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Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis

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Abstract The induction of apoptosis in Jurkat T-lymphocytes with 50 μM hydrogen peroxide was associated with caspase activation. Caspase activity was first detected 3 h after treatment, and the morphological features of apoptosis were apparent by 6 h. At higher concentrations of hydrogen peroxide there was no detectable caspase activity, and the cells died by necrosis. Cells treated with hydrogen peroxide were impaired in their ability to undergo Fas-mediated apoptosis. This appeared to be the result of direct inhibition of the cysteine-dependent caspases. The cells were able to recover and undergo apoptosis at later times. Therefore, hydrogen peroxide has two distinct effects. It initially inhibits the caspases and delays apoptosis. Then, depending on the degree of the initial oxidative stress, the caspases are activated and the cells die by apoptosis, or they remain inactive and necrosis occurs. We discuss the physiological implications of cells having to maintain a reducing environment during apoptosis to allow the caspases to function.

Key words: Apoptosis; Necrosis; Reactive oxygen species; Hydrogen peroxide; Caspase

1. Introduction

The caspases are a novel class of at least 10 cysteine proteases that play a critical role during apoptosis. Their name reflects the active cysteine group, and the characteristic cleavage of their targets at aspartate residues [1]. Caspases are present in the cell cytoplasm in an inactive proform. Pro-caspases become activated during apoptosis by proteolytic processing at specific sites, followed by assembly of the active form. The active enzyme can cleave a number of defined substrates, and lead to the eventual dismantling of the cell (reviewed in [2,3]). One example is poly-ADP ribose polymerase, which is cleaved at a specific Asp-Glu-Val-Asp (DEVD) sequence. Caspase-3 has been identified as the caspase responsible for much of this activity [4]. The ability of some caspases to cleave and activate the pro-caspase form of other family members suggests a defined cascade of proteolytic events [2,3]. Attempts are being made to elucidate these networks as inhibition of selected caspases can block many of the subsequent morphological and biochemical changes associated with apoptosis.

Reactive oxygen species (ROS) have also been implicated as mediators of apoptosis. While a high degree of oxidative stress can cause necrosis, lower levels will trigger apoptosis [5,6]. In addition, increased ROS levels have been detected emanating from apoptotic cells, and antioxidants can block apoptosis in a variety of systems (reviewed in [7,8]). The ROS hydrogen peroxide is generated during normal metabolism, and is also produced in large amounts by phagocytic cells at inflammatory sites [9]. Hydrogen peroxide is known to modulate a variety of cell functions [10], and its lower biological reactivity compared to many ROS, combined with its capacity to cross membranes and diffuse away from the site of generation, makes it an ideal signalling molecule. In this study we have investigated the effect of hydrogen peroxide on caspase activity in Jurkat T-lymphocytes. Addition of hydrogen peroxide activated the caspases and led to apoptosis. However, activation occurred several hours after consumption of the hydrogen peroxide by the cells, and these concentrations initially impaired the ability of the cells to undergo Fas-mediated apoptosis.

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Abbreviations: ROS, reactive oxygen species; AMC, aminomethylcoumarin; PS, phosphatidylserine

2. Materials and methods

2.1. Materials

Jurkat cells were obtained from the Microbiology and Tumour Biology Centre, Karolinska Institutet. DEVD-AMC was from Bachem (Bubendorf, Switzerland), and anti-Fas IgM (CH-11) was from Medical and Biological Laboratories (Nagoya, Japan). The Apoptest-FITC kit was from Nexins Research B.V. (Hoeven, The Netherlands). All other chemicals were from Sigma (St. Louis, MO).

2.2. Cell culture

Cells were cultured in RPMI 1640 with 10% heat-inactivated foetal calf serum and 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C in humidified air with 5% carbon dioxide, and kept in logarithmic phase by routine passage every 2–3 days. Before use cells were centrifuged at $500\times g$ for 5 min and resuspended in fresh medium at 1×10^6/ml.

