FEBS Letters 500 (2001) 114–118 FEBS 25007

Caspases are reversibly inactivated by hydrogen peroxide

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Received 28 March 2001; accepted 6 June 2001

First published online 18 June 2001

Edited by Veli-Pekka Lehto

Abstract Hydrogen peroxide (H_2O_2) is known to both induce and inhibit apoptosis, however the mechanisms are unclear. We found that H_2O_2 inhibited the activity of recombinant caspase-3 and caspase-8, half-inhibition occurring at about 17 μM H_2O_2. This inhibition was both prevented and reversed by dithiothreitol while glutathione had little protective effect. 100–200 μM H_2O_2 added to macrophages after induction of caspase activation by nitric oxide or serum withdrawal substantially inhibited caspase activity. Activation of H_2O_2-producing NADPH oxidase in macrophages also caused catalase-sensitive inactivation of cellular caspases. The data suggest that the activity of caspases in cells can be directly but reversibly inhibited by H_2O_2.

Key words: Apoptosis; Caspase; Hydrogen peroxide; Macrophage

1. Introduction

Hydrogen peroxide (H_2O_2) plays a variety of diverse roles in the body including (i) a signalling molecule, (ii) an intermediate in metabolism, (iii) a cytotoxic agent in host defence, and (iv) a cytotoxic agent in pathology [1–5]. H_2O_2 can be produced directly or indirectly (after dismutation of superoxide) by (a) a plasma membrane NAD(P)H oxidase, physiologically activated by growth factors (e.g. EGF, platelet-derived growth factor), (b) a related plasma membrane NADPH oxidase, activated in phagocytes by chemotactic factors to produce the 'respiratory burst', (c) a number of metabolic oxidases, mostly located in peroxisomes, and (d) the mitochondrial respiratory chain [2,6–9]. Low concentrations of H_2O_2 can modify proteins by reversibly oxidizing thiol residues (RSH) to sulfenic acid (RSOH) or dithiols (RSSR) [10].

Caspases are a family of cysteine–aspartate proteases responsible for executing apoptosis (and regulating inflammation) [11,12]. The active-site cysteine residue is susceptible to oxidation, resulting in caspase inactivation and thus potentially inhibition of apoptosis. Nitric oxide (NO) may regulate apoptosis by *S*-nitrosation of the caspase cysteine residue [13–15]. Reactive oxygen species (ROS, including superoxide and H_2O_2) are thought to be involved in both apoptotic and necrotic cell death, but the mechanisms are unclear [5,16–18]. Apoptotic cells have been found to produce higher levels of ROS [19–21]; pro-oxidants and redox cycling agents, such as H_2O_2, diamide or semiquinones, can induce apoptosis [5,22,23]; and antioxidants can prevent apoptosis [24–26]. However, although H_2O_2 can induce apoptosis, there is also evidence that it can inhibit this process [5,17,27], and thus H_2O_2 is potentially an important dual regulator of apoptosis. The mechanism by which H_2O_2 inhibits apoptosis is unclear; it might do so by: (a) inducing necrosis, (b) preventing caspase activation, or (c) directly inhibiting caspase activity. We set out to test the later possibility.

2. Materials and methods

2.1. Assay of activity of recombinant caspase-3 and -8

Human, recombinant caspase-3 and caspase-8 were obtained from Biomol Research Laboratories, Inc. Before an experiment, stock solutions of caspases in a buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol (DTT) were diluted to 2 U/μl with the buffer A (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol) plus 400 μM DTT. Activity of enzymes (final concentration 40 U/ml for caspase-3 and 50 U/ml for caspase-8) was measured in 1 ml buffer A (without addition of DTT) following the release of fluorescent amino-methyl-coumarin (amc) from the synthetic substrates 50 μM DEVD-amc for caspase-3 or 50 μM IETD-amc for caspase-8 with a Shimadzu RF-1501 spectrofluorimeter. Final concentration of DTT in the assay buffer was about 10 μM to avoid spontaneous inactivation of caspases. All measurements were done at 37°C.

