Hypoxia prolongs neutrophil survival in vitro

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Abstract Neutrophil apoptosis represents a major mechanism involved in the resolution of inflammation. Since hypoxia induces apoptosis in several cell lines and is of particular relevance in many disease states, we studied the effect of oxygen concentration on neutrophil survival in vitro. Hypoxia caused a dramatic decrease in neutrophil apoptosis (% apoptosis 20 h: 78.7 ± 2.2% in 21% O_2, 61.4 ± 6.5% in 2.5% O_2, 23.1 ± 3.2% in 0% O_2, $n = 5$). This was additive to the effect of GM-CSF (50 U/ml), not associated with induction of *bcl*-2 expression, and was not mimicked by methionine (5 mM), superoxide dismutase (200 μg/ml) or Trolox (10 mM) but was mimicked by catalase (250 μg/ml). Hence, hypoxia has a *bcl*-2-independent effect on neutrophil apoptosis that may adversely affect the clearance of these cells from an inflammatory focus.

Key words: Hypoxia; Neutrophil; Apoptosis; Bcl-2; Oxidant; Antioxidant

1. Introduction

During a bacterial invasion the body mounts an immune response involving recruitment of inflammatory cells into the area of infection [1]. One of the principal cells involved in this response is the neutrophil, which, when stimulated by a pathogen, degranulates and undergoes respiratory burst activity, to release a variety of histotoxic enzymes and short-lived reactive oxygen species (ROS) [2]. Many of the antiproteinases that protect the host against neutrophil-mediated injury [3] are inactivated by oxidation, and hence local oxidant-antioxidant balance appears to be a critical factor determining the extent of tissue damage [4]. The many deleterious contents of neutrophils necessitate that for complete resolution of inflammation these cells should be removed in a way that limits cell activation, and neutrophil apoptosis represents an important mechanism wherby this may be achieved [5,6,7].

When neutrophils die by apoptosis they retain their granule contents but lose chemotactic and secretory responsiveness [8] and thereafter are recognised and phagocytosed intact by macrophages [9] which fail to release pro-inflammatory mediators in response to this interaction [10]. Although neutrophils undergo constitutive apoptosis in vitro [11], their survival may be increased by the addition of certain cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and bacterial products such as lipopolysaccharide (LPS) [12,13]. However, the intracellular signalling pathways that regulate neutrophil survival are unclear.

Studies on the nematode, *C. elegans*, have shown that individual cell survival is signalled by the gene, *ced*-9 [14]. The mammalian homologue of the Ced-9 protein is Bcl-2 [15], which is capable of causing survival of a number of cell-lines and B-lymphocytes [16,17], an effect potentially mediated by the ability of Bcl-2 to inhibit oxidant-mediated cell damage [18,19]. This hypothesis arose from studies in which oxidant treatment of FL5.12 cells, an IL-3 dependent pro-B lymphocyte cell-line, caused an increase in apoptosis that could be counteracted by over-expression of *bcl*-2 [18], and studies in GT1–7 neural cells where *bcl*-2 expression prevented death induced by glutathione depletion [19]. Bcl-2 did not prevent free radical production but did appear to block the damaging effects of these agents. In contrast, Jacobson and Raff [20], and Shimizu et al. [21], have shown that the protective effects of Bcl-2 are observed even under extreme hypoxic conditions where the generation of ROS is negligible. These latter studies also demonstrate that hypoxia alone can induce apoptosis in certain cells [20,21].

To date, all the studies examining the potential involvement of reactive oxygen intermediates in regulating apoptosis have been undertaken in cell lines to allow manipulation of *bcl*-2 levels. This is the first report to examine the effects of hypoxia on apoptosis in the neutrophil, a terminally differentiated cell that lacks detectable Bcl-2 [22]. In contrast to the above studies, we demonstrate a profound inhibition of neutrophil apoptosis by hypoxia. This effect of hypoxia on neutrophils may be of considerable relevance to conditions in vivo where the oxidant potential at sites of inflammatory disease may be extremely low [23,24].

