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Hydrogen peroxide triggers the proteolytic cleavage and the inactivation of calcineurin

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Abstract

Increases in the levels of reactive oxygen species (ROS) are correlated with a decrease in calcineurin (CN) activity under oxidative or neuropathological conditions. However, the molecular mechanism underlying this ROS-mediated CN inactivation remains unclear. Here, we describe a mechanism for the inactivation of CN by hydrogen peroxide. The treatment of mouse primary cortical neuron cells with $A\beta_{1-42}$ peptide and hydrogen peroxide triggered the proteolytic cleavage of CN and decreased its enzymatic activity. In addition, hydrogen peroxide was found to cleave CN in different types of cells. Calcium influx was not involved in CN inactivation during hydrogen peroxide-mediated cleavage, but CN cleavage was partially blocked by chloroquine, indicating that an unidentified lysosomal protease is probably involved in its hydrogen peroxide-mediated cleavage. Treatment with hydrogen peroxide triggered CN cleavage at a specific sequence within its catalytic domain, and the cleaved form of CN had no enzymatic ability to dephosphorylate nuclear factor in activated T cells. Thus, our findings suggest a molecular mechanism by which hydrogen peroxide inactivates CN by proteolysis in ROS-related diseases.

Keywords: calcineurin, hydrogen peroxide, lysosomal protease, nuclear factor of activated T cells, reactive oxygen species.

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Calcineurin (protein phosphatase 2B), a calcium-dependent serine/threonine protein phosphatase, couples calcium/calmodulin signaling to a variety of cellular responses in immune, neuronal, and muscular cells (Aramburu *et al.* 2000; Crabtree 2001). CN is also known to be a pivotal component for the regulation of nuclear factor of activated T cell (NFAT) transcription factor, because of its ability to alter the phosphorylation states of NFAT during T-cell activation and to induce the apoptosis of immature T cells (Youn *et al.* 2000; Feske *et al.* 2003). Furthermore, the orchestration of CN-dependent NFAT activation appears to be intimately involved in heart valve development and in myocardial hypertrophy (Molkentin *et al.* 1998; de la Pompa *et al.* 1998; Ranger *et al.* 1998; Sussman *et al.* 1998; Meguro *et al.* 1999), axonal guidance (Chang *et al.* 1995), and in neuronal memory and learning (Mansuy *et al.* 1998; Winder *et al.* 1998). Despite the fact that calcium principally regulates the activity of CN, other molecules like complexes of immunosuppressive drugs (FK506, Cyclosporin A) with cognate partners (FK506 binding protein, cyclophilin), A-kinase-anchoring protein 79, cabin1/cain, calcineurin-homologous protein, and Down syndrome critical region protein 1/myocyte-enriched calcineurin-interacting protein 1 have been revealed to inhibit CN activity (Liu *et al.* 1991; Coghlan *et al.* 1995; Lin and Barber 1996; Lai *et al.* 1998; Sun *et al.* 1998; Fuentes *et al.* 2000; Rothermel *et al.* 2000).

Reactive oxygen species (ROS) have also been demonstrated to inhibit CN (Wang *et al.* 1996; Carballo *et al.* 1999; Furuke *et al.* 1999; Sommer *et al.* 2000). However, the manner in which ROS inactivates CN remains debatable.

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Abbreviations used: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid; CN, calcineurin; CNA, calcineurin A; DMEM, Dulbecco's modified Eagle's medium; HEK 293, human embryonic kidney 293; NAC, N-acetylcysteine; NFAT, nuclear factor of activated T cells; ROS, reactive oxygen species; WCLs, whole cell lysates.