2.2. Cell culture

Murine macrophage J774 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% streptomycin +1% penicillin at 37°C in a humidified atmosphere containing 5% CO_2. Two models of induction of apoptosis in macrophages were used: serum withdrawal or incubation of cells with the NO-donor *S*-nitrosoglutathione (GSNO). For the serum withdrawal experiments, cells ($2–3\times10^7$) were suspended in 8 ml of DMEM and incubated for 16 h in Falcon culture flasks, then two additions of 100 μM H_2O_2 (with a 15-min interval between them) were made and after another 15 min, cells were scraped from the flasks and used for assay of caspase activity. In experiments on NO-induced apoptosis, cells were incubated with 1 mM GSNO for 6 h, and then the NO-donor was removed by washing cells with DMEM. Cells were resuspended in DMEM and then treated with 100 μM H_2O_2 as in experiments on serum withdrawal described above. The viability of cells was examined by counting cells excluding trypan blue.

2.3. Measurement of caspase activity in cells extracts

Cells were sedimented at $700\times g$ for 3 min, washed with Krebs–HEPES buffer (25 mM HEPES, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, 1 mM $CaCl_2$) and resuspended in 200 μl of lysing buffer containing 100 mM HEPES (pH 7.4), 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 5 μl protease

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Abbreviations: amc, amino-methyl-coumarin; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species

0014-5793/01/$20.00
PII: S0014-5793(01)02593-5

inhibitor cocktail (Sigma). After 20 min on ice, cells were disrupted by passing through a 27-G needle 15 times and centrifuged at 13 000 rpm × 10 min. Supernatants (50–100 μg total protein) were incubated with 100 μM DEVD-amc for 60 min. Substrate cleavage was determined fluorometrically (excitation at 380 nm, emission at 460 nm). DEVD-CHO, 1 μM, was used to inhibit caspase activity and to test specificity of the reaction. 10–200 nM 7-amino-4-methyl-coumarin was used for calibration of the fluorescence signal.

Protein concentrations in cytosolic extracts were determined by the Bradford method using bovine serum albumin as a standard.

3. Results

It has been reported that excessive generation of ROS [17,27] including H_2O_2 might inhibit apoptosis by interfering with some steps in the caspase activation cascade. We investigated whether H_2O_2 can directly inhibit caspases. Fig. 1A shows the measurement of caspase-3 activity following the increase in fluorescence due to cleavage of caspase-3 substrate DEVD-amc. The rate of substrate cleavage by caspase-3 in the presence of about 10 μM DTT was linear for at least 15–20 min, and only negligible loss of activity was observed during further incubation up to 30 min (data not shown). After addition of H_2O_2 the rate of DEVD-amc cleavage gradually decreased and reached a new steady-state rate within 4 min. Thus, in further experiments caspase-3 activity was measured after 5 min incubation of the enzyme with different concentrations of H_2O_2. To exclude the possibility that H_2O_2 interacts with the fluorogenic caspase-substrate rather than with the enzyme itself, in some experiments catalase was added after 5 min incubation of caspase-3 with H_2O_2 to remove residual H_2O_2 and then DEVD-amc was added. Such pretreatment had no effect on the H_2O_2-inhibited enzyme rate (data not shown). As can be seen in Fig. 1B, H_2O_2 in a concentration-dependent manner inhibited caspase-3 activity, and half-inhibition of the enzyme was achieved at about 17 μM H_2O_2. 5 mM reduced glutathione (GSH) had no protective effect in the case of caspase-3, however 5 mM DTT substantially reduced the sensitivity of the enzyme to H_2O_2: half-inhibition of the enzyme was obtained at about 41 μM H_2O_2 in the presence of DTT. Similar inhibition by H_2O_2 and protection by DTT was observed with isolated caspase-8, however in contrast to caspase-3, GSH exerted some protection from H_2O_2-induced inhibition of caspase-8 (Fig. 1C).

The ability of DTT to reactivate H_2O_2-inhibited caspase-3 activity is shown in Fig. 2. In these experiments, caspase-3 was

