Interaction with substrate sensitises caspase-3 to inactivation by hydrogen peroxide

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Abstract **Caspases have an active site cysteine whose oxidation blocks catalytic activity. Caspase activity, measured in lysates of apoptotic cells, was inhibited by H_2O_2 with an IC_{50} of 7 μM. Recombinant caspase-3 was directly inhibited by H_2O_2, with an estimated second-order rate constant of 750 M^{-1} s^{-1}. These values were determined when H_2O_2 was added while the caspases were cleaving a peptide substrate. There was a 40-fold decrease in sensitivity to inactivation if the substrate was absent at the time of H_2O_2 addition. These results rationalise conflicting reports of the sensitivity of caspase-3 to H_2O_2, and identify a novel mechanism for sensitising a thiol enzyme to oxidative inactivation.**

Key words: Thiol; Cysteine; Hydrogen peroxide; Oxidation; Caspase; Apoptosis

1. Introduction

Cysteine residues play a fundamental role in protein structure and catalytic activity. Oxidation and reduction of these residues can regulate a variety of signal transduction pathways in cells [1]. Proteins whose function can be altered by cysteine oxidation include protein kinases and phosphatases, transcription factors, membrane receptors and channel proteins. H_2O_2 is one of the oxidants speculated to regulate cell function via its interaction with thiol proteins. While cysteine itself has a low reactivity with H_2O_2 at neutral pH, the local protein environment can enhance the reactivity of selected cysteine residues. One common example is the presence of positively charged residues that stabilise the thiolate anion. This enables some form of selectivity during redox signaling.

The caspases are a family of cysteine proteases that play an essential role in the execution of apoptosis [2]. They are expressed as inactive zymogens, and become proteolytically active during apoptosis. Caspases have an active site cysteine that mediates nucleophilic attack on its target substrate. The thiol has to be reduced for the enzyme to function, and dithiothreitol (DTT) is regularly included in activity assays [3]. In a study with apoptotic cells it was observed that H_2O_2, depending on the time of its addition, could delay the onset of caspase activation or impair the activity of those effector caspases present immediately prior to harvest [4]. This suggested that oxidation could directly inhibit caspase activity, and observations of oxidative stress during apoptosis raised the possibility of a physiological and pathological role for caspase oxidation [4]. Consistent with these observations, inactivation of recombinant caspases at low concentrations of H_2O_2 has been reported [5], as has the effect of other thiol oxidants and reductants [6–8]. Reactive nitrogen species have also been shown to inhibit caspase-3 activity in vitro via thiol modification [9–12], and one report provided evidence that the pro-form of caspase-3 is *S*-nitrosylated in resting cells [13].

However, there are contradictory reports with cell extracts and purified caspases showing minimal inactivation by H_2O_2 [14,15]. In the present study, we have undertaken a thorough kinetic analysis of the sensitivity of caspase-3 in cell lysates and its recombinant form to oxidation by H_2O_2. We have discovered an unusual phenomenon whereby caspase-3 becomes more sensitive to oxidative inactivation in the presence of its substrate. This explains contradictory results in the literature, and it identifies a novel mechanism for sensitising a thiol enzyme to oxidative inactivation.

2. Materials and methods

2.1. Materials

The Jurkat T-lymphocyte cell line was obtained from the American Type Culture Collection. Anti-Fas IgM (CH-11) was from Upstate Biotechnology (Lake Placid, NY, USA), Ac-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (DEVD-AMC) was from Peptide Institute (Osaka, Japan), and Ac-DEVDGI-NH_2 was synthesised by AusPep (Parkville, Australia). Recombinant caspase-3 was a generous gift from Dr D. Nicholson of Merck Frosst Canada. All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cell culture and preparation of lysates

Cells were cultured in RPMI 1640 with 10% heat-inactivated foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C in humidified air with 5% CO_2. Cells were stimulated at 1×10^6/ml with 250 ng/ml anti-Fas antibody. After 2 h the cells were harvested, washed and pelleted. DTT was not routinely included in the caspase assay. Therefore, cells were either assayed immediately, or stored at −80°C and assayed within 6 h of harvesting, to prevent spontaneous caspase oxidation.

