Nitrone spin traps and a nitroxide antioxidant inhibit a common pathway of thymocyte apoptosis

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Oxidative stress has recently been suggested to be a mediator of apoptotic cell death [Buttke and Sandstrom (1994) *Immunology Today* 15, 7–10], although evidence that this phenomenon is a widespread component of apoptosis is lacking. When rat thymocytes were exposed to the glucocorticoid methylprednisolone (MPS), a progressive increase in intracellular peroxides and a decrease in glutathione (GSH) were observed to accompany the onset of apoptosis. Using Percoll density gradients to isolate subpopulations of thymocytes at different stages of apoptosis, the increase in peroxide content was found to be restricted to apoptotic cells, while a significant depletion of GSH and reduced protein thiol was detected in both pre-apoptotic and fully apoptotic cells. To investigate the biological significance of these redox changes, the free radical spin traps 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrroline-

1-oxide (TMPO), and the related nitroxide-radical antioxidant 2,2,6,6-tetramethyl-1-piperidiny-1-oxyl (TEMPO) were tested as inhibitors of thymocyte apoptosis. The cell shrinkage and DNA fragmentation induced by four different initiators of apoptosis were reduced by each compound. TEMPO inhibition of both etoposide- and MPS-induced thymocyte DNA fragmentation was also found to correlate with an increase in intracellular GSH, providing support for the proposal that its antioxidant properties were responsible for the observed protective activity. We conclude that some form of intracellular oxidation (here measured indirectly by changes in intracellular GSH and peroxide levels) is required during thymocyte apoptosis even when this process is initiated by an agent that does not exert a direct oxidant action.

INTRODUCTION

The capacity to die is an inherent property of cells. It permits the selective elimination of excess cells during development of a multicellular organism, the homeostasis of tissue size, and, in mammals, the removal of both autoreactive immature and clonally expanded mature lymphocytes. The majority of these cell deaths share a common morphology, including a reduction in cell volume, blebbing of the plasma membrane, chromatin aggregation (often followed by fragmentation of DNA into nucleosome-sized remnants), and finally the break-up of the cell into discrete membrane-bound fragments that are prone to rapid phagocytosis by neighbouring cells. This morphologically distinct form of cell death is called apoptosis [1,2].

Apoptotic cell death can be induced experimentally in many different ways. Examples include ligation of cell-surface receptors, elevation of intracellular Ca^{2+} , chelation of intracellular Zn^{2+} , irradiation, inhibition of either topoisomerase II or protein kinase C, exposure to oxidative stress, deprivation of growth factors and glucocorticoid treatment (reviewed in [3] and [4]). A complex array of initial intracellular disturbances can therefore engage apoptosis in any given cell. However, the biochemical mechanisms underlying the common morphological changes that subsequently occur are poorly characterized.

At the molecular level, one of the most reproducible features of apoptosis is the ability of the Bcl-2 protein to inhibit or at least

retard the process [5–7]. Hockenberry et al. and Kane et al. have independently demonstrated that Bcl-2 possesses an antioxidant property and proposed that this is causally related to the ability of the protein to inhibit cell death [8,9]. If correct, this implies that undefined oxidative events are necessary if a cell is to successfully undergo apoptosis. The ability of thiol-containing antioxidants such as *N*-acetyl cysteine (NAC) or *N*-(2-mercaptoethyl)-1,3-propanediamine to similarly confer protection against apoptosis supports this hypothesis [10–12]. In addition, Flomerfelt et al. report elevated expression of a glutathione *S*-transferase gene at an early stage in dexamethasone-induced lymphocyte apoptosis [13]. As this gene is known to be transcriptionally up-regulated by oxygen radicals, it provides further indirect evidence for the increased presence of oxidants in cells undergoing apoptosis. However, direct biochemical evidence that cells are oxidatively stressed at an early stage in the apoptotic pathway is lacking.