(i) ROS may inhibit CN via the oxidation of the metal center of CN. It was initially suggested that active CN possesses a superoxide-sensitive Fe^{2+}-Zn^{2+} center. This was predicated based on the finding that Cu/Zn-superoxide dismutase protects CN activity (Wang *et al.* 1996). Later, however, the Ullrich group in a series of kinetic and spectroscopic experiments, uncovered evidence which indicated that CN does in fact possess a Fe^{2+}-Zn^{2+} center (Namgaladze *et al.* 2002). The Rusnak group, on the contrary, presented contradictory Electron Paramagnetic Resonance (EPR) spectroscopic data and concluded that active CN is characterized by a redox-insensitive Fe^{3+}-Zn^{2+} center, (Yu *et al.* 1995). This group later suggested that native CN *in vivo* might have a redox-sensitive Fe^{3+}-Fe^{2+} center, resembling that of purple acid phosphatase (Yu *et al.* 1997). (ii) ROS may also inhibit CN activity via the oxidation of cysteine residues near the active sites of CN (Bogumil *et al.* 2000). However, mutations of putative cysteine residues were determined to exert no relevant effect on the redox-sensitivity of CN (Reiter *et al.* 1999). Thus, the question as to which ROS inhibit CN at the intracellular level remains to be answered. It has been reported that superoxide can reduce CN activity *in vitro* in a more efficient manner than hydrogen peroxide (Namgaladze *et al.* 2002). Nonetheless, hydrogen peroxide has been reported to inhibit intracellular CN activity *in vivo* only (Reiter and Rusnak 2002).

Considering the findings of these previous works, we speculated that the hydrogen peroxide-mediated inactivation of CN at the intracellular level could differ form the *in vitro* process. Therefore, we attempted to determine the mechanism by which hydrogen peroxide inactivates the activity of CN at the intracellular level. In this study, we suggest a mechanism, by which hydrogen peroxide mediates the proteolytic cleavage of CN within its catalytic domain, and ultimately abrogates CN enzymatic activity.

Materials and methods

Cell culture

For primary cortical neuron cultures, embryos were surgically removed from ICR mice (E.15.5). Cortices were dissected and cultured as described previously (Koh *et al.* 1995). Human embryonic kidney 293 (HEK 293) cells and human neuroblastoma SK-N-SH cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, and 50 U/mL of streptomycin and penicillin.

Transfection and DNA constructs

SK-N-SH cells were transfected using Lipofectamine reagent (Life Technologies, Carlsbad, CA, USA), as described by the manufacturer. HEK 293 cells were transfected using the calcium phosphate method. To produce expression vectors containing full-length or truncated CNAβ2 mutants, PCR fragments were subcloned into pSG5-HA vector (Stratagene, La Jolla, CA, USA) or pcDNA-Flag (Clontech, Palo Alto, CA, USA). GFP-CNAβ2 was made by subcloning the full-length cDNA of CNAβ2 into pEGFP-C2 vector (Clontech). Point mutations were carried out using a QuickChange Site-Directed Mutagenesis kit (Stratagene). Detailed information concerning the DNA constructs used in this study is available on request.

Purification of recombinant $(His)_6$-calcineurin

Recombinant CN was purified as described previously (Mondragon *et al.* 1997). Briefly, pET15-CNα was transformed into *Escherichia coli* BL21(DE3), and the transformed *E. coli* was grown in LB broth and treated with 1 mmol/L IPTG to an OD600 of ~0.6. After 3 h of further growth, cells were harvested and sonicated. Cell lysates were precipitated with 45% (w/v) ammonium sulfate, and precipitated proteins were resuspended and dialyzed at 4°C overnight. CN was further purified by passing the dialyzed samples through TALON Metal Affinity Resin (Clontech) and then CaM-Sepharose (Amersham Biosciences, Uppsala, Sweden). CN purity was confirmed by Coomassie Blue staining and aliquots of purified CN were kept at −70°C until required.