2.3. Caspase assay

Pellets (10^6 cells) were resuspended in 100 μl of caspase buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 10^{-4}% NP-40 and 50 μM DEVD-AMC at pH 7.25). Samples were placed in a 100 μl cuvette

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Abbreviations: AMC, 7-amino-4-methylcoumarin; DTT, dithiothreitol

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from Hellma Cells (New York, NY, USA) and substrate cleavage was monitored continuously at excitation 370 nm and emission 445 nm using a 37°C heat-jacketed fluorescent spectrophotometer (F-4500 Hitachi). Fluorescent units were converted to pmol of free AMC using a standard curve generated with reagent AMC.

Recombinant caspase-3 was supplied at a stock concentration of 40 U/μl in caspase buffer with 5 mM DTT. The enzyme was originally recovered as separate p17 and p12 subunits from inclusion bodies of *Escherichia coli*, where it constitutes >99% of the total protein [16]. The stock was assayed at a 1 in 1000 dilution into 100 μl of DTT-free caspase buffer (4 U final). 1 U is defined as the release of 1 pmol AMC per min at 25°C.

Concentrations of stock H_2O_2 solutions were determined by measuring A_{240} ($\varepsilon = 43.6$). The rate equation for inactivation of recombinant caspase-3 with H_2O_2 can be represented as:

$$-\mathrm{d[caspase]}/\mathrm{d}t = k\,[H_2O_2]\,[\mathrm{caspase}]$$

where [caspase] represents the concentration of active enzyme and k is the second-order rate constant. Provided H_2O_2 is in substantial excess, the pseudo first-order rate constant with respect to caspase activity ($k' = k\,[H_2O_2]$) can be obtained by fitting an exponential curve to the data measuring caspase activity versus time after treatment with H_2O_2. The reaction was performed over a range of H_2O_2 concentrations (2–20 μM), and k was determined from the slope of the plot of k' versus H_2O_2 concentration.

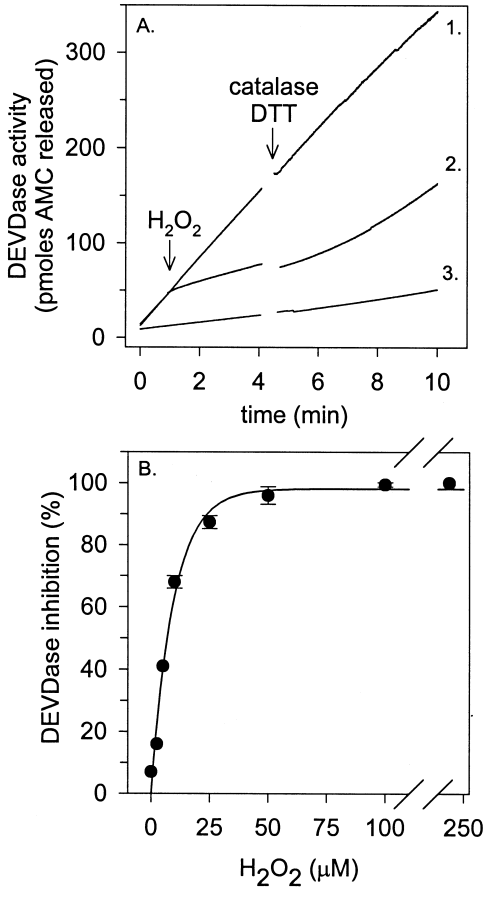

Fig. 1. Caspase inactivation in cell lysates by H_2O_2. A: Continuous measurement of DEVD-AMC cleavage in Jurkat cell lysates (10^6 cells/100 μl). 1, Lysate from apoptotic cells, treated with catalase (20 μg/ml) and 10 mM DTT at 4 min; 2, lysate from apoptotic cells treated with 100 μM H_2O_2 after 1 min, and then with catalase and DTT at 4 min; 3, lysate from non-apoptotic cells, treated with catalase and DTT at 4 min. B: Apoptotic cell lysates were treated with varying concentrations of H_2O_2 for 2 min. The subsequent linear rate of DEVDase activity was measured over the next minute. The H_2O_2-insensitive activity present in non-apoptotic cells was subtracted from all samples, and the percent inhibition as compared to untreated lysates was calculated. The mean and S.E.M. of three experiments are plotted. An exponential curve was fitted to the means of the data points and an IC_{50} of 7 μM was calculated.