Superoxide, hydroxyl radical and lipid radicals each react with nitrones such as 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrroline-1-oxide (TMPO) to form more stable nitroxide radical products [14]. Although this property is usually exploited in e.s.r. studies, in any situation where the same radicals are essential biochemical mediators their effects will be ameliorated by reaction with the spin traps. For example, both compounds have been shown to protect neurons against *N*-methyl-D-aspartate-induced cell death by scavenging superoxide

Abbreviations used: BSO, buthionine sulfoximine; DCF, 2',7'-dichlorofluorescein; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; MEM, minimum essential medium; MPS, methylprednisolone; NAC, *N*-acetyl cysteine; TBARS, thiobarbituric acid-reactive substances; TEMPO, 2,2,6,6-tetramethyl-1-piperidiny-1-oxyl; TMPO, 3,3,5,5-tetramethyl-1-pyrroline-1-oxide; TNF α , tumour necrosis factor α ; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine.

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Figure 1 Inhibition of thymocyte apoptosis by nitron free radical spin traps

Thymocytes were incubated at 37 °C for 6 h in the presence or absence of 25 μ M etoposide (a) or 500 nM MPS (b), and various concentrations of DMPO (a) or TMPO (b). DNA fragmentation in untreated (●) or treated (■) cells was assayed with diphenylamine, and is expressed as percentage of DNA in a soluble form. Data points show mean ($\pm$ S.D.).

[15]. The nitroxide radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) possesses a superoxide dismutase-mimicking activity [16], and can maintain iron in its ferric form such that the potential for hydroxyl radical generation via the Haber-Weiss reaction is lowered [17]. Iron-dependent reduction of TEMPO also generates a potent hydroxylamine-containing antioxidant that can redox cycle back to its original state by reducing a second radical species [18]. In this paper we demonstrate that both types of antioxidant provide rat thymocytes with protection against apoptosis induced by multiple stimuli. Pre-apoptotic and apoptotic cells were found to be depleted of GSH and reduced protein thiols, and the latter cells also had an elevated peroxide content. Prevention of these changes by extracellular addition of antioxidants is demonstrated to correlate with an inhibition of apoptosis. These results identify intracellular oxidation as an early biochemical event common to many forms of thymocyte apoptosis.

MATERIALS AND METHODS

Materials

The following materials were purchased from the manufacturer listed. DMPO, TMPO and TEMPO were from Aldrich; DNA molecular-mass marker VI from Boehringer Mannheim; RPMI 1640, 10 $\times$ Eagle's minimum essential medium (MEM), HEPES and thapsigargin from Gibco; Percoll from Pharmacia; bicinchoninic acid (BCA) protein assay reagent from Pierce; dansyl chloride, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), etoposide, 6 α -methylprednisolone (MPS), and N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) from Sigma; 2',7'-dichlorofluorescein (DCF) diacetate from Molecular Probes.

Thymocyte preparation and culture

Thymic glands were obtained from 6- to 8-week-old male Sprague-Dawley rats and thymocytes prepared as described by McConkey et al. [19]. For experiments, cells were incubated at 5×10^6 cells/ml in RPMI 1640 supplemented with 2% (v/v) fetal-calf serum and 10 μ g/ml gentamicin in an atmosphere containing 5% CO₂. Cell viability, as determined by Trypan Blue exclusion, was greater than 95% at the start of each experiment, and remained above 80% after 6 h of culture in all cases.

Percoll fractionation

Isotonic 100% Percoll was prepared by diluting 9 vol. of Percoll in 1 vol. of 10 $\times$ Eagle's MEM, and then supplemented to 20 mM HEPES, 24 mM NaHCO₃, 10 μ g/ml gentamicin and 0.1% Pluronic F68. Percoll solutions of various densities were prepared by diluting this 95% Percoll stock solution in similarly supplemented RPMI 1640. Discontinuous Percoll gradients were prepared as described by Wyllie and Morris [20], with the addition of 55 and 65% layers of Percoll. Cells suspended in supplemented medium were layered on the top of the gradient and centrifuged at 2000 *g* for 15 min. The cells at each interface were collected, washed and resuspended on ice in isotonic buffer (110 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl₂, 24 mM NaHCO₃ and 1 mM MgSO₄, pH 7.2). Cells were maintained at 37 °C between their initial harvest and final collection from the Percoll gradient (approx. 1 h). Five different cell populations were routinely obtained: fractions 1 (55–60% Percoll interface), 2 (60–65%), 3 (65–70%), 4 (70–80%) and 5 (80–95%). The 60% Percoll layer was omitted in one series of experiments (see Table 5), and in this case cells in fractions 1 and 2, as defined above, were collected together.

