

Inactivation of cellular caspases by peptide-derived tryptophan and tyrosine peroxides

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Abstract Peroxides generated on peptides and proteins within cells, as a result of radical attack or reaction with singlet oxygen, are longer-lived than H₂O₂ due to their poor removal by protective enzymes. These peroxides readily oxidize cysteine residues and can inactivate thiol-dependent enzymes. We show here that Trp- and Tyr-derived peptide peroxides, generated by singlet oxygen, inhibit caspase activity in the lysates of apoptotic Jurkat cells. *N*-Ac-Trp-OMe peroxide was the most effective inhibitor, and was 30-fold more effective than H₂O₂ under identical conditions. As such, protein peroxides could modulate the progression of apoptosis in cells in which they are generated. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Exposure of a wide range of amino acids, peptides and proteins to a variety of radical sources (e.g. γ - and X-rays, Fenton systems, thermolabile azo compounds, peroxyinitrite, activated white cells) in the presence of O₂ generates high yields of unstable amino acid, peptide and protein peroxides (reviewed in [1,2]). Singlet oxygen (molecular oxygen in its ¹ Δ_g state; ¹O₂), which is generated by a number of cellular, enzymatic and chemical reactions, by UV exposure, and by visible light in the presence of a number of exogenous or endogenous cellular sensitizers, also generates amino acid, peptide and protein peroxides, though with a somewhat different selectivity (reviewed in [3]). As a result of the widespread exposure of humans to these oxidizing systems, protein oxidation and protein peroxides have been suggested to play a key role in the development of a number of human pathologies including cataract, sunburn, some skin cancers and aging (reviewed in [3,4]). While H₂O₂ is rapidly consumed by cellular catalases and peroxidases, protein peroxides are poor substrates for these enzymes ([5], P.E. Morgan, R.T. Dean and M.J. Davies, unpublished data), and can be readily detected within cells ([6], A. Wright, C.L. Hawkins and M.J. Davies, submitted).

Previous studies have shown that protein peroxides can play a key role in the propagation of oxidative chain reactions within proteins [2,7] and can oxidize other biomolecules, including lipids, antioxidants and DNA via both radical-mediated, and non-radical, reactions [1,8–10]. It has been shown that peptide and protein peroxides can inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in the absence of added metal ions, via reaction of the peroxide with the active site thiol group [5]. This inhibition is concentration, peroxide, and time dependent, with peptide peroxides more efficient than H₂O₂ [5]. These studies demonstrate that peroxide formation on one protein can result in subsequent selective damage to other proteins. As activated (low pKa) thiol residues are favored targets for these peroxides [5], we hypothesized that these materials may also inhibit cysteine proteases, and in particular the caspase family.

The caspase family of cysteine proteases play a key role in apoptosis [11,12]. They are divided into two general classes: the initiator or amplification caspases and the effector caspases. They exist as inactive zymogens in normal cells. Pro-caspase forms of the initiator caspases have some catalytic activity, and their enforced localization leads to autocatalytic processing and activation. The initiator caspases cleave and activate the effector caspases, which proteolytically degrade an array of structural and regulatory proteins at Asp-Glu-Val-Asp (DEVD), or closely related sequences, leading to the irreversible dismantling of the cell. This activity can be readily measured in crude cell lysates by cleavage of a fluorescent tag from a peptide substrate [13].

The caspases have an active site cysteine that mediates nucleophilic attack on the target substrate [14]. Oxidizing species, including H₂O₂, have already been shown to be potent inhibitors of caspase activity [15,16]. Excessive oxidative stress can delay apoptosis until a reducing environment is restored, and sustained oxidation (including exposure to UV radiation [17]) precludes caspase-dependent apoptosis [18,19]. In this study we have therefore investigated the ability of a range of amino acid and protein peroxides, generated by ¹O₂-mediated oxidation of Trp and Tyr residues, to inhibit the action of effector caspases, and compared their action to that of H₂O₂.

2. Materials and methods

2.1. Materials

The Jurkat T-lymphoma 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-methylcoumarin (DEVD-AMC) was from Peptide Institute, Inc.