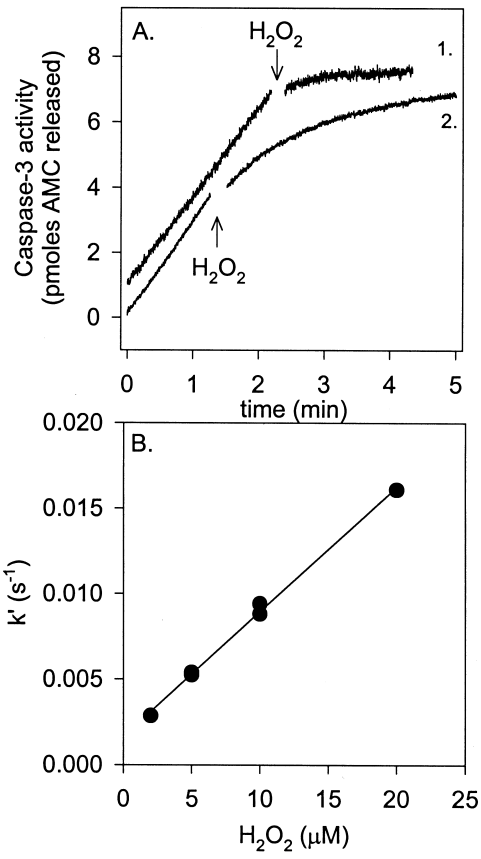

Fig. 2. Inactivation of recombinant caspase-3 by H_2O_2. Caspase-3 was diluted 1:1000 from a stock of 40 U/μl into caspase buffer. A: Continuous measurement of DEVD-AMC cleavage, with 100 μM H_2O_2 (trace 1) and 20 μM H_2O_2 (trace 2) added at the indicated times. B: Exponential curves were fitted to the experimental data obtained after treatment of caspase-3 with H_2O_2 as in A. Pseudo first-order rate constants (k') were calculated from these curves, and were plotted at various concentrations of H_2O_2 to allow estimation of the second-order rate constant.

3. Results

3.1. Inactivation of lysate and recombinant caspase-3 by H_2O_2 added to the assay system

To obtain cell lysates containing active caspases, Jurkat T-lymphocytes were cultured in the presence of anti-Fas antibody. After 2 h the cells were pelleted and lysed in buffer containing the tetrapeptide Ac-DEVD-AMC, and caspase activity was monitored continuously by the generation of fluorescent AMC (Fig. 1A, trace 1). Lysates of apoptotic cells typically contained greater than 10 times more activity than lysates of resting cells (trace 3). Much of the caspase activity in Fas-treated Jurkat cells is attributable to caspase-3 [17], but it is described as DEVDase activity to acknowledge the potential role of other effector caspases.

Addition of H_2O_2 to the lysate/substrate mixtures resulted in immediate inhibition of activity (trace 2). Control experiments indicated that H_2O_2 does not react with the substrate