2.3. Caspase activity

The measurement of DEVD-AMC cleavage was modified from Nicholson et al. [4]. Cells were pelleted and frozen on microtitre plates at 1×10^6 cells per 25 μl. 50 μl of buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, 10^{-6}% NP-40, and 0.1% CHAPS at pH 7.25) was added to each well along with 50 μM of DEVD-AMC. Substrate cleavage to release free aminomethylcoumarin (AMC) (excitation 355 nm, emission of 460 nm) was monitored with time at 37°C. Fluorescent units were converted to pmoles of AMC using a standard curve generated with free AMC.

2.4. Cell blebbing and nuclear fragmentation

The number of cells with large membrane protrusions was counted by light microscopy. Samples were also centrifuged at $500\times g$ for 5 min and resuspended in 4% paraformaldehyde before being spread on slides coated with 3-aminopropyltriethoxysilane, stained with 10 μg/ml Hoechst 33342, and sealed with Eukit and a coverslip. The fluorescent nuclei were viewed with a Leitz Diaplan fluorescent microscope.

2.5. Phosphatidylserine (PS) exposure

PS exposure was measured by the binding of annexin V-FITC ac-

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cording to the protocol outlined by the manufacturers in the Apoptest-FITC kit. Cells were also stained with 100 μg/ml propidium iodide, before being analysed with a Becton Dickinson FACScan flow cytometer with a 15 mW 488 nm argon laser.

2.6. Hydrogen peroxide consumption

Hydrogen peroxide concentrations were measured using the ferrous oxidation of xylenol orange assay [11]. At specific intervals the cells were pelleted and supernatants were added to the FOX reagent. An increase in A_{560} nm was measured, and concentration was calculated with a standard curve generated with reagent hydrogen peroxide.

3. Results

Oxidants are known to induce both apoptosis and necrosis in cells [5,6], with the concentrations required dependent on the cell type being investigated. We therefore characterised the response of Jurkat T-lymphocytes to hydrogen peroxide. Induction of apoptosis was slow with hydrogen peroxide, but by 6 h distinctive apoptotic changes were detectable. At 50 μM hydrogen peroxide flow cytometry with annexin-FITC showed that PS, normally constrained to the inner leaflet of the plasma membrane [12], appeared on the outer surface of 25% of the cells (Fig. 1B). Membrane blebbing and nuclear fragmentation was detected in 20 ± 4% and 13 ± 3% (S.E. of $n = 3$) of the cells respectively. The exclusion of propidium iodide or trypan blue showed that the cells maintained their plasma membrane integrity at this stage (Fig. 1B,D); however, higher concentrations of hydrogen peroxide led to the appearance of necrotic cells (Fig. 1C,D).

Caspase activity in the hydrogen peroxide-treated cells was measured using DEVD peptide conjugated to the fluorophore AMC [4]. At 50 μM hydrogen peroxide there was a 10-fold increase in the rate of DEVD-AMC cleavage compared to control cells. Cleavage was inhibited by addition of DEVD-CHO to the wells, indicating that it was specific for caspases (not shown) [4]. Optimal caspase activation occurred at 50 μM hydrogen peroxide, followed by a decline in activity at higher concentrations (Fig. 1D). Above 200 μM, caspase activity dropped below the background activity seen in control cells. There was a 25% decrease in the ability of the cells to exclude trypan blue at 500 μM (Fig. 1D), and these necrotic cells could also be detected by flow cytometry (Fig. 1C). The dye exclusion assays are insensitive markers. Most cells showed gross morphological damage when viewed under the light microscope, yet could still exclude the dyes. At the higher concentrations of hydrogen peroxide, all cells eventually became necrotic (not shown), however there were no signs