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Abbreviations: AMC, 7-amino-4-methylcoumarin; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ¹O₂, singlet oxygen

(Osaka, Japan). Amino acids and peptides were commercial samples of high purity and L-stereochemistry (with the exception of Trp which was DL) obtained from Sigma-Aldrich (St Louis, MO, USA), Bachem (Bubendorf, Switzerland) and Auspep (Parkville, Victoria, Australia).

2.2. Generation of amino acid, peptide and protein peroxides

Peroxides were generated on amino acids, peptides (all 2.5 mM), and ovalbumin (50 mg/ml) by photolysis with visible light (from a Kodak S-AV 2050 slide projector) through a 345 nm cut-off filter in the presence of 10 μ M rose bengal [5,20]. Samples were made up in water that had been passed through a four-stage Milli Q system equipped with a 0.2 μ m-pore-size final filter. The pH of all solutions was checked prior to photolysis and adjusted where necessary to pH 7.0–7.5. Solutions were kept on ice during photolysis (60 min, except for Tyr which was photolysed to 120 min), and were continually aerated. After cessation of photolysis, catalase (Sigma-Aldrich, bovine liver, 50 μ g/ml ovalbumin, 250 μ g/ml for amino acids and peptides) was added to remove H₂O₂ and the samples incubated for 30 min at room temperature before freezing (–80°C) in aliquots. Peroxide concentrations were determined by a modified FOX (FeSO₄/xylenol orange) assay, using H₂O₂ standards [21]. This assay gives similar values to iodometric analysis [20]. The yield of peroxides formed on the various amino acids, peptides and proteins examined are given in Table 1. Peroxide concentrations of all control (non-photolysed) samples were also determined, and found to be negligible.

2.3. Cell culture and preparation of lysates

Cells were cultured in RPMI-1640 with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, at 37°C in humidified air with 5% CO₂. 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. Dithiothreitol (DTT) was not included during the assay of caspase activity, therefore cells were either assayed immediately, or stored at –80°C and assayed within 6 h, to prevent spontaneous caspase oxidation.

2.4. Caspase assay

Pellets (10⁶ cells) were resuspended in 100 μ l of caspase buffer [100 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)), 10% sucrose, 0.1% CHAPS (3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate), 10^{–4}% NP-40 and 50 μ M DEVD-AMC at pH 7.25]. Samples were placed in a 100 μ l cuvette from Hellma Cells, Inc. (NY, USA) and substrate cleavage was monitored continuously at λ_{ex} 370 nm (band width 5 nm) and λ_{em} 445 nm (band width 10 nm) using a 37°C heat-jacketed fluorescence spectrophotometer (F-4500 Hitachi). Fluorescence units were converted to pmol of free AMC using a standard curve generated with reagent AMC.

Table 1

Peroxides formed on amino acids, peptides and proteins by photolysis with visible light in the presence of rose bengal

Parent compound	Peroxide concentration (μ M)
Tyr	290 $\pm$ 10
<i>N</i> -Ac-Trp-OMe	1010 $\pm$ 40
Trp-OMe	390 $\pm$ 50
Tyr-OMe	290 $\pm$ 20
Trp	1560 $\pm$ 100
<i>N</i> -Ac-Trp	870 $\pm$ 60
<i>N</i> -Ac-Tyr	540 $\pm$ 10
<i>N</i> -Ac-Tyr-OEt	280 $\pm$ 20
<i>N</i> -Ac-Asp-Glu-Tyr-Asp-OH	360 $\pm$ 30
<i>N</i> -Ac-Asp-Glu-Trp-Asp-OH	270 $\pm$ 20
Ovalbumin	260 $\pm$ 20

Amino acids, peptides (both 2.5 mM) and proteins (50 mg/ml) were photolysed at 4°C with continuous aeration in the presence of 10 μ M rose bengal, through a 345 nm cut-off filter, for 120 min (Tyr) or 60 min (all other substrates). Following photolysis, H₂O₂ was removed by incubation at room temperature for 30 min with catalase (50 μ g/ml for proteins, 250 μ g/ml for amino acids and peptides). Peroxide concentrations were determined by a modified FOX assay, using H₂O₂ standards.