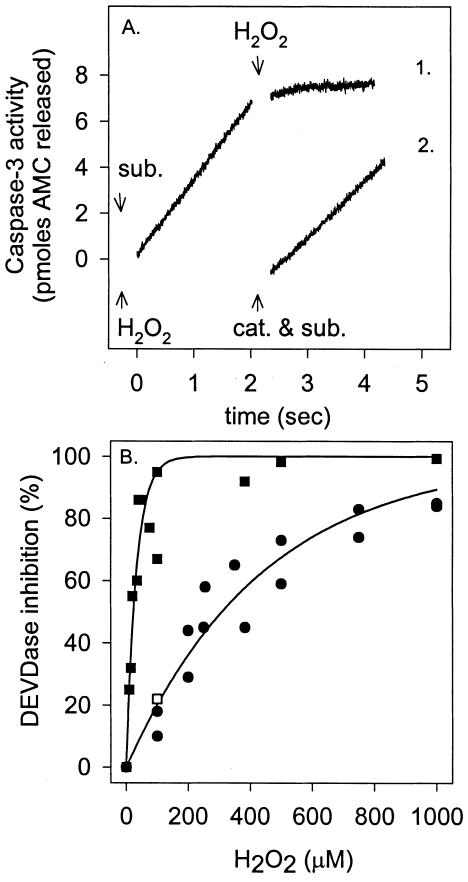

Fig. 3. Effect of substrate on caspase inactivation by H_2O_2. A: Recombinant caspase-3 was either incubated with its substrate (sub) for 2 min before addition of 100 μM H_2O_2 (trace 1), or pre-treated with 100 μM H_2O_2 for 2 min before catalase (cat) and then substrate was added (trace 2). B: Lysates from apoptotic Jurkat cells were treated with H_2O_2 for 2 min in the presence (■) or absence (●) of 10 μM Ac-DEVDGI-NH_2, before catalase was added and the amount of remaining DEVDase activity measured as described in Fig. 1B. The peptide was added at a concentration below that of the fluorescent substrate so as to not significantly interfere with the measurement of caspase activity. Exponential curves were fitted to the data, and IC_{50}s of 23 and 310 μM were calculated, respectively. (□) 10 μM angiotensin II (DRVYIHPF).

or the fluorescent AMC (not shown). Measurement of DEVDase activity 2 min after addition of H_2O_2 showed increasing inhibition with increased concentrations of H_2O_2, with an IC_{50} of 7 μM (Fig. 1B). Concentrations in excess of 100 μM H_2O_2 decreased DEVDase activity to that present in non-apoptotic cells. After catalase was added to remove any remaining H_2O_2, the thiol reductant DTT was able to regenerate caspase activity (Fig. 1A). However, recovery was slow and only 50% was regained after 5 min with 10 mM DTT.

To establish whether caspase inhibition involved a direct reaction with the enzyme we tested the effect of H_2O_2 on recombinant caspase-3. Higher concentrations (>100 μM) of H_2O_2 resulted in rapid and complete caspase-3 inactivation (Fig. 2A, trace 1). At lower concentrations (2–20 μM; trace 2) there was a gradual decrease in activity over a few minutes. Exponential curves were fitted to caspase-3 activity traces, and pseudo first-order rate constants (k'_1) for the loss of activity were calculated. A plot of k'_1 versus H_2O_2 concentration gave an estimated second-order rate constant (k_1) of 750 M^{-1} s^{-1} (Fig. 2B).

3.2. Caspase inactivation by H_2O_2 in the absence of substrate

In the experiments above, we kept the substrate present the entire time and continuously monitored the loss of enzyme activity upon addition of H_2O_2. This approach might be expected to underestimate the rate constant, since the substrate could protect the active site cysteine from oxidation. Therefore, we also performed experiments in which the enzyme was incubated with H_2O_2 for selected times, catalase was added to remove H_2O_2, and DEVD-AMC was finally added to measure the enzyme activity remaining. Under these conditions recombinant caspase-3 was considerably more resistant to oxidation than when treated with H_2O_2 in the absence of substrate. Whereas 100 μM H_2O_2 completely inhibited caspase-3 activity within seconds (Fig. 3A, trace 1), significant activity remained after a 2 min treatment in the absence of substrate (Fig. 3A, trace 2).

This phenomenon was explored further in lysates from apoptotic cells. Lysates were treated with H_2O_2 for 2 min, then catalase and substrate were added. A plot of caspase activity versus H_2O_2 concentration (Fig. 3, solid circles) gave an IC_{50} of 310 μM, more than 40 times that measured in Fig. 1 in the presence of the fluorometric substrate. We then included a peptide substrate of caspase-3, Ac-DEVDGI-NH_2, during preincubation so that the enzyme would be catalytically active at the time of adding H_2O_2. The peptide substrate dramatically enhanced the sensitivity of the caspases to H_2O_2, dropping the IC_{50} to 25 μM (Fig. 3, solid squares). A control non-substrate peptide had no effect at 100 μM H_2O_2 (Fig. 3, hollow square).