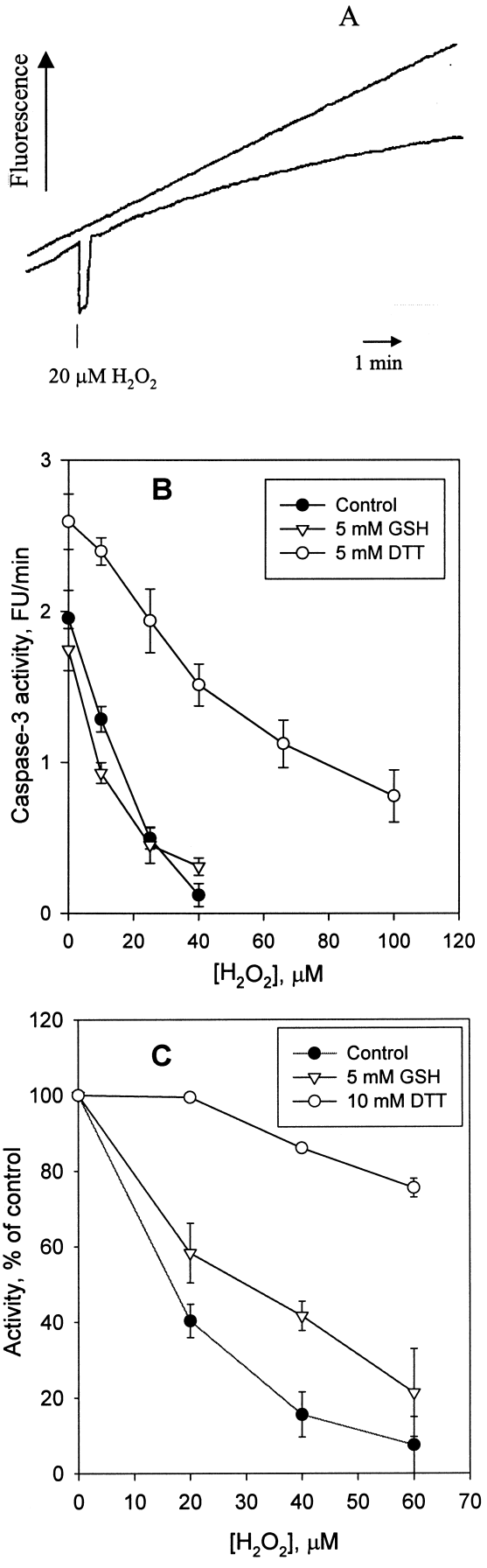

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Fig. 1. H_2O_2 inhibits caspase-3 (A, B) and caspase-8 (C). A: Fluorimetric measurement of caspase-3 activity. 40 U caspase-3 was incubated in 1 ml buffer A containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 50 μM DEVD-amc. Upper trace represents control, the lower trace enzyme inhibition by 20 μM H_2O_2. B: 40 U recombinant caspase-3 was incubated 5 min in 1 ml buffer A with different concentrations of H_2O_2 and then enzyme activity was measured. Where indicated, incubation buffer was supplemented with 5 mM DTT or 5 mM GSH. C: 50 U recombinant caspase-8 was incubated 5 min in 1 ml buffer A in the presence of different concentrations of H_2O_2. Where indicated, the incubation buffer was supplemented with 10 mM DTT or 5 mM GSH. Activity of caspase-8 in the absence of H_2O_2 (1.02 ± 0.31 FU/min, 0.95 ± 0.17 FU/min and 1.75 ± 0.40 FU/min in the absence of DTT, presence of 5 mM GSH and presence of 10 mM DTT, respectively) was taken as 100%.