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and enzymes were purchased from Sigma (Dorset, UK), and all antibodies obtained from Dako (Bucks., UK). The plasticware was supplied by Becton Dickinson (Leics., UK) or Nunc (Paisley, UK), and the culture media were obtained from Life Technologies (Paisley, UK). Percoll and dextran were purchased from Pharmacia (Milton Keynes, UK), and GM-CSF (specific activity 1.25×10^7 U/mg) from Genzyme Diagnostics (Kent, UK). Trolox (6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich (Dorset, UK).

2.2. Isolation of neutrophils

Peripheral venous blood was collected from healthy volunteers and placed in citrated polypropylene tubes (0.38% (v/v) final concentration

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Abbreviations: ROS, reactive oxygen species; GM-CSF, granulocyte-macrophage colony-stimulating factor; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; SOD, superoxide dismutase.

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of sodium citrate solution). The whole blood was centrifuged ($300 \times g$) for 20 min and the plasma removed. The leukocyte-rich fraction was separated from the erythrocytes in the remaining portion by dextran sedimentation [25]. Neutrophils in the leukocyte-rich fraction were separated from mononuclear cells using a discontinuous plasma/Percoll gradient and collected from the 42%/51% plasma/Percoll interface. The isolated neutrophils were >95% pure (<0.1% mononuclear cells) and >99% viable as determined by Trypan-blue dye exclusion. Previous studies have demonstrated that neutrophils isolated by this method are non-activated since they display <5% basal shape change, minimal basal superoxide or lysozyme generation and, unlike cells prepared using Ficoll-Hypaque gradients, exhibit normal chemotactic responses to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) [25].

2.3. Neutrophil culture

Freshly harvested neutrophils were suspended at a density of 5×10^6/ml in Iscove's DMEM supplemented with 10% autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Neutrophils (6.75×10^5) were cultured in a final volume of 150 μl in flat-bottomed 96-well polypropylene flexiwell inserts. The plates were placed in sealed air-tight plastic boxes that were flushed through for 15 min with gas mixtures containing either 2.5% O_2/5% CO_2 or 21% O_2/5% CO_2 (control), with the balance made up with nitrogen. The boxes were then incubated at 37°C for the time periods indicated. More extreme hypoxic conditions were achieved by placing cultured neutrophils in an MK3 anaerobic incubator (0% O_2, Don Whitley Scientific Ltd., Yorkshire, UK) fitted with a palladium catalyst. The level and stability of pO_2 achieved using the 2.5% O_2 mixture was measured using a blood-gas analyser (model ABL-330 Radiometer, Copenhagen, Denmark). The pO_2 values obtained were 8.69 ± 0.73 kPa, 8.6 ± 0.32 kPa and 9.55 ± 1.66 kPa after 0, 6 and 20 h, respectively, in culture in 2.5% O_2. Equilibrium of the pO_2 and pCO_2 in the medium took less than 30 min with the boxes (2.5% O_2) and less than 1 h with the anaerobic incubator (0% O_2). To assess whether the effects of hypoxia influenced the known ability of GM-CSF to prolong neutrophil survival, cells were also incubated under hypoxic or normoxic conditions in the presence of absence of GM-CSF (50U/ml).

2.4. Morphological assessment of apoptosis

At the stated time points, neutrophils were gently resuspended, harvested and cytocentrifuged. The resulting slides were fixed and stained with Diff-Quick. Cell recovery and viability by Trypan-blue dye exclusion were measured in parallel. Cell morphology was examined under a 100× objective and apoptotic cells defined as cells containing darkly stained pyknotic nuclei [26]. Triplicate slides were prepared for each condition and at least 500 neutrophils counted per slide in random fields.

2.5. Treatment with oxidants and antioxidants

Freshly isolated neutrophils were suspended at a density of 5×10^6/ml in Iscove's DMEM supplemented with 10% autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and dispensed into flat-bottomed 96-well polypropylene flexiwell inserts. The neutrophils were treated with 200 μg/ml bovine erythrocyte SOD (750 U/ml), 250 μg/ml bovine liver catalase (4750 U/ml), 200 μg/ml SOD and 250 μg/ml catalase or 5 mM methionine. Control, untreated cells were also prepared. The cells were cultured for 6 or 20 h at 37°C under normoxic (5% CO_2:air) conditions, after which cytocentrifuge preparations were made and apoptosis assessed morphologically.