Quantification of apoptosis

DNA fragmentation was quantified with diphenylamine [19]. The agarose gel electrophoresis protocol described by Aw et al. [21] was used to detect nucleosome-sized DNA ladders, except that whole-cell homogenate was processed without prior fractionation (2×10^6 cells per sample). The distribution of cell volumes in a sample of 5×10^5 thymocytes was measured in a Z1 Coulter counter (Coulter Electronics Ltd., Luton, U.K.), and results presented either as average cell volumes or as a frequency distribution of cells in each 10 fl size class from 50–200 fl.

Thiol analysis

After Percoll fractionation, GSH in each cell population was estimated as total acid-soluble thiol using DTNB [22] after

Table 1 Inhibition of cell shrinkage by nitron free radical spin traps

Thymocytes were incubated at 37 °C in the presence of DMPO and TMPO (both 40 mM). Apoptosis was induced by MPS (500 nM), etoposide (25 μ M) or thapsigargin (100 nM) and cell volume measured after 6 h. Results are presented as means ($\pm$ S.D.).

	Mean cell volume (fl)		
	Untreated	DMPO	TMPO
Untreated	105 $\pm$ 2 (11) [†]	106 (2)	107 $\pm$ 5 (6)
MPS	96 $\pm$ 6* (9)	104 $\pm$ 7 (3)	105 $\pm$ 3 (4)
Etoposide	93 $\pm$ 4* (4)	108 (1)	99 (2)
Thapsigargin	99 (2)	107 (1)	109 (1)

* *t*-test, *P* < 0.01 when compared with untreated control.

[†] Number of independent samples.

precipitation of proteins in 5% (w/v) trichloroacetic acid. Trichloroacetic acid-precipitated proteins from the same samples were washed three times and reduced thiol groups (protein-SH) measured as described by Di Monte et al. [23]. In each case results are expressed as nmol of reduced thiol per mg of protein in the original cell extract.

In four experiments, thymocytes (1.5×10^7 per assay) were incubated for 1–6 h in the presence of 500 nM MPS or 25 μ M etoposide, and either 0, 0.5, 1 or 2 mM TEMPO. The cells were washed twice in isotonic buffer, extracted with 1 M perchloric acid and derivatized with 6 mM iodoacetic acid at pH 9.0 for 1 h. Dansyl chloride (2 mM) was added, and the samples left for 24 h to form *S*-carboxymethyl-*N*-dansyl-GSH and *N,N'*-bis-dansyl-GSSG. The adducts were separated by h.p.l.c. essentially as described by Reed et al. [24] for the corresponding dinitrophenyl adducts, and quantified relative to standards using a fluorescence detector [excitation wavelength (Ex) 335 nm; emission wavelength (Em) 515 nm].

α -Tocopherol analysis

α -Tocopherol was measured by h.p.l.c. on a C₁₈ CT-sil column (Chromtech AB, Norsborg, Sweden) using electrochemical detection in the oxidative mode (0.5–0.7 V) [25].

Analysis of intracellular peroxides and peroxidation

Intracellular peroxides were measured following formation of a fluorescent derivative of DCF [26,27]. Briefly, thymocytes suspended in phosphate-buffered saline (pH 7.3) were incubated at 37 °C for 15 min with 10 μ M DCF diacetate (0.05% ethanol final concentration). The cells were washed twice, and then lysed by sonication in 1 ml of 50 mM potassium phosphate, pH 7.0, with 0.1% CHAPS and 0.1 mM EDTA. Insoluble material was removed by centrifugation (15000 *g* for 10 min at 4 °C), and fluorescence in the supernatant determined (Ex 488 nm; Em 520 nm). Thiobarbituric acid-reactive substances (TBARS) were also measured after one experiment [28].

Statistical analysis

Sample means were compared by a two-tailed *t*-test after log₁₀ transformation of the data.

