ORIGINAL ARTICLE

4-OH-TEMPO prevents the morphological alteration of rat thymocytes primed to apoptosis by oxidative stress inducer ButOOH

Ewa Kossowska[1], Agata Zauszkiewicz[2], Jolanta Kubasik-Juraniec[2], Cecylia Tukaj[2], Jan Henryk Spodnik[3], Anna Hallman[4], Jerzy Klimek[4], Emilia Syta[5], Adam Figarski[6], Takashi Wakabayashi[6], Michał Woźniak[6]

[1]Department of Biochemistry, Medical University, Gdańsk, Poland
[2]Laboratory of Electron Microscopy, Medical University, Gdańsk, Poland
[3]Department of Anatomy and Neurobiology, Medical University, Gdańsk, Poland
[4]Department of Pharmaceutical Biochemistry, Medical University, Gdańsk, Poland
[5]Department of Biophysics, Medical University, Gdańsk, Poland
[6]Department of Medical Chemistry, Medical University, Gdańsk, Poland

Thymocytes exposed to the pro-oxidant tert–butyl-hydroperoxide (ButOOH) display a number of dramatic changes in morphology similar to those observed in the case of dexamethasone-treated cells. Both reagents induce nuclear chromatin peripheral aggregation below the nuclear membrane. Some nuclei themselves break up producing two or more fragments. ButOOH-treated cells are morphologically characterised by cell shrinkage, extensive surface blebbing and, finally, fragmentation into membrane–bound apoptotic bodies composed of cytoplasm and tightly packed with or without nuclear fragments. An increased level of lipid hydroxyperoxides was detected after exposure of thymocytes to ButOOH. Both oxidative stress markers and morphological damage to cells were prevented by the antioxidant 4-OH-TEMPO.

Key words: 4-OH-TEMPO, apoptosis, thymocytes, oxidative stress

INTRODUCTION

Oxidative stress has been implicated in the pathophysiology of cell damage and death [8, 10, 22]. All aerobic cells generate reactive oxygen species (ROS), which are potentially cytotoxic [7, 13, 20]. When the subtle redox balance of the cell is finally disturbed, the level of ROS can be significantly raised, resulting in oxidative stress.

Among the most important mechanisms in the first line of cell defence against oxidative stress is the glutathione–glutathione peroxidase system. Glutathione peroxidases neutralise hydroxyperoxides by initiating their two-electron reduction to the oxidation of glutathione [5]. Hydroperoxides are generated as intermediates of lipid peroxidation, and have been implicated as causative agents of thymocyte apoptosis induced by glucocorticoids [23].

Tert-butylhydroperoxide (ButOOH) has been used as a substitute for glutathione peroxidase as well as a model hydroperoxide, being a water-soluble and cell-permeable mimic of natural hydroperoxide [18].

ButOOH induces lipid peroxidation and mitochondrial permeability transition [14, 16], resulting in cell death [14]. The underlying molecular mechanism responsible for these effects is only poorly understood.

Address for correspondence: Prof. Michał Woźniak MD, PhD, Department of Medical Chemistry, Medical University, ul. Dębinki 1, 80–211 Gdańsk, Poland, tel/fax: +48 58 349 14 50, e-mail: mwozniak@amg.gda.pl

It is possible that some of the observed effects of ButOOH on cell death processes are indirect [14]. ButOOH free radical chemistry largely depends on cytochrome c-catalysed biotransformation into a peroxyl methyl radical ($\bullet$OOCH$_3$). The formation of an alkyl carbon centred radical, namely the methyl radical ($\bullet$CH$_3$), has been found to precede the formation of the peroxyl radical species during cytochrome c-dependent biotransformation of ButOOH [1]. Both alkyl and peroxyl radicals are engaged in the propagation component of free radical-mediated lipid peroxidation [21].

4-OH-TEMPO belongs to the aliphatic class of aminoxyls, which are extremely efficient in the neutralisation of carbon centred radicals [2, 4], while being ineffective in the neutralisation of the peroxyl species [3, 6]. Taking into account this unique ability of 4-OH-TEMPO to neutralise the alkyl radical species, it is possible to evaluate the participation of particular free radical species in the pro-apoptotic machinery of ButOOH biotransformation.