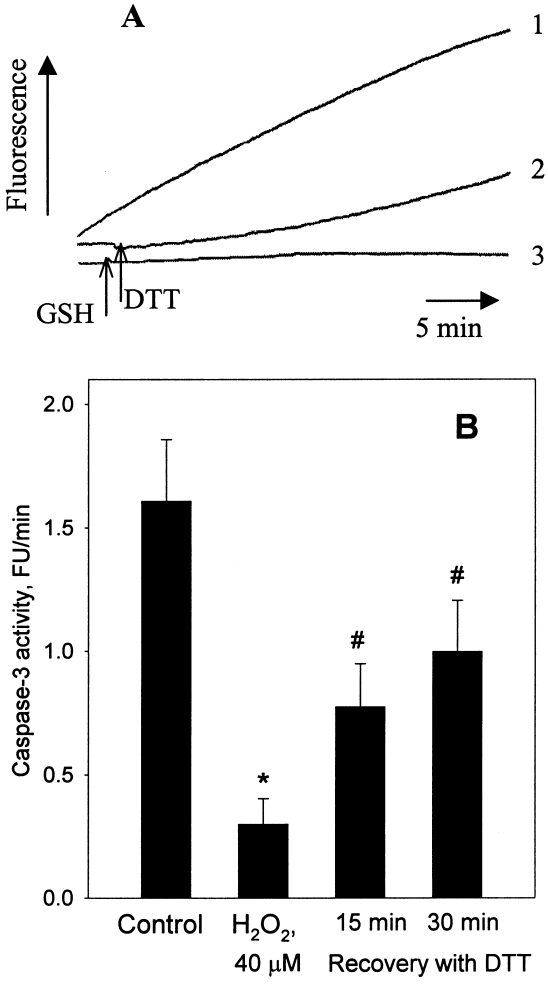

Fig. 2. Reversal of H_2O_2-inhibited caspase-3 activity by DTT. A: representative traces of fluorimetric measurement of caspase-3 activity. Trace 1: control, caspase-3 activity in the absence of H_2O_2. Traces 2 and 3: caspase-3 was preincubated for 5 min with 70 μM H_2O_2 and then either 10 mM DTT (trace 2) or 5 mM GSH (trace 3) were added where indicated. B: caspase-3 was treated with 40 μM H_2O_2 for 5 min followed by incubation with 10 mM DTT for 15 min and 30 min. Data are means ± S.E.M. of four experiments. *$P < 0.001$ compared to control; #$P < 0.05$ compared to H_2O_2-inhibited caspase activity.

treated with 40 or 70 μM H_2O_2 for about 5–10 min, then 10 mM DTT was added and changes in fluorescence due to cleavage of DEVD-amc were followed (Fig. 2A). As one can see, incubation of caspase-3 with DTT resulted in a slow, gradual increase in caspase activity. After 15 min and 30 min incubation caspase-3 activity was recovered to 48 and 62% of control level, respectively (Fig. 2B). DTT revived caspase-3 even when it was inhibited by higher (100–200 μM)

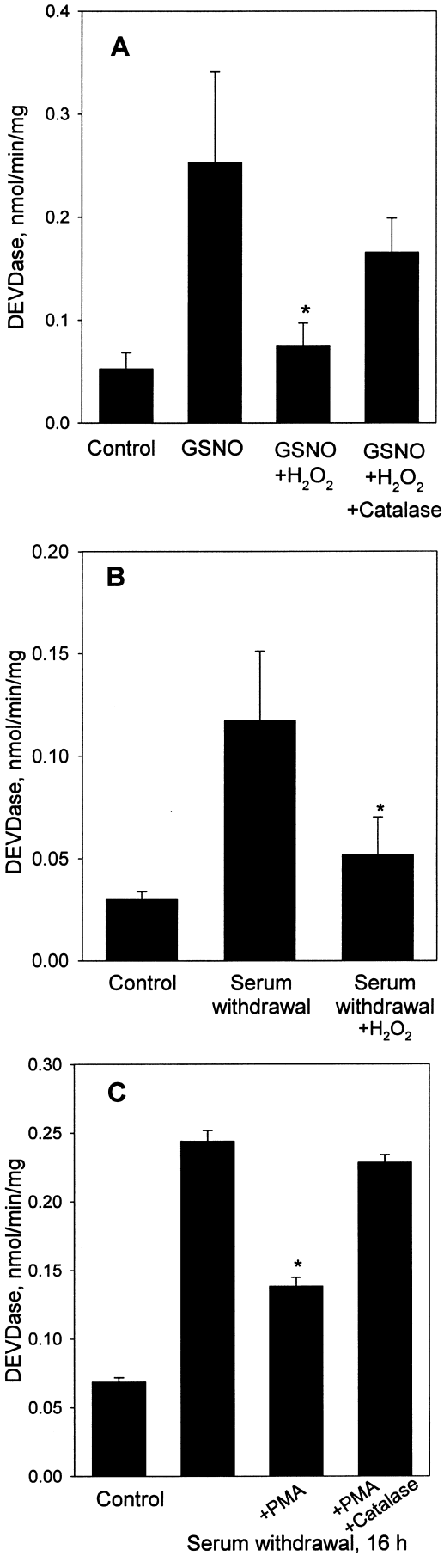

Fig. 3. H_2O_2 inhibits GSNO- or serum withdrawal-induced caspase-3-like activity in macrophages. A: Cells were incubated 6 h with 1 mM GSNO, then washed and two additions of 100 μM H_2O_2 to the cells were made in a 15-min interval in the absence or presence of 500 U/ml catalase. B,C: Apoptosis was induced by serum withdrawal for 16 h. Then cells were treated with H_2O_2 as in A or incubated with 5 μg/ml PMA for 30 min in the presence or absence of catalase.

concentrations of H_2O_2 (data not shown). In contrast to DTT, GSH did not reverse H_2O_2-inhibited caspase-3 activity. These findings indicate that H_2O_2-induced inhibition of caspases is reversible by DTT.