Protease inhibitors and other chemicals

Cell-permeable protease inhibitors were pretreated with mouse primary cortical neuronal cells prior to H_2O_2 treatment. To observe the effects of inhibitors on CN cleavage *in vitro*, cells were lysed with a lysis buffer containing 20 mmol/L Tris-HCl (pH 6.0), 150 mmol/L NaCl, and 0.5% NP-40. Cell lysates were then centrifuged at 15 000 *g* for 5 min and supernatants were used as whole cell lysates. E-64d and chloroquine were purchased from Sigma (St Louis, MO, USA); calpain inhibitor III, caspase inhibitor I, BACE1 inhibitor (β-secretase inhibitor II), DAPT (γ-secretase inhibitor IX), PME (pepstatin A Methyl Ester), 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid (BAPTA)-AM, cathepsin inhibitor I, cathepsin G inhibitor, and MG132 from Calbiochem (San Diego, CA, USA). Protease inhibitor cocktail was from Roche (Mannheim, Germany); amyloid β-protein fragment 1-42 ($Aβ_{1-42}$) from Sigma and Bachem (Bubendorf, Switzerland), amyloid β-protein fragment 42-1 ($Aβ_{42-1}$) from Bachem, and N-acetylcysteine (NAC), catalase, cycloheximide, glutamate, hydrogen peroxide (30%), and ionomycin were from Sigma. DAPI was purchased from Calbiochem.

Calcineurin activity assays

Calcineurin activities in cell lysates were measured using CN assay kit (Calbiochem). Briefly, cells were lysed and immunoprecipitated with anti-CNA(C) or anti-HA monoclonal antibody. Reactions were started by adding immunoprecipitate to phospho-RII substrate-containing mixtures and terminated by adding malachite green. Released free phosphates were detected by measuring absorbance at 620 nm. For NFAT mobility-shifting assays, SK-N-SH cells were transiently transfected with mammalian expression vectors of HA-NFAT1 (1-460) along with various CNAβ2 mutants. Transfected cells were harvested and directly boiled in sodium dodecyl sulfate (SDS) sample buffer. Samples were then subjected to 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with anti-HA monoclonal antibody (Covance, Richmond, CA, USA).

Reporter gene assay

SK-N-SH cells were transfected with vectors containing NFAT-promoter-driven luciferase and various CNAβ2 mutants. Luciferase

activities were measured using a Tropix TR717 microplate luminometer.

Confocal microscopy

HEK 293 cells were transfected with mammalian expression vectors for GFP-fused NFAT4 (1-351) and various HA-tagged CNAβ2 mutants. Transfected cells were fixed with 3% (w/v) formaldehyde. Ectopically expressing CN mutants were immunostained with anti-HA-mAb followed by rhodamine-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Protein localization was observed by confocal microscopy (Model LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany).

Western blotting

Endogenous CN was detected using anti-CN monoclonal antibody recognizing the autoinhibitory domain (Pharmingen, San Diego, CA, USA). To detect CN fragment, cells were directly boiled in SDS sample buffer to avoid fragmentation during the preparation of cell lysates. Anti-CNA monoclonal antibody was purchased from Pharmingen, anti-$(His)_6$ monoclonal antibody from Amersham-Pharmacia, anti-HA monoclonal antibody from Covance, and anti-Flag(M2) and anti-β-actin antibodies from Sigma-Aldrich.

Statistics

Data are presented as mean ± SD and were analyzed by ANOVA. *p*-values of <0.05 were considered statistically significant.