Assessment of the effect of hydrogen peroxide on the rate of neutrophil apoptosis was studied by treating freshly isolated neutrophils (1×10^6/ml of Iscove's DMEM supplemented with 2% autologous serum, 100 U/ml penicillin and 100 μg/ml streptomycin) with hydrogen peroxide (0.1–5 mM) in the presence or absence of the soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 mM). Control, untreated cells, and cells treated with antioxidant alone were also examined. The cells were cultured for 8 h at 37°C under normoxic (5% CO_2:air) conditions, after which cytocentrifuge preparations were made and apoptosis assessed morphologically.

2.6. Immunocytochemistry

Cultured cells (1×10^5) were transferred into round-bottomed 96-well polypropylene, flexiwell inserts. The cells were simultaneously fixed and permeabilised with 50 μl 0.01% (w/v) L-α-lysophosphatidylcholine in PBS containing 3.7% (v/v) formaldehyde for 15 min at 37°C [27]. The cells were then washed with PBS containing 0.5% (w/v) BSA and incubated (4°C) for 1 h with a saturating concentration of a mouse monoclonal antibody raised against human Bcl-2 protein. As a positive control, parallel samples were stained with MAC 387, a mouse monoclonal antibody which recognises p8,14, an intracellular calcium binding protein complex found in neutrophils. The amount of primary antibody binding was detected using a FITC-conjugated goat anti-mouse polyclonal antibody. Negative control cells were stained with second layer antibody alone. The samples were analysed using an EPICS profile II (Coulter Electronics, Luton, UK).

2.7. Statistics

All data are presented as mean ± S.E.M. for (n) separate experiments. Values were compared using the Student's t-test for paired data with $P < 0.05$ considered to be significant.

3. Results and discussion

Incubation of neutrophils under hypoxic conditions resulted in a dramatic inhibition of apoptosis (Fig. 1a) to the extent that cells incubated for 44 h in 0% O_2 were >95% viable and <25% apoptotic compared to nearly 100% apoptosis/ necrosis under normoxic (21% O_2) conditions (Fig. 1b). These findings are in complete contrast to those of Jacobson and Raff [20] and Shimizu et al. [21] who have shown that hypoxia induces apoptosis in SV-40 transformed human fibroblasts (that lacked mitochondria) and a rat pheochromacytoma cell line, respectively. A similar induction of apoptosis by hypoxia has been reported in WEHI 7.1 T-lymphoma cells [28], adenocarcinoma HT29 cells [29] and superior cervical ganglia cells [30]. In the studies of Jacobson and Raff [20] and Shimizu et al. [21], apoptosis could be inhibited under both normoxic and hypoxic conditions by transfected *bcl*-2. Since we were unable to detect any Bcl-2 protein in neutrophils aged under either hypoxic or normoxic conditions (Table 1), it is unlikely that the enhanced survival of neutrophils under hypoxic conditions was *bcl*-2-mediated.

We have previously demonstrated that cytokine growth factors such as GM-CSF are capable of extending the life of the neutrophil [31], and hence we investigated whether the effects of GM-CSF and hypoxia were mediated by a common mechanism. In fact, GM-CSF- and hypoxia-mediated survival of neutrophils were additive (Fig. 2), suggesting that the hypoxia and GM-CSF effects are independent.