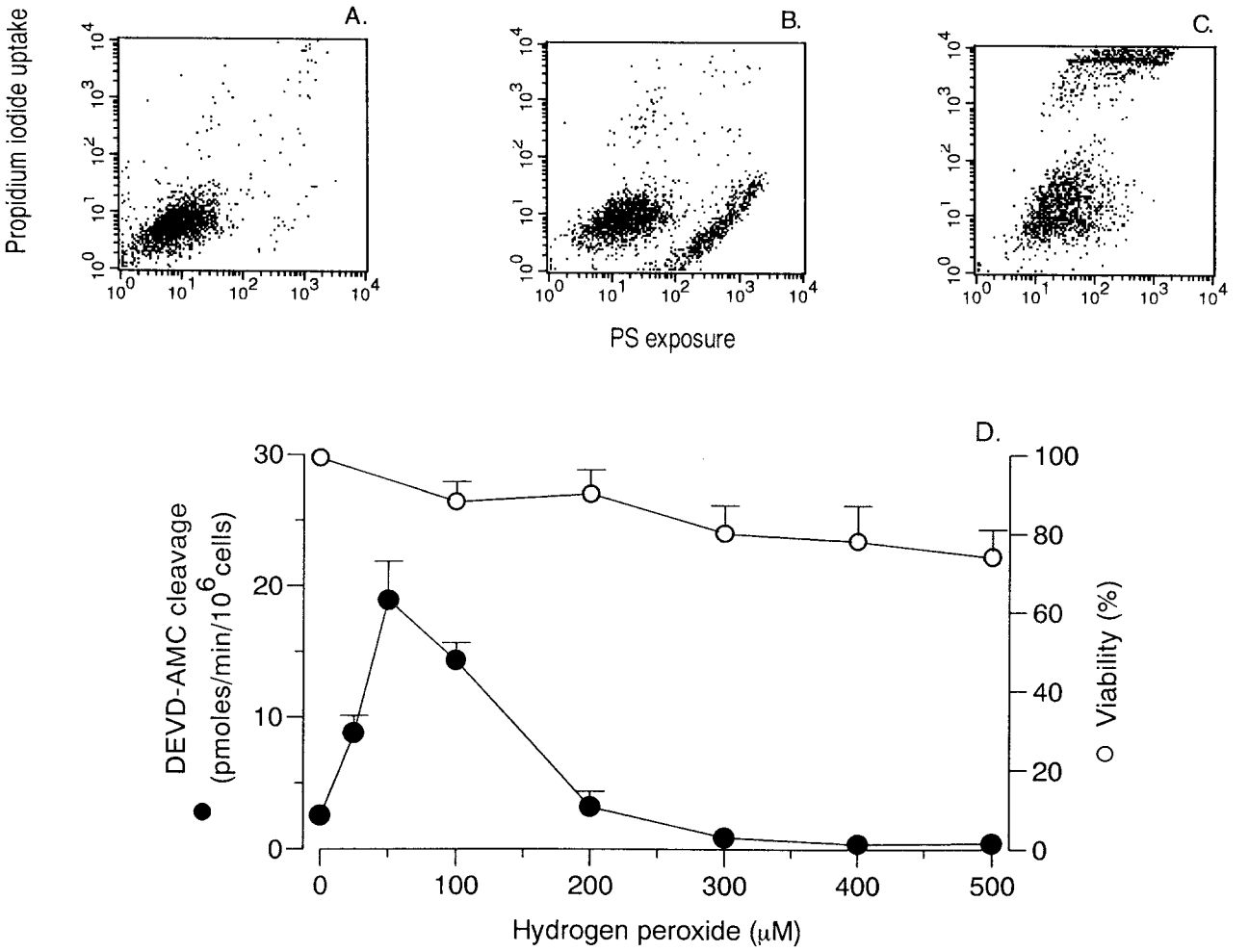

Fig. 1. Caspase activation and apoptosis in Jurkat cells treated with hydrogen peroxide. Jurkat cells (1×10^6/ml) were treated with varying concentrations of hydrogen peroxide for 6 h. After this time samples were taken and PS exposure, viability, and caspase activity were determined. A representative experiment of PS exposure after (A) 0 μM, (B) 50 μM, or (C) 500 μM hydrogen peroxide treatment is shown. (D) DEVD-AMC cleavage (●) and trypan blue exclusion (○) were measured, and the means and S.E. of at least three experiments are shown.

