iNOS is upregulated in stromal cells and a sufficient amount of NO is produced to form peroxynitrite, PARS is likely to be cleaved as a result of TCR and CD28 signalling-induced apopain activation. From the above, it also follows that during thymocyte negative selection, PARS cleavage may be a mechanism that maintains apoptosis by ensuring that sufficient cellular levels of ATP are maintained. Furthermore, since apopain inhibition suppressed DNA fragmentation of PARS-deficient thymocytes, it is also evident that pro-apoptotic effect of apopain is not confined to cleavage of PARS.

In a recent preliminary report, sensitivity of different cell types, (hepatocytes, neural cells, thymocytes) obtained from PARS-deficient and wild-type mice, to different apoptotic stimuli was reported to be essentially the same in all apoptotic models investigated. This investigation prompted the authors to conclude that cleavage of PARS in the course of apoptosis is nothing else but an epiphenomenon.[29] This may be true in many of the apoptotic models, and is also supported by our finding with FAS-antibody- and dexamethasone-induced thymocyte apoptosis. However, during apoptosis induced by oxidative agents that are capable of activating PARS (e.g. peroxynitrite or H_2O_2), cleavage of PARS makes physiological sense, since it prevents consumption of the cellular energy

pools, as hypothesized before by Earnshaw,[43] allowing peroxynitrite to cause a more 'favourable' form of thymocyte death, which is apoptosis instead of necrosis. This hypothesis is supported by recent findings that PARS-deficient splenocytes are more susceptible to apoptosis induced by DNA-damaging alkylating agents[44] and PARS inhibition results in increased susceptibility of leukaemia cells to apoptosis induced by a methylating agent, temozolomide.[45]

Based on previous studies and our current findings, we summarized our hypothesis for a possible scenario for thymocyte apoptosis with the participation of peroxynitrite, as follows. According to this model, TCR/MHC–peptide interaction and other cell surface costimulatory signals (e.g. B7/CD28) ignites the apoptotic machinery of thymocytes which, on the one hand, leads to the activation of apopain which in turn cleaves PARS and, on the other hand, leads to the upregulation of cytokine genes in the thymocytes. The cytokines will then activate stromal cells to upregulate iNOS and NADPH-oxidase resulting in the production of superoxide and NO which then recombine to form peroxynitrite contributing to completion of thymocyte apoptosis. This hypothesis implies that iNOS-deficient animals might have a defective negative selection unless selection mechanisms are redundant. Although negative selection in the absence of iNOS has not yet been investigated in detail, it may be worthwhile to note that iNOS-expressing stromal cells have been found to be less abundant in the thymi of the autoimmune prone Lewis rats, as compared to Sprague–Dawley or Fisher rats, which has been proposed to contribute to the sensitivity of Lewis rats to autoimmune diseases.[46]

In recent studies, it has been demonstrated that pharmacological inhibition of PARS during oxidant stress in response to various forms of inflammation and shock exerts beneficial effects as it improves survival, prevents the loss of vascular contractility, reduces the degree of peroxynitrite-induced endothelial and epithelial dysfunction, and inhibits neutrophil-related organ injury,[14,46–51] Based on the results of the current study, the question arises as to whether in addition to its role in mediating tissue injury under pathophysiological conditions, peroxynitrite might also be produced in the lymphoid organs and may serve as a physiologic mediator of apoptosis.

ACKNOWLEDGMENTS

The authors wish to thank Alvin G. Denenberg for the HPLC analysis. The authors acknowledge Dr Z. Q. Wang (Institute of Molecular Pathology, Vienna, Austria) for providing the PARS knockout animals, Dr V. Laubach (Glaxo–Wellcome Research Institute, Research Triangle Park, NC, USA) for providing the iNOS knockout animals, and Dr H. Ischiropoulos (Institute of Environmental Medicine, University of Pennsylvania, PA, USA) for providing peroxynitrite for these studies. L.V. was supported by the Ida and Zoltán Dobsa Foundation. This study was supported by a Grant from the National Institutes of Health (R29 GM54773) to C.S.