To examine whether the effect of hypoxia was mediated by inhibition of ROS generation, we investigated whether exogenous addition of antioxidant enzymes and methionine, an essential thiol containing amino acid, would mimic the hypoxic

Table 1
Effect of hypoxic culture on Bcl-2 in neutrophils

% oxygen	Amount of detectable Bcl-2 (Relative Mean Fluorescence)	
	-GMCSF	+GMCSF
0%	1.14 ± 0.14	1.01 ± 0.24
21%	1.09 ± 0.12	1.01 ± 0.014

Neutrophils were incubated in the presence or absence of GMCSF (50 U/ml) for 20 h in atmospheres containing 0% or 21% O_2. The amount of Bcl-2 in the cells was measured as detailed in section 2 and expressed as mean fluorescence relative to negative control. In all cases, Bcl-2 could not be detected in the neutrophils. HL60 cells, which are known to express Bcl-2, were used as a positive control (relative mean fluorescence = 4.43). MAC 387, which recognises an intracellular antigen in neutrophils, was used confirm the efficient permeabilisation of the neutrophils (relative mean fluorescence > 50).

effect. These agents had no effect on cell viability after 20 h of culture as measured by Trypan-blue exclusion which supports a previous study [32]. Methionine and SOD did not influence the rate of neutrophil apoptosis (Fig. 3) suggesting that hypochlorous and superoxide radicals do not play a significant role in regulating neutrophil apoptosis. This supports a previous a study which demonstrated that SOD was ineffective in preventing cell death in human lung fetal fibroblasts [33]. In contrast, catalase, both alone, and in combination with SOD, caused a significant inhibition of neutrophil apoptosis (Fig. 3). It is probable, however, that this effect of catalase is independent of its effects on hydrogen peroxide levels since a similar anti-apoptotic effect of catalase has been demonstrated in WEHI 231 cells [34] and catalase has now been identified as the anti-apoptotic factor present in the conditioned medium of CCRF-CEM T-cell leukaemia cells [35]. This conclusion is supported by the lack of effect of SOD and Trolox. Of interest, we also observed that the endogenous SOD and catalase levels in neutrophils measured by standard methods [36,37], was significantly increased under hypoxic culture conditions (SOD activity after 20 h culture in 21% O_2 was 10.05 ± 0.287 U/mg protein and in 0% O_2 was 18.68 ± 3.32 U/mg protein ($P < 0.05$). Catalase activity after 20 h culture in 21% O_2 was 125 ± 13.83 U/mg protein and in 0% O_2 was 263 ± 32.71 U/protein ($P < 0.05$)). It is likely that this induction of antioxidant enzymes represents a stress response to the hypoxia since Das et al. have shown that Mn SOD and catalase are two of a number of stress-related genes up-regulated in cardiac biopsy tissue following repeated ischaemia [38]. Whether the anti-apoptotic effect of hypoxia relates to the induction of other stress-related proteins or changes in poly-ADP-ribose transferase activity and hence ATP levels [39,40], lipid oxidation [41], regulation of NFκB activation [42], or some alternative mechanism, requires further investigation.

Having shown that neutrophil survival in vitro can be prolonged by hypoxia where the cells' capacity to generate ROS, at least in 0% O_2, is severely diminished [18] we investigated the direct effect of ROS on the rate of neutrophil apoptosis. Hydro-