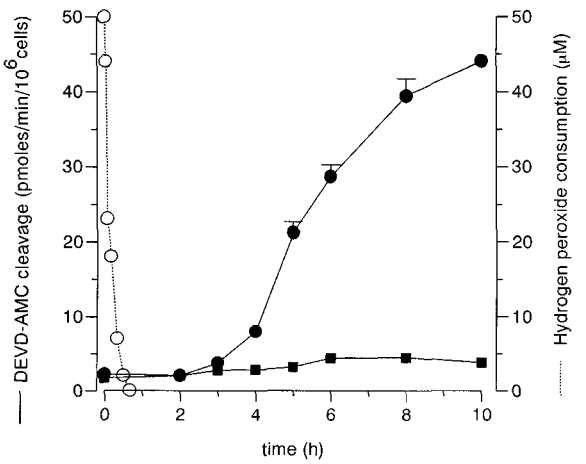

Fig. 2. Time course of caspase activation and hydrogen peroxide consumption. Jurkat cells (1×10^6/ ml) were treated with 0 (■) or 50 μM (●) hydrogen peroxide, and samples were harvested at various times and assayed for their ability to cleave DEVD-AMC. The rate of DEVD-AMC cleavage is presented as triplicate determinations from one of three representative experiments. The consumption of 50 μM hydrogen peroxide is shown (○). In experiments without cells, the media alone after 30 min only consumed 15% of the hydrogen peroxide added.

of apoptosis – PS exposure did not occur (Fig. 1C), and there was no blebbing or nuclear fragmentation.

A time course of cells treated with 50 μM hydrogen peroxide showed a 3 h delay in caspase activation, before a rapid increase in activity after this time (Fig. 2). Measurement of the consumption of hydrogen peroxide by the cells showed that the majority of the hydrogen peroxide disappeared within 30 min (Fig. 2).

As high concentrations of hydrogen peroxide appeared to inhibit the caspases, and activating concentrations only did so hours after consumption of the initial trigger, we were interested in the ability of the cells to respond to other apoptotic triggers at the same time as being treated with apoptosis-inducing concentrations of hydrogen peroxide. We investigated Fas (CD95)-mediated apoptosis, a well characterised system in which major apoptotic changes are detectable within 1–2 h, and these changes are dependent on caspase activity [13]. Low concentrations of hydrogen peroxide inhibited the activation of caspases in Jurkat cells treated with anti-Fas antibody. This effect was most apparent when hydrogen peroxide was added 30–60 min after the initial trigger (Fig. 3). There was up to a 40% inhibition of caspase activity at 50 and 100 μM, levels that go on to stimulate apoptosis. Inhibition was not due to impairment of the Fas signalling pathway. Extensive inhibition was seen when the peroxide was added at 90 min, which is just before the cells were harvested, washed, and caspase activity measured. This indicates a direct effect on the caspases active at this time. In contrast, hydrogen peroxide had little or no effect on caspase activation when added 30 min before triggering with anti-Fas antibody (Fig. 3). If hydrogen peroxide was acting by disrupting signal transduction of the Fas receptor its effect should have been most apparent at this time.

The cells appeared to recover from the early hydrogen peroxide treatment to have normal caspase levels. A time course of caspase activation following anti-Fas antibody stimulation confirmed this. The addition of hydrogen peroxide 30 min after Fas activation delayed the subsequent induction of caspase activity for a further 30 min, but did not alter the net responsiveness obtained (Fig. 4). Membrane blebbing and nuclear fragmentation, which are dependent on caspase activity, were also delayed (not shown). The addition of hydrogen peroxide 30 min before the anti-Fas antibody had no detectable effect on the system, supporting the observation that the cells only required that amount of time to recover (Fig. 4).