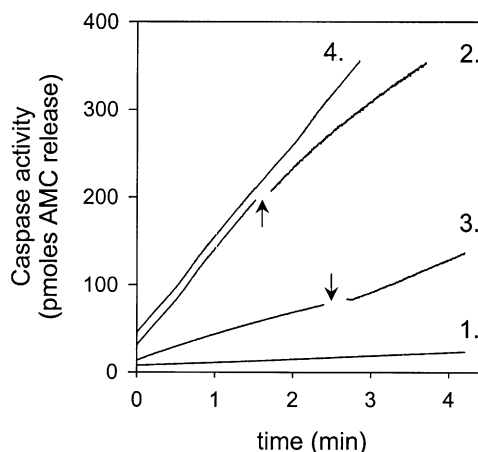


Fig. 1. Inhibition of caspase activity by *N*-Ac-Trp-OMe peroxides. DEVD-AMC (50 μ M) was added to lysates of control and apoptotic Jurkat cells and the release of AMC was monitored continuously in a fluorescence spectrophotometer. Trace 1: control lysate. Trace 2: apoptotic lysate, arrow indicates addition of 20 μ M *N*-Ac-Trp-OMe peroxide (35% inhibition was measured 2 min after addition compared with 80% if preincubated). Trace 3: apoptotic lysate preincubated with 20 μ M *N*-Ac-Trp-OMe peroxide, arrow indicates addition of 10 mM DTT. Trace 4: apoptotic lysate preincubated with non-photolysed (i.e. native) *N*-Ac-Trp-OMe.

3. Results

3.1. Inactivation of caspase activity in cell lysates

Apoptosis was induced in a Jurkat cells line by treatment with anti-Fas antibody. The cells were then pelleted and lysed in caspase buffer, and the tetrapeptide DEVD-AMC added to allow continuous monitoring of caspase activity. Lysates from apoptotic cells showed 20-fold more caspase activity than lysates from control cells (Fig. 1, traces 1 and 2). Preincubation of cell lysates for 2 min with 20 μ M *N*-Ac-Trp-OMe peroxide resulted in an 80% inhibition of caspase activity (Fig. 1, trace 3). The non-photolysed (native) peptide had no effect on caspase activity (Fig. 1, trace 4). DTT was able to restore caspase activity to some extent, indicating thiol-dependent inactivation (Fig. 1, trace 3). It was not possible to gauge the full extent of DTT-reversibility because we were unable to selectively remove the peptide peroxide from the cell lysates, and inhibition still occurred, albeit more slowly, if *N*-Ac-Trp-OMe peroxide was added while the substrate was present (Fig. 1, trace 2).

3.2. Selectivity of amino acids resulting in inhibition of caspase activity

A variety of oxidized amino acids and peptides containing Trp- and Tyr-derived peroxides were able to inhibit caspase activity, though the Trp-derived peroxides were the most effective (Fig. 2). Inhibition was greatest with the peroxide from *N*-Ac-Trp-OMe, suggesting that blocking both the N- and C-termini increases efficacy. To optimize access to the active site we also generated peroxides on peptides with structures similar to the preferred effector caspase substrate Asp-Glu-Val-Asp in which the Val was substituted with Trp or Tyr. These tetrapeptide peroxides were no more effective than the parent protected amino acid, and results with the non-photolysed peptides indicated that some portion of their inhibitory activity was due to competition with the caspase substrate (Fig. 2).

In contrast to the amino acids and peptide peroxides, photolysed ovalbumin (i.e. ovalbumin peroxides) had no effect (Fig. 2).