RESULTS

Rat thymocytes readily undergo apoptosis in response to various stimuli [29–33]. To investigate whether intracellular oxidation is a necessary component of this process, thymocytes were exposed to different inducers of apoptosis in the presence of the free radical spin traps DMPO and TMPO, and the degree of DNA fragmentation and cell shrinkage determined 6 h later. Both spin traps were observed to provide thymocytes with concentration-dependent protection against apoptosis. This was evident as a reduction in (1) the amount of soluble DNA produced (Figures 1a and 1b), (2) the degree of DNA laddering detectable on an agarose gel (data not shown), and (3) the number of shrunken cells present. Very similar results were obtained irrespective of whether apoptosis was induced by MPS (a glucocorticoid hormone), etoposide (a topoisomerase II inhibitor), thapsigargin (an inhibitor of endoplasmic reticular Ca²⁺-ATPase) or TPEN (an intracellular Zn²⁺ chelator). The results are summarized in Tables 1 and 2. At concentrations of 10 mM and above, both TMPO and DMPO were consistently observed to inhibit the shrinkage and DNA fragmentation that follows exposure of thymocytes to each initiator of apoptosis. As it is unlikely that the free radical spin traps prevent each of the four inducers from interacting with their different molecular targets within the cell, this result suggests that a shared component(s) of the apoptotic pathway is blocked in their presence.

A similar outcome was obtained when the experiments were repeated with the nitroxide radical antioxidant TEMPO. Apoptosis, as measured by both degradation of nuclear DNA and cell shrinkage, was reduced in all cases (see Figure 2). When DNA fragmentation at 6 h was compared with the four different initiators, EC₅₀ values in the range 0.5–1.8 mM TEMPO were obtained (Table 2). This approximate 10–20-fold increase in efficacy relative to the nitron spin traps probably relates to the higher membrane permeability and more reactive nature of the TEMPO radical as opposed to the DMPO and TMPO ions. In time-course experiments TEMPO (as well as DMPO and TMPO) was found to delay rather than completely prevent apoptotic DNA degradation (data not shown). No effect on cell viability, as measured by Trypan Blue exclusion, was detectable after 6 h at the concentrations of the antioxidants used, although exposure of thymocytes to TEMPO concentrations above 2 mM was observed to stress the cells and eventually cause some necrosis.

The higher inhibitory activity of TEMPO was also associated with a reduction in the spontaneous apoptosis of unstimulated

Table 2 Inhibition of thymocyte DNA fragmentation by nitron spin traps and nitroxide radical-containing antioxidants

Thymocytes were incubated at 37 °C in the presence of various concentrations of each antioxidant. Apoptosis was induced with MPS (500 nM), etoposide (25 μ M), thapsigargin (100 nM) or TPEN (12.5 μ M). Cells were harvested after 6 h and DNA fragmentation determined by the diphenylamine assay. For each experiment an EC₅₀ relative to fragmentation in the absence of antioxidant (100%) or inducer (0%) was calculated by linear regression. Results are presented as means ($\pm$ S.D.). Values in parentheses indicate the number of independent experiments. Abbreviation: n.d., not determined.

Antioxidant	Inhibition of DNA fragmentation [EC ₅₀ (mM)]			
	MPS	Etoposide	Thapsigargin	TPEN
DMPO	22.9 $\pm$ 9.4 (3)	22.6 $\pm$ 7.8 (3)	n.d.	26.0 (2)
TMPO	19.1 $\pm$ 4.2 (5)	30.7 $\pm$ 11.2 (3)	20.2 $\pm$ 2.4 (3)	n.d.
TEMPO	1.8 $\pm$ 0.8 (3)	1.6 $\pm$ 0.9 (3)	0.8 $\pm$ 1.0 (3)	0.5 $\pm$ 0.2 (3)

Figure 2 Inhibition of thymocyte apoptosis by the nitroxide radical antioxidant TEMPO

Thymocytes were incubated with various concentrations of TEMPO as described in Figure 1. (a) Thymocytes were either untreated or exposed to etoposide, MPS, thapsigargin or TPEN, and DNA fragmentation determined using diphenylamine. (b) Total DNA extracted from cells incubated with etoposide and/or TEMPO was electrophoresed through a 1.8% agarose gel, and the presence of nucleosome-sized DNA fragments visualized with ethidium bromide. For a size comparison, DNA molecular-mass markers (range 2176–154 bp) are run in the first lane. (c) Cell volume was measured in a Coulter counter, and is expressed as a frequency distribution for untreated or etoposide-treated cells incubated in the presence or absence of 2 mM TEMPO.