4. Discussion

We have shown that hydrogen peroxide has two distinct effects on caspase activity in Jurkat T-lymphocytes. Firstly, hydrogen peroxide can activate the caspases and cause apoptosis. However, higher concentrations did not trigger caspase activity, and there was a long lag phase before caspase activation with the lower concentrations, suggesting that hydrogen peroxide may also have an inhibitory effect on these cysteine-dependent proteases. Indeed, we found that Jurkat cells treated with concentrations of hydrogen peroxide that eventually caused apoptosis were initially impaired in their ability to undergo Fas-mediated apoptosis. This indicates a dual regulation, with the ability of oxidative stress to trigger caspases and apoptosis balanced by the oxidative inhibition of caspase activity. While the ability of oxidants to activate apoptosis is well known, the potential for inhibition of this process, and the physiological consequences thereof, have been largely ignored.

Other studies have also shown that the addition of ROS or redox-active compounds can induce apoptosis, and that high

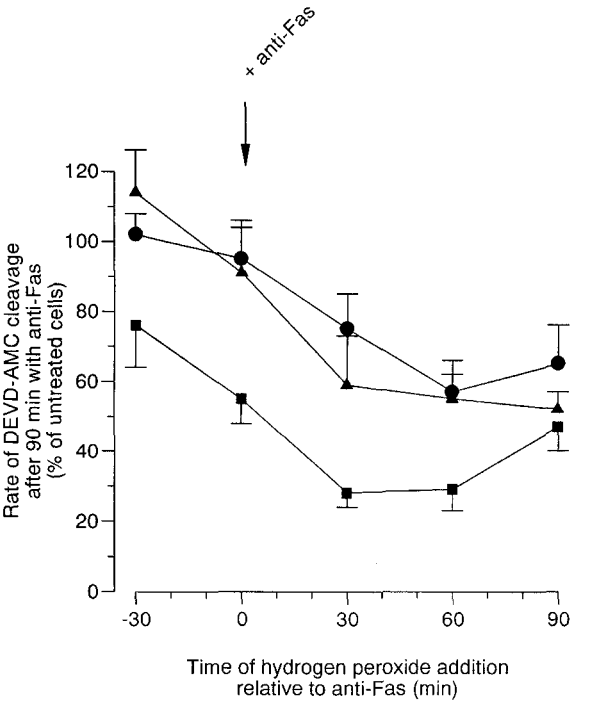

Fig. 3. Effect of hydrogen peroxide on Fas-mediated caspase activation. Jurkat cells were stimulated with 250 ng/ml anti-Fas antibody and caspase activity after 90 min was measured. The effect of adding hydrogen peroxide at the indicated times was determined. ● = 50 μM, ▲ = 100 μM, and ■ = 200 μM. The mean and S.E. of at least three experiments are shown.

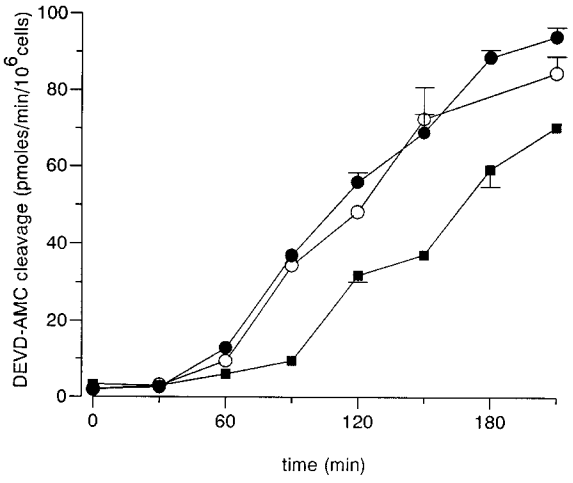

Fig. 4. Effect of hydrogen peroxide on the time course of caspase activation in Fas-treated cells. Jurkat cells (●) were stimulated with 250 ng/ml anti-Fas antibody, and the rate of DEVD-AMC cleavage was measured at various times after stimulation. ■ = addition 30 min after anti-Fas, ○ = addition of 200 μM hydrogen peroxide 30 min before the anti-Fas. Results are triplicate determinations from one of three representative experiments.

concentrations of these agents can cause necrotic cell death [5,6]. We propose that the necrosis-inducing effects are due to the sensitivity of the caspases to oxidative inactivation. If the oxidative challenge is sufficiently high, the cell cannot maintain a reducing environment, and the caspases will not function. An orderly disassembling of the cell would not occur, and necrosis will ensue. In our study, the increase in hydrogen peroxide to levels that did not activate the caspases correlated with the switch from apoptotic to necrotic death.