Next we investigated whether H_2O_2 can inhibit caspase activity in cells undergoing apoptosis. Apoptosis in macrophages was induced by incubating cells for 6 h with 1 mM GSNO or 16 h in the absence of serum. In both cases, 5–6-fold increases in caspase-3-like protease activity were observed compared to control (Fig. 3). Then cells were washed and two additions of 100 μM H_2O_2 were made with a 15-min interval between additions. Such treatment resulted in almost complete inactivation of caspase-3-like protease activity induced by GSNO or serum withdrawal (Fig. 3A,B). In the presence of exogenous catalase, treatment of cells with H_2O_2 had no effect on caspase activity in macrophages undergoing apoptosis (Fig. 3A).

Caspase activity was also inhibited by about 50% when cells after 16 h of serum withdrawal were exposed to a phorbol 12-myristate 13-acetate (PMA)-induced respiratory burst for 15–30 min (Fig. 3C). Addition of catalase before PMA completely protected caspase activity (Fig. 3C) indicating that inhibition was due to H_2O_2 formed during the respiratory burst. These data indicate that H_2O_2 can inhibit caspase activity within the cells.

4. Discussion

We have shown that H_2O_2 can inactivate caspases, when added to isolated caspases, when added to cells where the caspases have been activated during apoptosis, and when the H_2O_2 is generated endogenously from the NADPH oxidase. Physiological levels of glutathione are unable to prevent or reverse H_2O_2-induced inactivation of isolated caspase-3 and only minimally protect caspase-8. This suggests that cellular glutathione would not directly protect caspases from H_2O_2-induced inactivation. However, DTT both prevented and reversed H_2O_2-induced inactivation. This suggests that the inactivation is due to a reversible thiol oxidation, probably either to a RSSR or RSOH, which might be reversible by the cell.

It has previously been shown that H_2O_2 can inhibit Fas-mediated apoptosis in Jurkat T-cells, apparently by reversibly inactivating caspase activity within the cell [17]. Fas-mediated and spontaneous apoptosis in human neutrophils was found to be inhibited by PMA activation of the NADPH oxidase, apparently causing ROS-mediated inactivation of the caspases [28]. These reports are consistent with our findings of a direct, reversible inactivation of caspase-3 and caspase-8 by H_2O_2, and inactivation by H_2O_2 and PMA of caspase-3-like activity in J774 macrophages induced by NO and/or serum withdrawal. TNF-α-induced caspase activity in HepG2 cells was found to be potentiated by overexpression of catalase and inhibited by a catalase inhibitor (azide), suggesting that basal or TNF-α-induced cellular H_2O_2 production inhibited caspase activity or caspase activation [29]. Menadione inhibited caspase activity in HepG2 cells undergoing Fas-mediated apoptosis and this inhibition was prevented by catalase, suggesting that menadione-induced H_2O_2 was inactivating the caspases [5]. These reports are again consistent with a direct inactivation of the caspases by H_2O_2, and indicate that this inactivation may be relevant in a wide range of systems and cells.

H_2O_2 can also induce apoptosis causing caspase activation, but this induction is upstream of the caspases, resulting in caspase activation several hours after H_2O_2 addition to cells [17], whereas H_2O_2-induced caspase inactivation is direct and rapid. It has previously been shown in Jurkat T-cells that there is a bell-shaped dependence of H_2O_2-induced caspase activity on H_2O_2 concentration, so that high concentrations (>200 μM) of H_2O_2 apparently prevent H_2O_2-induced caspase activation [17]. However, these concentrations also induced necrosis, which might itself prevent apoptosis, or alternatively be a consequence of aborted apoptosis. It remains unclear whether physiological sources of H_2O_2 could inhibit apoptosis without inducing necrosis.

Activated macrophages and neutrophils can produce high levels of H_2O_2 that kill pathogens, with relatively little effect on the producer cells [30]. It is possible that H_2O_2-induced inactivation of the caspases within these cells prevents or delays self-induced apoptosis during the respiratory burst.

Acknowledgements: This work was supported by British Heart Foundation and BBSRC.

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