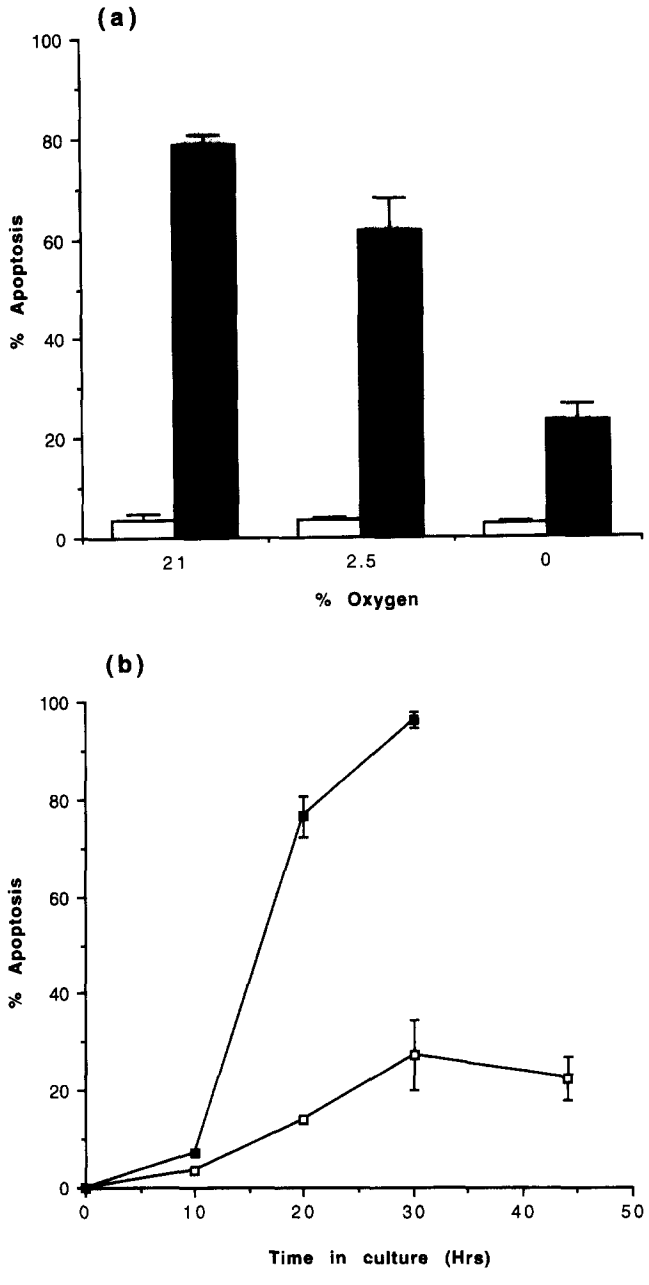

Fig. 1. Effect of hypoxia on neutrophil apoptosis. Neutrophils were incubated in atmospheres containing either 21%, 2.5% or 0% O_2 for 6 hrs (open bars) or 20 hrs (closed bars) in (a) or 21% (closed symbols) and 0% (open symbols) in (b). The percentage apoptosis was assessed morphologically as detailed in section 2 at the time points indicated. Data in (a) represent mean ± S.E.M. of $n = 5$ separate experiments, each performed in triplicate. In (b), culture of cells for 44 h in the presence of 21% O_2 caused a major increase in the percentage necrotic cells (as assessed by Trypan-blue dye exclusion) and therefore it was not possible to determine the percentage of apoptotic cells at this time point. The data in (b) represent mean ± S.E.M. of triplicate incubations from a single representative experiment.

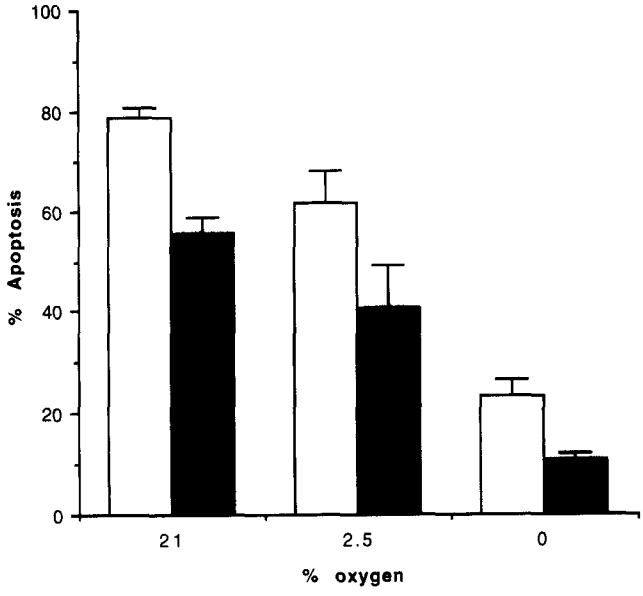

Fig. 2. Modulation of the GMCSF survival effect in neutrophils by hypoxia. Neutrophils were incubated with GMCSF (50 U/ml) for 20 h in atmospheres containing 21%, 2.5% or 0% O_2 (closed bars). The control cells were incubated under the same conditions but in the absence of cytokine (open bars). The data are expressed as the mean ± S.E.M. of $n = 5$ separate experiments each performed in triplicate. Under each O_2 condition tested, the addition of GM-CSF caused a significant inhibition of apoptosis ($P < 0.05$).