MATERIAL AND METHODS

Chemicals

ButOOH, thiobarbituric acid and dexamethasone were purchased from Sigma Chemicals Co. (St. Luis, USA). All other reagents were of the highest grade of purity commercially available.

Thymocyte preparation and culture

All the procedures concerning animals were approved by Local Ethical Committee and performed according to the instructions laid down by them. Rats were euthanised with diethyl ether. Thymocytes were isolated from male Wistar rats from between 4 and 8 weeks of age. The thymi were removed and put into culture plates containing 10 ml of RPMI-1640 medium supplemented with 2.5% foetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The thymi were cut into small pieces and homogenised between two microscopic glasses. The debris was removed and the cell suspension transferred to another 15 ml sterile tube and centrifuged at 800 $\times$ g for 5 min. The supernatant was discarded and the pellet was suspended in complete RPMI-1640 medium and centrifuged again. The pellet cells were resuspended in 10 ml of complete RPMI-1640 medium and the number of cells was determined. This method was applied to separate the thymocytes from the connective tissue and the thymic epithelium. The cells were incubated in 5% CO$_2$/95% air. Cell viability was routinely evaluated by Trypan blue exclusion [12] and was greater than 95%.

Cell suspension after dilution to a density of 5 $\times$ $\times$ 10^6/ml with complete RPMI-1640 medium was incubated in culture plates at 37°C in a humidified incubator under an atmosphere of 5% CO$_2$ in air. All the reagents to be tested were dissolved in PBS or, if water-insoluble, in ethanol as 500-fold concentrated solutions, and 2.5 μ/ml were added to the thymocyte suspensions. No effect was observed from the addition of the solvent on any of the parameters evaluated in the present study.

Assessment of apoptosis by electron microscopy

At the end of the incubation period the cells were washed in PBS, sedimented and quickly fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. Postfixation was performed in 1% OsO$_4$ in the same buffer. They were then dehydrated in a graded series of ethanol, immersed in propylene oxide and embedded in Epon. Semithin sections prepared for light microscopy were stained with 0.5% Toluidine blue. Ultrathin sections were double-stained with uranyl acetate and lead citrate before examination with a JEM 1200EX II electron microscope.

The morphological changes were assessed by electron microscopy. Cell shrinkage, chromatin condensation and the formation of "apoptotic bodies" were checked as hallmarks of the apoptotic process.