Table 3 TEMPO inhibition of spontaneous thymocyte DNA fragmentation

Freshly prepared thymocytes were centrifuged over a cushion of 70% Percoll to remove apoptotic cells, washed and then incubated for 3 h at 37 °C in the presence of 0, 0.5, 1 or 2 mM TEMPO. DNA fragmentation was determined using the diphenylamine assay. Data points are means ($\pm$ S.D.) from triplicate samples.

TEMPO concentration (mM)	DNA fragmentation (%)
0	10.5 $\pm$ 0.9
0.5	9.2 $\pm$ 0.9
1.0	6.9 $\pm$ 1.5*
2.0	6.1 $\pm$ 0.8*

* *t*-test, $P < 0.05$ when compared with cells cultured in the absence of TEMPO

thymocytes. Combining all the data, DNA fragmentation of untreated thymocytes after 6 h of culture was reduced by 18% in the presence of 2 mM TEMPO (average $17 \pm 3\%$ and $14 \pm 3\%$ DNA fragmentation with 0 and 2 mM TEMPO respectively; *t*-test, $P > 0.1$). However, the variable amount of background DNA fragmentation already present in thymocytes at the start of each experiment (range 2–10% soluble DNA) almost certainly reduces the magnitude of this difference. To investigate this issue further, thymocytes freshly obtained from a rat were centrifuged over a cushion of 70% Percoll (to remove contaminating high-density apoptotic cells), and the normal density thymocytes collected. When these cells were cultured in the presence of 2 mM TEMPO a significant decrease in their subsequent rate of spontaneous apoptosis was observed. An example after 3 h culture is shown in Table 3.

The observation that antioxidants protect thymocytes against apoptosis induced in different ways suggests that some form of intracellular oxidation is occurring in each case. Our attempts to obtain direct evidence for the production of free radicals during thymocyte apoptosis via detection of a DMPO or a *N*-tert-butyl- α -phenylnitron spin-trapped e.s.r. signal have so far been unsuccessful. This is not surprising given the relative insensitivity of this method coupled with the unsynchronized entry of individual thymocytes into apoptosis. GSH is one of the most abundant reductants present in mammalian cells, and determination of changes in its concentration provides an alternative method of monitoring oxidative events within cells. The intracellular GSH content of thymocytes undergoing apoptosis was observed to fall approximately coincident with an increase in DNA fragmentation (two experiments; see Figure 3). This decrease in GSH was not accompanied by a corresponding increase in intracellular GSSG (results not shown). GSH loss occurred faster in MPS-stimulated cells; after 6 h of culture the GSH levels in untreated cells had decreased 17%, while in the presence of MPS it had fallen by 46%. The corresponding increases in apoptotic DNA fragmentation were 9% and 38% respectively. This result suggests that thymocytes are oxidatively stressed as they undergo apoptotic DNA fragmentation irrespective of whether the apoptosis is spontaneous or induced by a glucocorticoid hormone.

To obtain more direct evidence that these oxidative events were confined to apoptotic cells, thymocytes were either untreated or exposed to MPS, washed and then centrifuged through a discontinuous Percoll density gradient to separate cells in different stages of apoptosis [20,34]. The pattern of recovery of cells from the Percoll gradients was similar irrespective of their incubation conditions, the only difference being that approxi-

Figure 3 Decrease in intracellular GSH during MPS-induced thymocyte apoptosis

(a) Thymocytes were incubated at 37 °C for various times in the presence (■) or absence (●) of 500 nM MPS, washed, and their intracellular GSH content measured by h.p.l.c. as described in the Materials and methods section. (b) DNA fragmentation was assayed using diphenylamine in a sample of the same cells, and is expressed as percentage of total DNA recovered in the soluble phase. Results show mean ($\pm$ S.D.) for triplicate samples.

mately 2-fold more cells were recovered in the two highest density fractions after MPS treatment. By incubating cells for the shortest time possible for MPS induction of apoptosis (2 h), viability remained high in all of the different cell populations collected (Table 4). Minimal soluble DNA was detectable in the cells recovered in fractions 1, 2 and 3 (identified by Sun et al. as healthy mature and immature thymocytes [34]), while those of fraction 4 had intermediate and those of fraction 5 consistently

Table 4 Percoll density gradient separation of thymocytes at different stages of apoptosis

Thymocytes were incubated at 37 °C in the presence or absence of 500 nM MPS, washed and centrifuged through a discontinuous Percoll density gradient as described in the Materials and methods section. Cells at each interface were collected, washed and cell viability (Trypan Blue exclusion), DNA fragmentation (diphenylamine assay) and cell volume determined.