gen peroxide (0.1–5 mM) induced a concentration-dependent increase in the rate of apoptosis observed at 8 hrs (Fig. 4a). Cell viability as judged by Trypan-blue dye exclusion was not affected by hydrogen peroxide treatments below 5 mM (half-life of the hydrogen peroxide in this system was 2.25 h as determined by the reduction of absorbance at 240 nm). In view of the potent antioxidant capacity of serum (in our donors equivalent to a Trolox concentration of 1.08–1.12 mM [43], data not shown), the serum concentration in the Iscove's DMEM these experiments was reduced from 10% to 2%. The ability of 1 mM hydrogen peroxide to increase the number of cells undergoing apoptosis could be reversed by the addition of the antioxidant Trolox (10 mM), a soluble vitamin E analogue (Fig. 4b). This suggests that a potential pathway exists for oxidants to induce neutrophil apoptosis. However, it is important to note that activation of the respiratory burst by agents such as fMLP and IL-8 does not increase the rate of neutrophil apoptosis [13]. This, together with the observation that only an extremely high, supraphysiological concentration of hydrogen peroxide increases neutrophil apoptosis, and the inability of SOD, methionine and Trolox to mimic the effect of withdrawal of oxygen (Fig. 4b), implies that generation of superoxide anion, hypochlorous and peroxyl radicals may not be a major regulator of neutrophil apoptosis in vivo.

In conclusion, hypoxia caused a profound inhibition of neutrophil apoptosis in vitro. This effect is directly opposite to the pro-apoptotic effect of hypoxia observed in a number of cell-lines [20–21,28–30]. The ability of hypoxia to prolong neutrophil survival is additive to that induced by GM-CSF, is not associated with *bcl*-2 expression, and is not mimicked by exogenous SOD, methionine or Trolox. However, apoptosis was inhibited by catalase, but the exact mechanism underlying this effect and the potential role of hydroxyl radicals and hydrogen peroxide require further investigation. These data also imply that examination of apoptosis under routine hyperoxic

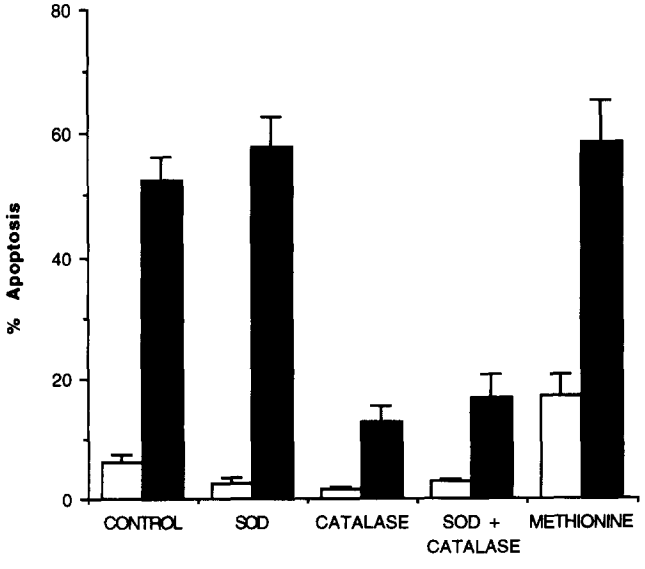

Fig. 3. Effect of SOD, catalase and methionine on neutrophil apoptosis. Neutrophils were cultured for 6 h (open bars) or 20 h (closed bars) in the presence of 200 μg/ml SOD, 250 μg/ml catalase, 200 μg/ml SOD and 250 μg/ml catalase, or 5 mM methionine. Control, untreated neutrophils were also prepared. Apoptosis was assessed morphologically. Data represent mean ± S.E.M. of $n = 3$ separate experiments, each performed in triplicate.