Measurement of lipid peroxidation

Control and 4-OH-TEMPO (500 μM) enriched cells (4 mg of protein) were incubated at 37°C under air in the dark in the presence of 500 μM ButOOH for 120 min. 4-OH-TEMPO had been added to the samples 45 min before the addition of ButOOH. After 120 min the reaction was stopped by the addition of BHT (100 μM). Lipid peroxidation was determined as TBAR-s formation at 532 nm by using E = 1.56 $\times$ $\times$ 10^5 M^{-1}cm^{-1} as described [11], evaluated using a known amount of malonodialdehyde-generated acid hydrolysis of 1,1,3,3 tetramethoxypropane in 20% acetic acid at pH 3.5. After the addition of 2 ml of TBA-TCA-HCl (0.375% w/v TBA, 15% w/v TCA, 0.1 M HCl) in particular the samples were vortexed and incubated at 90°C for 15 min. The samples were than cooled in ice, centrifuged for 4 min at 3000 $\times$ g and read at 532 nm.
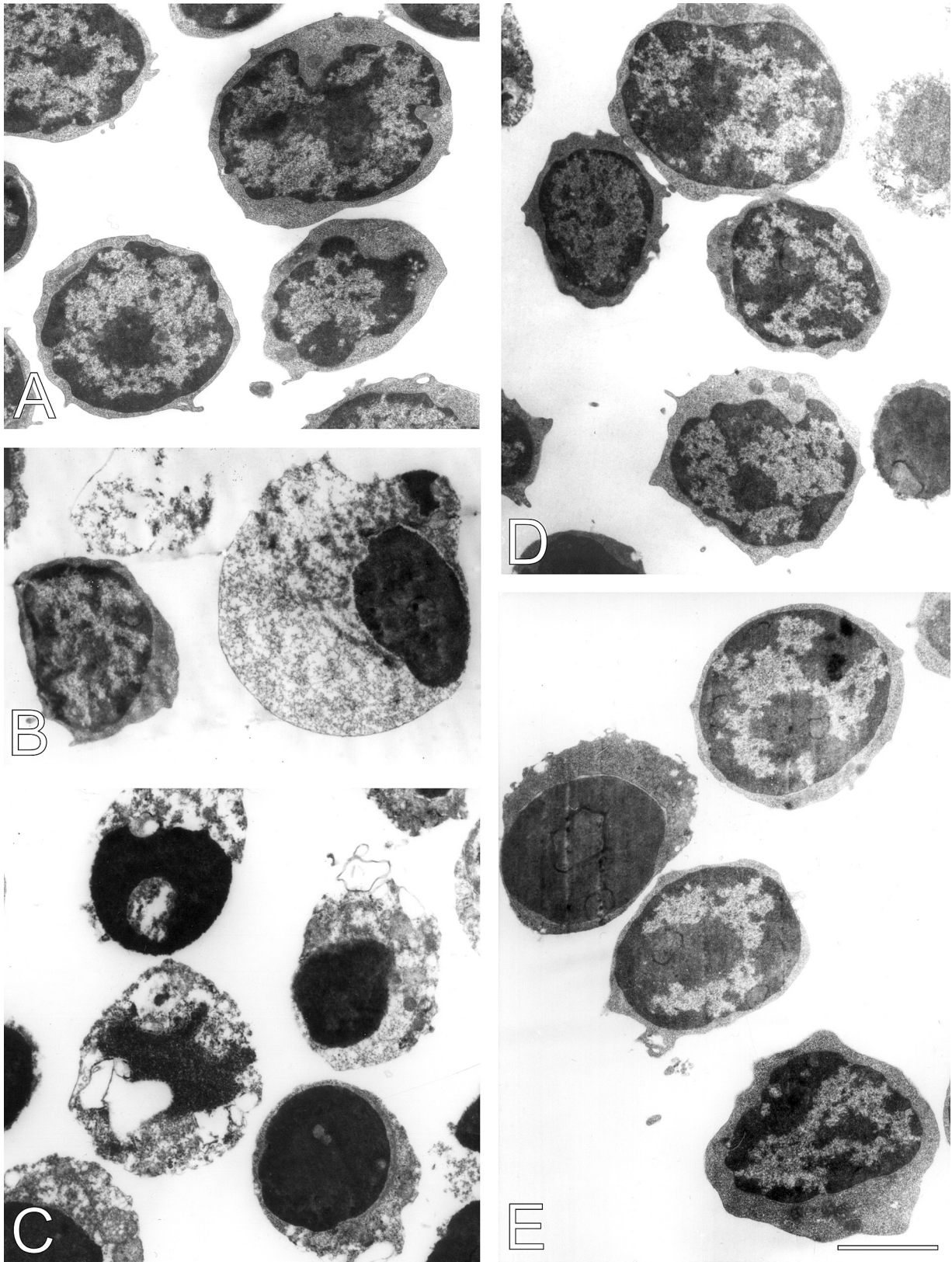
Figure 1. Electron microscopic study of the protective effect of 4-OH-TEMPO on ButOOH-induced apoptotic cell death in rat thymocyte derived from a Wistar rat (60 days of age). **A.** Rat thymocyte cultured 24 hours (negative control); **B.** Thymocyte treated with 500 μM ButOOH; **C.** Thymocyte treated with 1 μM dexamethason (positive control); **D.** Thymocyte exposed to 500 μM 4-OH-TEMPO and co-treated with **E.** 500 μM ButOOH (scale bar: 2 μm).