Percoll fraction	Cell viability (%)		DNA fragmentation* (% soluble)		Mean cell volume (fl)	
	Untreated (n = 2)	MPS* (n = 3)	Untreated (n = 8)	MPS (n = 12)	Untreated (n = 1)	MPS (n = 1)
1	97	98 $\pm$ 1	1 $\pm$ 1	3 $\pm$ 2	132	125
2	96	97 $\pm$ 1	1 $\pm$ 1	2 $\pm$ 1	109	110
3	98	98	2 $\pm$ 1	4 $\pm$ 4	105	105
4	97	95 $\pm$ 1	11 $\pm$ 10	23 $\pm$ 22	87	87
5	89	93 $\pm$ 7	73 $\pm$ 11	73 $\pm$ 13	76	79

* Mean $\pm$ S.D.

high amounts of soluble DNA (Table 4). Cell volume was also measured after one of the Percoll gradients, and as expected the thymocytes with intermediate and high levels of DNA fragmentation were both smaller than the other cells (Table 4). These results therefore indicate that each of the different cell populations used for biochemical analysis (see below) were intact, despite their differences in degree of shrinkage and DNA fragmentation.

A significant decrease in the concentration of trichloroacetic acid-soluble thiol (mainly GSH) was detectable in both the intermediate and fully apoptotic cells compared with normal thymocytes (Table 5). It is significant that this decline (approx. 40%) is detectable in the cells recovered from fraction 4, as it suggests that thymocytes experience an oxidative stress at an early stage of apoptosis prior to the formation of inter-nucleosomal DNA fragments. When results from untreated and MPS-stimulated thymocytes are compared, it is clear that cells of similar density and degree of apoptosis have similar levels of GSH. In each case the concentration of reduced protein thiol in the intermediate and fully apoptotic fractions was also decreased by about 30%, although the difference was only significant in one case (Table 5). In contrast, the α -tocopherol content of the cells was independent of their density and degree of DNA fragmentation (Table 5; although a non-significant trend for higher α -tocopherol in the mature thymocytes of fraction 1 and lower α -tocopherol in the apoptotic cells of fraction 5 can be seen). These results therefore indicate that both spontaneous and MPS-induced thymocyte apoptosis proceeds through a common mechanism that includes an early unidentified oxidative component.

In a separate series of experiments, the intracellular peroxide content of MPS-stimulated thymocytes at different stages of apoptosis was also determined (Table 6). Peroxide levels in apoptotic thymocytes (fraction 5) were approx. 3-fold higher than in normal immature thymocytes (fraction 3), providing further evidence that apoptotic thymocytes are oxidatively stressed. No change in the peroxide content of pre-apoptotic thymocytes was detected (fraction 4), while they were slightly elevated in the mature cells (fraction 1). In a single experiment, thymocytes incubated for 3 h with 25 μ M etoposide were again separated by Percoll density gradient centrifugation as described in Table 6, and aldehyde products of lipid peroxidation in each

Table 5 Determination of reduced trichloroacetic acid-soluble thiol, protein thiol and α -tocopherol in thymocytes at different stages of apoptosis

Thymocytes were harvested from Percoll density gradients as described in Table 4, washed and the levels of reduced trichloroacetic acid-soluble thiol, protein thiol and α -tocopherol determined. Data points are means $\pm$ S.D.