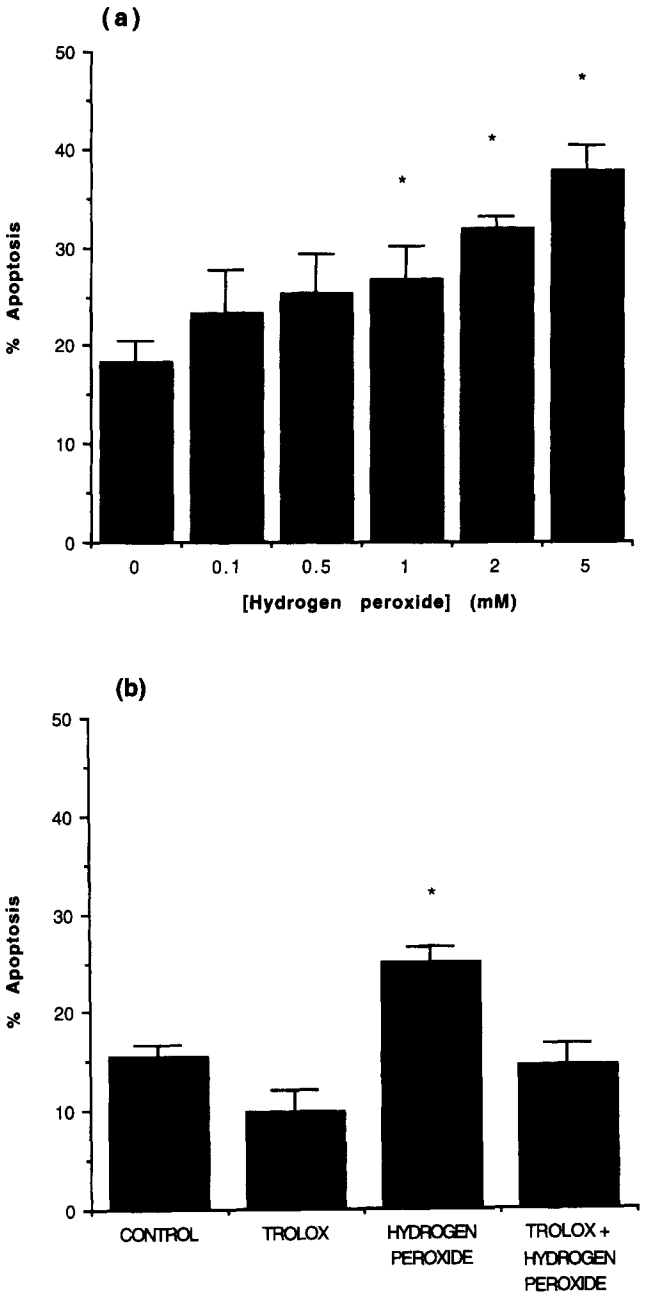

Fig. 4. Effects of hydrogen peroxide and Trolox on neutrophil apoptosis. Neutrophils were incubated for 8 h in the presence or absence of (a) increasing concentrations of hydrogen peroxide or (b) 10 mM Trolox ± 1 mM hydrogen peroxide. Apoptosis was assessed morphologically. Both sets of data represent mean ± S.E.M. of $n = 3$ separate experiments, each performed in triplicate. The rate of neutrophil apoptosis in response to 1, 2 and 5 mM hydrogen peroxide was significantly different from the control apoptosis ($^*P < 0.05$). In (b) the rate of apoptosis when cells were treated with both Trolox and hydrogen peroxide was significantly less than the rate of apoptosis when treated with hydrogen peroxide alone ($^*P < 0.005$); there was no significant difference between the rates of apoptosis of the control cells, Trolox treated cells and Trolox and hydrogen peroxide treated cells.

(21% O_2) culture conditions may alter the susceptibility of cells to apoptosis and not accurately reflect the O_2 environment in vivo. In addition, neutrophils may remain alive and viable for considerably longer periods when recruited to an inflammatory

focus where there is local, and often systemic, hypoxia [23,24]. The effect on neutrophil survival, and hence delay in their removal, could potentially exacerbate the disease state and result in additional neutrophil-mediated tissue destruction. This finding may also have the practical consequence of stimulating research into the role of hypoxic conditions for prolonged granulocyte storage prior to transfusion.

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