The most likely mechanism for the observed inhibition of both caspase activity and subsequent apoptosis is a direct effect of hydrogen peroxide on the caspases themselves. The active cysteine group makes all of the caspase family members potential targets. It would depend on the time that the hydrogen peroxide was added as to which caspase would be affected. Receptor trimerisation and association of the factors that bind to the intracellular death domain of the Fas receptor occurs within seconds [14]. Caspase-8, the first of the caspases proposed to be involved in this apoptotic pathway, should be activated shortly after this. Caspase-3 activity can be detected within 20 min, and this enzyme is a major contributor to DEVD cleavage measured after this time, although other family members such as caspase-7 are likely to contribute [3]. Addition of hydrogen peroxide directly before the cells were harvested, washed, and assayed confirmed that direct inactivation of the caspases present at the later times could occur. Hydrogen peroxide had little effect when added before or at the same time as the anti-Fas antibody. This indicates that transduction of the Fas signal was not being impaired. However, the caspases present at this time should still be inactivated. The reason that this inhibition was not detected 90–120 min later is that the effect appeared to be temporary, and given time, the cells were able to recover normal caspase function. The nature of this recovery is currently unclear, but may involve either repair or replacement of the damaged proteins. As the delay and recovery in Fas-mediated apoptosis lasted for only 30 min, caspase inhibition by hydrogen peroxide is unlikely to be responsible for the 3 h delay that we observed in hydrogen peroxide-mediated apoptosis. Other rate limiting steps in the pathway from hydrogen peroxide consumption to caspase activation seem to be involved. As yet, little is known about this transduction pathway.

Studies in our laboratory with the dithiocarbamate class of compounds support the conclusions presented here [15]. These compounds are commonly used as inhibitors of apoptosis or NFRB activation, an activity ascribed to their antioxidant capacity. However, it was shown that they function by inactivating the caspases at critical thiol-containing residues [15]. Similar effects would be predicted with other physiological oxidants including superoxide, nitric oxide, peroxynitrite, and hypochlorous acid. Indeed, Dimmeler et al. [16] have recently shown that caspase-1 and caspase-3 are inhibited by nitric oxide, and they provide evidence for nitrosylation of the active site thiol group.

An early burst of ROS production has been hypothesised to be involved in the triggering of apoptosis in a variety of models [7,8]. One prominent source of these ROS is the mitochondria [17]. Our results warn that excessive ROS production at an early stage in apoptosis will actually block apoptosis. A late stage apoptotic cell also displays signs of oxidative stress [7]. The stress is accentuated by the active efflux of reduced glutathione from the dying cell [18]. Glutathione is an important cellular antioxidant that will help maintain the caspases in their active form. At these late stages ROS may be important is downregulating caspase activity.

Oxidative inactivation of the caspases may be important in various pathological states. Phagocytic cells are a major source of reactive oxidants in the body, and while hydrogen peroxide is rapidly converted to more reactive species that are directed at invading pathogens, levels around 100 μM have been measured during inflammation [9]. While phagocytes may be more resistant to these levels, bystander lymphocytes and endothelial cells at inflammatory sites will be affected. Tumour cells have also been proposed to have a higher 'prooxidant status' [19]. Ras-transformed fibroblasts generate increased ROS [20], and Clement and Stamenkovic [21] recently proposed that increased intracellular superoxide levels were responsible for the resistance of some cell lines to Fas-induced apoptosis. We speculate that the mechanism is due to oxidative inactivation of the caspases. We note that this and most other studies have been performed with a single bolus of hydrogen peroxide. Enzymatic physiological sources, while potentially lower, will be maintained at continuous levels for much longer times. This is likely to prevent cell recovery and enhance the inhibitory phase of apoptosis by oxidants.

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