3.3. Effect of peroxide concentration upon caspase inhibition

Examination of the concentration dependence of inactivation induced by *N*-Ac-Trp-OMe peroxides after 2 min incubation indicated an IC_{50} of 10 μ M (Fig. 3A). Under identical conditions, we previously measured an IC_{50} of 310 μ M with H_2O_2 [16]. Furthermore, extending the incubation past 2 min with H_2O_2 does not increase the extent of inhibition because this oxidant is rapidly detoxified (removed) by the lysates. However, it was possible to see 20% inhibition of caspase activity with 2 μ M *N*-Ac-Trp-OMe peroxide by extending the preincubation time (e.g. to 4 min, Fig. 3B) as this material is poorly removed by enzymatic reactions ([5], P.E. Morgan, R.T. Dean and M.J. Davies, unpublished data).

4. Discussion

We have shown that Trp- and Tyr-derived peroxides are effective inhibitors of caspase activity in cellular lysates. Inhibition was apparent at low concentrations of peroxide (2–20 μ M), and was considerably more effective than H_2O_2 under identical conditions. This concentration range is within that which we have detected on cellular proteins extracted from viable cells after exposure to visible light in the presence of rose bengal (A. Wright, C.L. Hawkins and M.J. Davies, submitted). The ability of these species to oxidize GAPDH [5] and cellular caspases increases the likelihood that they target

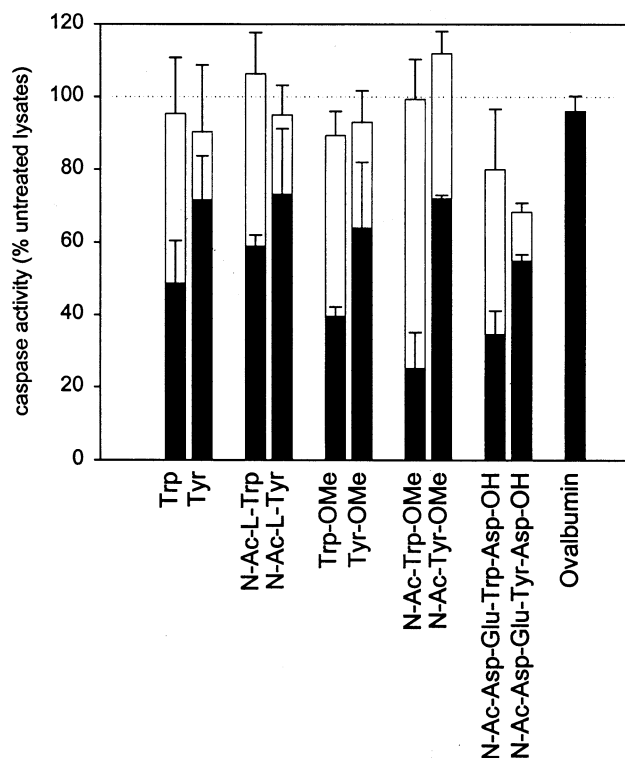


Fig. 2. Inhibition of caspase activity by amino acid and peptide peroxides. Lysates from apoptotic cells were preincubated for 2 min with the photolysed amino acid or peptide (solid bars) at a concentration of 20 μ M peroxide, or with the corresponding concentration of non-photolysed amino acid or peptide (hollow bar). Results are the mean $\pm$ S.D. of three to seven experiments.