Percoll fraction	Reduced trichloroacetic acid-soluble thiol (nmol/mg of protein)		Reduced protein thiol (nmol/mg of protein)		α -Tocopherol (pmol/mg of protein)	
	Untreated (<i>n</i> = 3)	MPS (<i>n</i> = 4)	Untreated (<i>n</i> = 3)	MPS (<i>n</i> = 4)	Untreated (<i>n</i> = 2)	MPS (<i>n</i> = 4)
1	21 $\pm$ 2	21 $\pm$ 3	100 $\pm$ 28	92 $\pm$ 25	216	205 $\pm$ 35†
2	22 $\pm$ 2	24 $\pm$ 5	83 $\pm$ 24	85 $\pm$ 26	225	174 $\pm$ 36
3	20 $\pm$ 2	20 $\pm$ 2	78 $\pm$ 17	81 $\pm$ 20	133	126 $\pm$ 22
4	13 $\pm$ 5*	14 $\pm$ 4*	48 $\pm$ 18*	60 $\pm$ 18†	120	136 $\pm$ 28
5	10 $\pm$ 3*	11 $\pm$ 6*	61 $\pm$ 23	57 $\pm$ 30†	116	117 $\pm$ 8†

**t*-test, *P* < 0.05 when compared with combined average of fractions 2 and 3 (over 70% of normal thymocytes are recovered in these two fractions).

†*t*-test, 0.1 *P* > 0.05 when compared as above.

Table 6 Peroxide content of thymocytes at different stages of apoptosis

Thymocytes were incubated at 37 °C for 2 h in the presence of 1 μ M MPS, washed and then centrifuged through a discontinuous Percoll density gradient at 4 °C as described in the Materials and methods section. The 60% Percoll layer was omitted from these gradients, and fraction 1 therefore corresponds to fraction 1 and 2 in the experiments described in Tables 2 and 4. The peroxide content of the cells was measured using DCF, and is expressed as relative fluorescence (RF) per mg of protein. Data points are means ($\pm$ S.D.) from four independent experiments.

Percoll fraction	Cell viability (%)	DNA fragmentation (%)	Peroxide content (RF per mg of protein)
1	96 $\pm$ 1	1 $\pm$ 1	98 $\pm$ 11*
3	95 $\pm$ 4	1 $\pm$ 1	66 $\pm$ 9
4	97 $\pm$ 0	3 $\pm$ 2	77 $\pm$ 13
5	89 $\pm$ 6	73 $\pm$ 23	208 $\pm$ 42*

**t*-test, *P* < 0.05 when compared with cells recovered in fraction 3 (normal immature thymocytes).

fraction measured. 1422, 1424, 1540 and 2360 pmol of TBARS/mg of protein were present in the thymocytes of fractions 1, 3, 4 and 5 respectively, indicating that more end-products of lipid peroxidation had accumulated in the apoptotic cells (fraction 5) than in those of any other fraction.

To investigate whether antioxidant inhibition of thymocyte apoptosis correlated with a prevention of oxidative changes within the treated cells, thymocytes were incubated for 5 h in the presence of increasing concentrations of TEMPO (0–2 mM) and their GSH levels and degree of DNA fragmentation subsequently determined. A concentration-dependent increase in the GSH content of unstimulated thymocytes was observed following a 5 h exposure to TEMPO (Figure 4a). Very similar results were obtained with both MPS- and etoposide-treated thymocytes, except that, as expected, initial GSH levels were about 30–40% lower. An example with etoposide-treated thymocytes is shown in Figure 4(a). The TEMPO-induced increase in intracellular GSH was accompanied by a steady reduction in apoptotic DNA fragmentation in these cells (Figure 4b).

Figure 4 TEMPO increases intracellular GSH levels in etoposide-treated thymocytes at similar concentrations to its inhibitory effect on DNA fragmentation

Thymocytes were incubated at 37 °C for 5 h with 0, 0.5, 1 or 2 mM TEMPO in the presence (hatched bar) or absence (open bar) of 25 μ M etoposide. Cells were washed, and intracellular GSH (a) and % DNA fragmentation (b) measured as described in the legend to Figure 3. Results show means ($\pm$ S.D.) from triplicate samples.