REFERENCES

1. Anderson G., Moore N.C., Owen J.J. & Jenkinson E.J. (1996) Cellular interactions in thymocyte development. *Annu Rev Immunol* **14,** 73.
2. Page D.M., Kane L.P., Allison J.P. & Hedrick S.M. (1993) Two signals are required for negative selection of $CD4^+CD8^+$ thymocytes. *J Immunol* **151,** 1868.
3. Anderson K.L., Anderson G., Michell R.H., Jenkinson E.J. & Owen J.J. (1996) Intracellular signaling pathways involved in the induction of apoptosis in immature thymic T lymphocytes. *J Immunol* **156,** 4083.
4. Punt J.A., Osborne B.A., Takahama Y., Sharrow S.O. & Singer A. (1994) Negative selection of $CD4^+CD8^+$ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. *J Exp Med* **179,** 709.
5. Drappa J., Brot N. & Elkon K.B. (1993) The Fas protein is expressed at high levels on $CD4^+CD8^+$ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL lpr/lpr. *Proc Natl Acad Sci* **90,** 10 340.
6. Hueber A.O., Raposo G., Pierres M. & He H.T. (1994) Thy-1 triggers mouse thymocyte apoptosis through a bcl-2 resistant mechanism. *J Exp Med* **179,** 785.
7. Lerner A., Clayton L.K., Mizoguchi E. *et al.* (1996) Cross-linking of T-cell receptors on double-positive thymocytes induces a cytokine-mediated stromal activation process linked to cell death. *EMBO J* **15,** 5876.
8. McGowan A.J., Ruiz-Ruiz M.C., Gorman A.M., Lopez-Rivas A. & Cotter T.G. (1996) Reactive oxygen intermediate (s) (ROI): Common mediator (s) of poly (ADP-ribose) cleavage and apoptosis. *FEBS Lett* **392,** 299.
9. Fehsel K., Kröncke K.-D., Meyer K.L., Huber H., Wahn V. & Kolb-Bachofen V. (1995) Nitric oxide induces apoptosis in mouse thymocytes. *J Immunol* **155,** 2858.
10. Tai X.G., Toyo-Oka K., Yamamoto N. *et al.* (1997) Expression of an inducible type of nitric oxide (NO) synthase in the thymus and involvement of NO in deletion of TCR-stimulated double-positive thymocytes. *J Immunol* **158,** 4696.
11. Beckman J.S. & Koppenol W.H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad and the ugly. *Am J Physiol* **271,** 1424.
12. Szabó C. (1996) The role of peroxynitrite in the pathophysiology of shock, inflammation and ischemia-reperfusion injury. *Shock* **6,** 79.
13. Lin K.-T., Xue J.-Y., Nomen M., Spur B. & Wong P.Y.-K. (1995) Peroxynitrite-induced apoptosis in HL-60 cells. *J Biol Chem* **270,** 16 487.
14. Szabó C., Cuzzocrea S., Zingarelli B., O'Connor M. & Salzman A.L. (1997) Endothelial dysfunction in endotoxic shock: importance of the activation of poly (ADP ribose) synthetase (PARS) by peroxynitrite. *J Clin Invest* **100,** 723.
15. Salgo M.G., Squadrito G.L. & Pryor W.A. (1995) Peroxynitrite causes apoptosis in rat thymocytes. *Biochem Biophys Res Commun* **215,** 1111.
16. Szabó C. (1996) DNA strand breakage and activation of poly-ADP ribosyltransferase: a cytotoxic pathway triggered by peroxynitrite. *Free Rad Biol Med* **21,** 855.
17. Szabó C., Zingarelli B., O'Connor M. & Salzman A.L. (1996) DNA strand breakage, activation of poly-ADP ribosyl synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite. *Proc Natl Acad Sci USA* **93**, 1753.
18. Zingarelli B., O'Connor M., Wong H., Salzman A.L. & Szabó C. (1996) Peroxynitrite-mediated DNA strand breakage activates poly-ADP ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipopolysaccharide. *J Immunol* **156,** 350.
19. Cochrane C. (1991) Mechanisms of oxidant injury of cells. *Mol Aspects Med* **12,** 137.
20. Nicholson D.W., Ali A., Thornberry N.A. *et al.* (1995) Identification and inhibition of the ICE/CED-3 protease necessery for mammalian apoptosis. *Nature* **376,** 37.
21. Tewari M., Quan L.T., O'Rourke K. *et al.* (1995) Yama/CPP. 32β, a mammalian homolog of CED-3, is a crmA-inhibitable

protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell* **81,** 801.
22. Kaufmann S.H. (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res* **49,** 5870.
23. Kaufmann S.H., Desnoyers S., Ottaviano Y., Davidson N.E. & Poirier G.G. (1993) Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* **53,** 3976.
24. Miura M., Zhu H., Rotello R., Hartwieg E.A. & Yuan J. (1993) Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homologue of the C. elegams cell death gene ced-3. *Cell* **75,** 653.
25. Nicholson D.W. & Thornberry N.A. (1997) Caspases: killer proteases. *TIBS* **22,** 299.
26. Kumar S. (1997) The apoptotic cysteine protease CPP. 32. *Int J Biochem Cell Biol* **29,** 393.
27. Dixit V.M. (1996) Role of ICE-proteasees in apoptosis. *Adv Exp Med Biol* **406,** 113.
28. Tanigawa Y. & Shimoyama M. (1983) Mg^{2+}-dependent/poly (ADP-ribose) -sensitive endonuclease. *J Biol Chem* **258,** 9184.
29. Leist M., Single B., Künstle G., Volbracht C., Hentze H. & Nicotera P. (1997) Apoptosis in the absence of poly-(ADP-ribose) polymerase. *Biochem Biophys Res Commun* **233,** 518.
30. Wang Z.-Q., Stingl L., Morrison C., Jantsch M. *et al.* (1997) PARP is important for genomic stability but dispensible in apoptosis. *Genes Dev* **11,** 2347.
31. Vermes I., Haanen C. & Steffens-Nakken H. & Reutelingsperger C. (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.
32. Martin S.J., Reutelingsperger C.P.M., McGahon A.J., Rader J.A., van Schie R.C.A.A., LaFace D.M. & Green D.R. (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.
33. Eastman A. (1995) Assays of DNA fragmentation, endonucleases, and intracellular pH and Ca^{2+} associated with apoptosis. *Methods Cell Biol* **46,** 41.
34. Polverino A.J. & Patterson S.D. (1997) Selective activation of caspases during apoptotic induction in HL-60 cells. Effects of a tetrapeptide inhibitor. *J Biol Chem* **272,** 7013.
35. Vanags D.M., Pörn-Ares M., Coppola S., Burgess D.H. & Orenius S. (1997) Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J Biol Chem* **271,** 31 075.
36. Bonfoco E., Krainc D., Ankarcrona M., Nicotera P. & Lipton S.A. (1995) Apoptosis and necrosis: Two distinct event induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci* **92,** 7162.
37. Coppola S., Nosseri C., Maresca V. & Ghibelli L. (1995) Different basal NAD levels determine opposite effects of poly (ADP-ribosyl) polymerase inhibitors on H2O2-induced apoptosis. *Exp Cell Res* **221,** 462.
38. Leist M., Single B., Castoldi A.F., Kuhnle S. & Nicotera P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* **185,** 1481.
39. Richter C., Schweizer M., Cossarizza A. & Francheschi C. (1996) Control of apoptosis by the cellular ATP level. *FEBS Lett* **378,** 107.
40. Kaushal G.P., Ueda N. & Shah S.V. (1997) Role of caspases (ICE/CED 3) proteases in DNA damage and cell death in response to a mitochondrial inhibitor, antimycin A. *Kidney Int* **52,** 438.
41. Yang J., Liu X., Bhalla K., Kim C.N. *et al.* (1997) Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. *Sci* **275,** 1129.
42. Kluck R.M., Bossy-Wetzel E., Green D.R. & Newmeyer D.D. (1997) The release of cytochrome c from mitochondria: a primary site for bcl-2 regulation of apoptosis. *Sci* **275,** 1132.
43. Earnshaw W.C. (1995) Apoptosis: Lessons from *in vitro* systems. *Trends Cell Biol* **5,** 217.
44. De murcia J.M., Niedergang C., Trucco C. *et al.* (1997) Requirement of poly (ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. *Proc Natl Acad Sci USA* **94,** 7303.
45. Tentori L., Orlando L., Lacal P.M. *et al.* (1997) Inhibition of O^6-alkyltransferase or poly(ADP-ribose) polymerase increases susceptibility of leukemic cells to apoptosis induced by temozolomide. *Mol Pharmacol* **52,** 249.
46. Downing J.E.G., Virág L., Salzman A.L. & Szabó C. (1998) Deficiency of Lewis rat thymus for nitrergic cells. *FASEB J* **12,** A601.
47. Szabó C., Wong H.R., Bauer P.I. *et al.* (1997) Regulation of components of the inflammatory response by 5-iodo-6-amino-1,2-benzopyrone, and inhibitor of poly (ADP-ribose) synthetase and pleiotropic modifier of cellular signal pathways. *Int J Oncol* **10,** 1093.
48. Szabó C., Lim L.H.K., Cuzzocrea S. *et al.* (1997) Inhibition of poly (ADP-ribose) synthetase attenuates neutrophil recruitment and exerts antiinflammatory effects. *J Exp Med* **186,** 1041.
49. Cuzzocrea S., Zingarelli B., Costantino G. *et al.* (1997) Beneficial effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase in a rat model of splanchnic artery occlusion and reperfusion. *Br J Pharmacol* **121,** 1065.
50. Eliasson M.J.L., Sampei K., Mandir A.S. *et al.* (1997) Poly (ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischaemia. *Nature Med* **3,** 1089.
51. Szabó C. & Dawson V.L (1998) Role of poly(ADP-ribose) synthetase activation in inflammation and reperfusion injury. *Trends Pharmacol Sci*, in press.