RESULTS

Figure 1A shows the characteristic ultrastructural aspect of rat thymocytes displaying a high nuclear/cytoplasmic ratio and strongly condensed chromatin. ButOOH-treated cells are characterised by significant cell shrinkage, extensive surface bleb formation and fragmentation of the cell nuclei (Fig. 1B). Dexamethasone-treated cells appeared mostly apoptotic with typical dark condensed chromatin aggregations inside the nuclei in cells swelling with more pale cytoplasm (Fig. 1C). Apoptosis-induced morphological changes were almost completely prevented if thymocytes were cultured in the presence of 4-OH-TEMPO (Fig. 1E) and the antioxidant-treated cells appeared closely comparable to the controls, showing only occasional apoptotic features (Fig. 1D).

Thymocytes were incubated at 37°C for 120 min with increasing concentrations of ButOOH. 500 μM ButOOH induced maximal accumulation of TBAR-s (Fig. 2). To evaluate whether ultrastructural safeguarding of thymocytes by 4-OH-TEMPO protection against ButOOH induced apoptosis, we checked the effect of this antioxidant on ButOOH-induced lipid peroxidation. ButOOH 4-OH-TEMPO significantly inhibited formation of TBAR-s all concentrations (Fig. 2).

DISCUSSION

Cell damage as a result of oxidative stress generation has been implicated in several pathologies including apoptosis and necrosis. Apoptosis permits selective elimination of excessive cells during development and the removal of autoreactive immature and clonally expanded mature lymphocytes. Apoptotic cell death shares a common distinct form of morphology including a reduction of cell volume, nuclear chromatin margination, aggregation and,
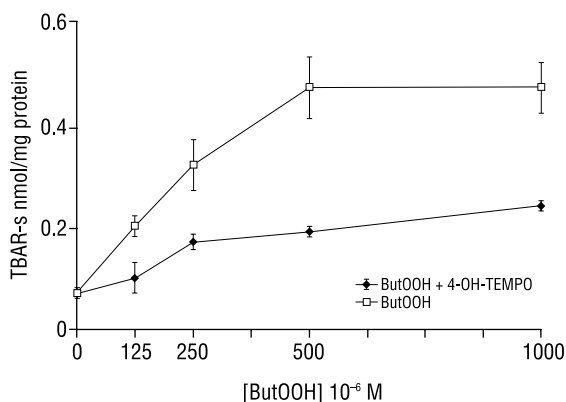
finally, fragmentation of the cell into membrane-bound fragments which are prone to rapid phagocytosis by neighbouring cells.

Oxidative stress has been suggested as a common mediator of apoptotic cell death. When rat thymocytes were exposed to glucocorticoid, a progressive increase of markers of oxidative stress was observed prior to the onset of apoptosis and isolated subpopulations of thymocytes at different stages of apoptosis displayed a high peroxide content [23].

The oxidant tert-butyl-hydroperoxide, which is a synthetic substrate for glutathione peroxidase, has been used in several studies as a model hydroperoxide compound. It induces lipid peroxidation in thymocytes [19], endothelial cells [18] and hepatocytes [14]. Metabolic activation of ButOOH to free radicals is associated with mitochondrial permeability transition leading to cell death [9, 17].

It has been found that ButOOH undergoes single-electron reduction to free radicals in the mitochondria of endothelial cells [18]. Free radical generation from ButOOH has been found to be completely attenuated by respiratory chain inhibitor antimycin indicating that electron transfer from the mitochondrial respiratory chain downstream of complex III is responsible for free radical generation inside the mitochondria.

The great novelty of our studies is finding that a small amount of synthetic hydroperoxide can mimic the pro-apoptotic activities of glucocorticoids at structural and molecular (lipid peroxidation) level. The efficient protection of the structural integrity of a cell by a small non-peptidic analogue of superoxide dismutase 4-OH-TEMPO [15], which is able to intercept and scavenge carbon-centred radicals, has shed light on the way in which the unwanted apoptotic process may be prevented by these compounds.

Figure 2. The effect of 4-OH-TEMPO on ButOOH-induced lipid peroxidation in rat thymocytes. ButOOH and 4-OH-TEMPO were added as ethanol solutions and appropriate amounts of ethanol were added to control samples (n = 6).

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