DISCUSSION

Several reports that oxidants can induce apoptosis when directly applied to cells exist. For example exposing cultured cells to low concentrations of H_2O_2 or lipid hydroperoxides can lead to apoptosis [35–38], while oxidized low-density lipoproteins have been shown to elicit apoptotic DNA fragmentation in lymphocytes [39]. The induction of apoptosis in response to tumour necrosis factor- α (TNF α ; [40,41]) may also relate to the increased generation of oxygen radicals that follows exposure to this cytokine [42–44]. Antioxidants have been found to inhibit apoptosis in many of these situations. Pretreating mouse thymocytes with a water-soluble analogue of α -tocopherol reduces H_2O_2 -induced apoptotic DNA fragmentation below control levels [38], while in various different cell systems the thiol reductant NAC has been reported to inhibit apoptosis following either exposure to TNF α or ligation of the T-cell receptor [10,11,45,46]. Overexpression of superoxide dismutase has also been observed to protect cells against several forms of apoptosis, while its down-regulation or inhibition promotes apoptotic cell death in both T lymphocytes and neuronal cells [47–50]. These findings have led to the suggestion that apoptosis is activated by oxidative events within a cell [51,52], although biochemical evidence that apoptotic cells experience an oxidative stress has been lacking.

The results presented in this paper indicate that oxidative events occur at an early stage during thymocyte apoptosis even when initiated by agents such as MPS or etoposide that are not, in themselves, oxidants. These events were manifest as a depletion of intracellular GSH and reduced protein thiol in both pre-apoptotic and apoptotic thymocytes, as well as in a significant increase in the endogenous peroxide levels of apoptotic cells. The GSH depletion was not accompanied by a corresponding increase in the intracellular concentration of its oxidized form GSSG. However, as GSSG is known to be rapidly transported out of various cell types and also to form mixed disulphides with reduced protein thiols, measurements of its intracellular concentration is an insensitive indicator of its rate of formation [53,54].

It is unlikely that GSH depletion alone is responsible for activating apoptosis. Although inhibition of GSH biosynthesis with buthionine sulfoximine (BSO) has been shown to sensitize HL-60 cells to necrosis, no effect of GSH depletion on the extent of apoptosis induced by either etoposide or camptothecin was observed [55]. Similarly we have recently found BSO treatment to have no effect on apoptosis in a T-cell hybridoma induced by either MPS or anti-CD3 antibody (D. P. Jones, E. Maellaro, S. Jiang, A. F. G. Slater and S. Orrenius, unpublished work). It is therefore more likely that the changes observed in the peroxide and GSH contents of apoptotic thymocytes were a consequence of other more specific oxidative modifications that occurred during initiation of the apoptotic programme.

In this study, millimolar concentrations of the nitron spin traps and nitroxide-containing antioxidants were needed to observe an inhibitory effect on apoptosis, while similar concentrations of NAC (1–25 mM) and α -tocopherol are reported to be required in other cell systems [10,11,38,46]. As these reagents are all used in high concentration, it is possible that they exert other cellular effects in addition to their known antioxidant action. For example, the lipid-soluble spin traps DMPO and TMPO may perturb membrane structure such that cell shrinkage (and other apoptotic changes) are somehow non-specifically prevented. In the absence of direct e.s.r. evidence for spin-trapped radicals, this issue remains unresolved. However, the observation that TEMPO increased intracellular GSH levels at approximately the same

concentrations as it inhibited apoptosis (Figure 4) suggests that the antioxidant interpretation of its action is correct. We have also recently observed that physiological concentrations of natural antioxidants such as ascorbic acid and dihydrolipoic acid inhibit both MPS- and etoposide-induced thymocyte apoptosis (E. Maellaro, J. Bustamante, S. Nobel, A. F. G. Slater, M. Comporti and S. Orrenius, unpublished work).

In summary, the common protective activity of antioxidants as structurally different as DMPO, TEMPO, NAC, α -tocopherol and (possibly) bcl-2 provides strong, albeit indirect, evidence that oxidants are involved in apoptotic cell death. Our results show that thymocytes experience an oxidative shift in their intracellular redox at an early stage in apoptosis prior to any detectable loss in their viability. Prevention of these changes was found to correlate with an inhibition of the subsequent events such as cell shrinkage and internucleosomal DNA degradation that define apoptosis in these cells. As similar results were obtained irrespective of the initiating stimulus, intracellular oxidation is probably an essential biochemical component of thymocyte apoptosis. Although the mechanism of formation, identity and targets of the oxidants involved are unknown, we believe their discovery is necessary for a more complete understanding of this complex topic.

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