4. Discussion

Our results show caspase-3 to be highly sensitive to oxidative inactivation by H_2O_2. We obtained a rate constant of 750 M^{-1} s^{-1} for the reaction of recombinant caspase-3 with H_2O_2, and an IC_{50} of less than 10 μM H_2O_2 in concentrated cell lysates, despite the presence of catalase and peroxidases that would consume much of the H_2O_2. The sensitivity to oxidation was dependent on one predominant factor, the presence of a caspase substrate at the time of H_2O_2 addition. Otherwise, there was a 40-fold decrease in sensitivity. This was not an artefact of the fluorescent substrate since a DEVDGI peptide, which represents the target sequence of the classic caspase-3 substrate poly-ADP ribose polymerase, also increased the sensitivity of the enzyme to inactivation. The mode of inactivation is consistent with thiol oxidation, rather than suicide inhibition through covalent attachment of the substrate, since it was able to be reversed by DTT, albeit slowly.

This phenomenon may explain contradictory reports in the literature. While we and others [5] have observed direct caspase inactivation by H_2O_2, it has been reported that H_2O_2 is a poor inhibitor of caspases in lysates or in its purified form [14,15]. While the former studies used a continuous assay to monitor caspase activity, the latter investigators treated lysates or recombinant protein with H_2O_2, before measuring the amount of substrate cleaved over 30–60 min. They included DTT in the assay buffer, which would reduce some of the oxidised caspases and mask the full extent of inhibition. In the continuous assay, activity is measured over a short time and DTT is not required to prevent spontaneous oxidation. A more important factor, however, is likely to be the absence of

substrate during H_2O_2 treatment. Indeed, the report of 20% inhibition by 100–200 μM H_2O_2 [15] is very similar to what we observed in the absence of substrate (Fig. 3).

The mechanism by which substrate enhances caspase-3 inactivation by H_2O_2 is currently unclear. The active site Cys-163 of caspase-3 is the most likely target of H_2O_2. In caspases, the basic amino acids surrounding the active site cysteine lower its pK_a to allow reaction with target substrates at physiological pH [18,19]. H_2O_2 only reacts with the thiolate anion, therefore a lower pK_a will also considerably enhance its reactivity with the active site [20]. We have observed that only one cysteine residue in caspase-3 is able to react with iodoacetic acid at physiological pH, and this reactive thiol is detectable whether or not the substrate is present (unpublished data). Therefore, the caspase substrate is unlikely to be responsible for stabilising the thiolate anion or exposing the active site. Instead, we propose that the presence of the substrate in the active site induces conformational changes that make the thiolate anion more reactive to H_2O_2. Further studies are required to elucidate the mechanism of enhanced reactivity.

The initiation, signaling and execution pathways of apoptosis are all subject to redox regulation [21], and the cysteine-dependent caspases are obvious redox-sensitive targets. When apoptosis is delayed or prevented under conditions of sustained oxidative stress [4,22], caspase-3 is not processed and remains in its inactive pro-form. Our results suggest that because pro-caspase-3 does not interact with substrate, it will be considerably more resistant to oxidation than the catalytically active form. Under these conditions, the initiator caspases-8 and -9 may be more likely targets. Whether their sensitivity to oxidation can be modulated by interactions with substrates, or other protein components of the apoptotic machinery, warrants further investigation.

Our results also have implications for redox signaling in general. To the best of our knowledge, the phenomenon whereby the sensitivity of a thiol protein to oxidation can be considerably enhanced by a substrate has not been previously reported. H_2O_2 has been shown to react with the active site cysteine of various thiol enzymes, including the protein tyrosine phosphatases [23]. Rate constants for the phosphatases, at 10–20 M^{-1} s^{-1} [23], are no faster than the individual cysteine thiolate [20], yet these proteins are proposed to be a direct primary target of H_2O_2 in cells [24]. These low rate constants are similar to caspase-3 in the absence of substrate, but we observed a 40-fold increase in reactivity while substrate was present. If a similar enhancement in reactivity can occur in other cellular thiol proteins, whether via substrate or other physical interactions, it may provide a means for target selectivity during redox signaling.

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