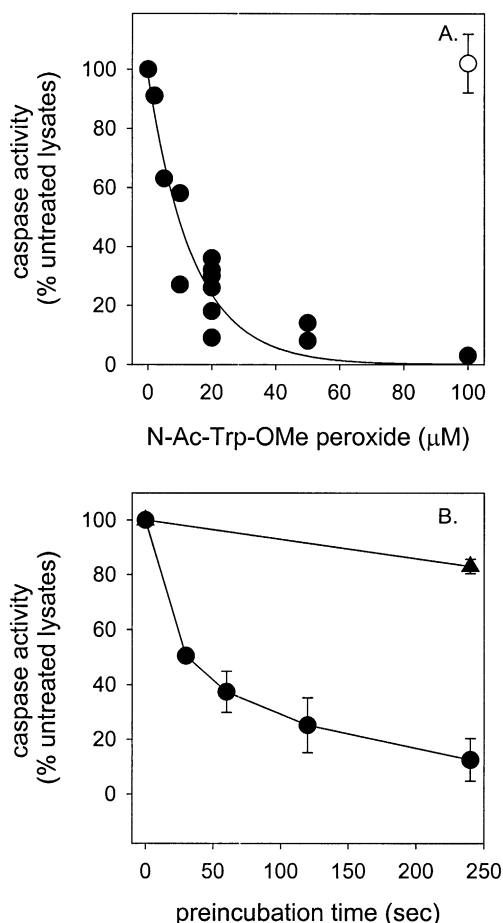


Fig. 3. Concentration dependence and time course of caspase inhibition by *N*-Ac-Trp-OMe peroxides. A: Lysates from apoptotic cells were treated for 2 min with a range of concentrations of photolysed *N*-Ac-Trp-OMe, and the caspase activity remaining was measured. An exponential curve was fitted to the data and an IC_{50} of 10 μ M was calculated. The hollow symbol represents the amount of non-photolysed compound corresponding to 100 μ M peroxide (mean $\pm$ S.D. of three experiments). B: Preincubation times were varied at 20 μ M *N*-Ac-Trp-OMe (circles) and 2 μ M *N*-Ac-Trp-OMe (triangle). Results are the mean $\pm$ S.D. of three experiments.

a range of thiol proteins, and have a significant impact on cell function.

The Trp-derived peroxides were consistently better inhibitors than Tyr-derived peroxides. This may reflect the nature of these species, with the Trp peroxide group being present at the C-3 site on the indole ring of Trp [22], whereas the major peroxide generated on Tyr residues is a ring-derived, C-1, dieneone hydroperoxide [20]. While thiol reactivity is important, accessibility to the active site will also be a crucial factor in their efficacy as caspase inhibitors. Blocking the N- and C-termini of the Trp and Tyr residues enhanced their inhibitory effect, and the tetrapeptide peroxides were also effective inhibitors. However, peroxides generated on photolysed ovalbumin did not inhibit caspase activity. While many different proteins are substrates for the caspases, the hydrophobic Tyr and Trp residues on which the peroxide groups are likely to be present may be buried in ovalbumin and hence inaccessible for reaction. Peroxides that form on residues close to a caspase target sequence in a protein may however be extremely effective inhibitors. Whilst protein-bound peroxides have been

shown to be relatively poor inhibitors of caspase activity, proteolysis of peroxidized proteins (which does not result in the removal of peroxide groups) would be expected to release peptides with bound peroxide groups which may then induce caspase inhibition.

Whilst caspase activity was inhibited by both H_2O_2 and *N*-Ac-Trp-OMe peroxides, there were significant differences between these oxidants. Firstly, the *N*-Ac-Trp-OMe peroxide was 30 times more effective at inhibiting caspase activity than H_2O_2 . A major reason for the difference is likely to be the presence of catalase and peroxidases in the cell lysates that rapidly consume H_2O_2 . It is also possible that the Trp-derived peroxide reacts more rapidly with the caspases. Indeed, studies with purified GAPDH showed *N*-Ac-Trp-OMe peroxide to be a more effective inhibitor than H_2O_2 [5]. More detailed studies with recombinant caspases will be necessary to determine comparative reactivities. Secondly, the presence of substrate considerably enhances the ability of H_2O_2 to inhibit caspase activity [16], a phenomenon that has also been reported with the cysteine protease papain [23]. The mechanism is unclear, but the substrate may be enhancing the reactivity of the active site cysteine. In contrast, the substrate impaired the action of the *N*-Ac-Trp-OMe peroxide. This suggests that the larger peroxide was targeting the active site, but any enhanced reactivity due to the presence of substrate was negated by restricted access.

In summary, we have shown that caspases are sensitive to oxidation by low micromolar concentrations of Trp- and Tyr-derived peroxides. The caspases are central mediators of apoptosis, and therefore the formation of these potent inhibitory peptide and protein oxidation products within cells could have a significant impact on this pathway. Such peroxide mediated inhibition of the apoptotic pathways may contribute to the observed resistance of some UV-exposed cells to undergo programmed cell death [17]. Protein peroxides are able to damage DNA [9,10], and in combination with impaired apoptosis, these species may promote the accumulation of transformed cells.

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