Results

Hydrogen peroxide cleaved calcineurin and reduced its enzymatic activity

To determine the effects of hydrogen peroxide (H_2O_2) on residual CN activity at the intracellular level, we assessed the levels of its enzymatic activities using CN immunoprecipitated from the cell lysates of H_2O_2-treated primary cortical neurons. H_2O_2 (250 μmol/L) was found to reduce residual CN activity in a time-dependent manner (Fig. 1a left panel). Unexpectedly, we found that the amount of precipitated CN decreased gradually as cells were exposed to higher H_2O_2 concentrations, implying that H_2O_2 can affect CN protein content. Thus, we assessed the CN contents of cell lysates via western blotting using CN monoclonal antibody. As was expected, the exposure of primary cortical neurons to H_2O_2 reduced the amount of full-length CN, H_2O_2 mediated the initial cleavage of CN. The size of the C-terminal fragment produced, as detected using anti-CN monoclonal antibody recognizing an autoinhibitory domain of the CNA subunit, was *ca.* 28 kD (Fig. 1a right panel). $A\beta_{1\text{-}42}$ peptide is a primary constituent of the amyloid plaques found in the brains of Alzheimer's disease patients, and has been shown to aggregate and cause neuronal death via the generation of H_2O_2 (Yankner 1996). We thus treated mouse primary cortical neurons with either $A\beta_{1\text{-}42}$ or $A\beta_{42-1}$ peptide at the indicated concentration. Treatment with $A\beta_{1\text{-}42}$ was found to reduce CN activity and to cleave CN, in a manner similar to that exhibited by H_2O_2 (Fig. 1b). However, $A\beta_{42\text{-}1}$ did not affect CN activity or cleave CN. Moreover, H_2O_2 treatment resulted in CN cleavage in all cell lines tested (Fig. 1c).

We verified that the pretreatment of media with catalase blocked CN cleavage by H_2O_2, and protected CN activity (Fig. 2a). In addition, to confirm that H_2O_2 induces CN cleavage, we examined the effect of NAC, a cell-membrane permeable antioxidant. It has been reported that NAC enhances or protects the phosphatase activity of CN from cell lysates (or of purified CN) from oxidative stress (Furuke *et al.* 1999; Sommer *et al.* 2000), and in the present study, NAC pretreatment completely blocked CN cleavage by H_2O_2, and protected its enzymatic activity (Fig. 2b upper). Moreover, the effect of NAC on CN activity correlated with the pattern of CN cleavage (Fig. 2b lower).

Mapping of the hydrogen peroxide cleavage site in calcineurin

To confirm the pattern of CN cleavage by H_2O_2, we transiently transfected SK-N-SH cells with NH_2-terminally HA-tagged CNAβ2 or with COOH-terminally Flag-tagged CNAβ2. When H_2O_2 was treated with either of CNAβ2-transfected cells, each of antibodies (anti-HA and anti-Flag) mainly detected a single cleavage fragment (Fig. 3a). The molecular size of HA-tagged NH_2-terminal fragment of CN was *ca.* 33 kD, and that of the Flag-tagged COOH-terminal fragment was *ca.* 29 kD. Moreover, the sum of the molecular sizes of these fragments almost precisely matched that of full-length CN, indicating that H_2O_2 initially cleaved CN at one position.

To precisely identify the location of this cleavage site in CNA, we generated a series of COOH-terminally truncated CNAβ2 mutants, and compared these with the H_2O_2-cleaved CN fragment. The molecular size of CNAβ2 fragment by H_2O_2 treatment was found to be similar to that of the CNAβ2 (1-280) mutant (Fig. 3b). We then generated several alanine-scanned point mutants near the Asn280 residue (Fig. 3c). Both CNAβ2 (N282A) and CNAβ2 (L283A) appeared relatively resistant to H_2O_2-mediated cleavage, indicating that H_2O_2 targets a sequence between the N282 and L283 residues. In fact, like CNAβ2, CNAα was also cleaved by H_2O_2 (Fig. 3b), which is reliable on the basis that sequences are conserved throughout all the CN isoforms (Fig. 3d).

Calcineurin A possesses a variety of functional domains (Fig. 3d), which are profoundly conserved among CNA isoforms. The N282-L283 residues are located within the catalytic domain of CNA (Fig. 3d). In particular, this cleavage site is positioned upstream of His290, which is critical for zinc coordination at the active site. Based on the three-dimensional structure of CN, these N282-L283 residues are located at the end of the protruding α-helix10 (α10), which is accessible to proteases (Fig. 3e) (Griffith *et al.* 1995; Kissinger *et al.* 1995).

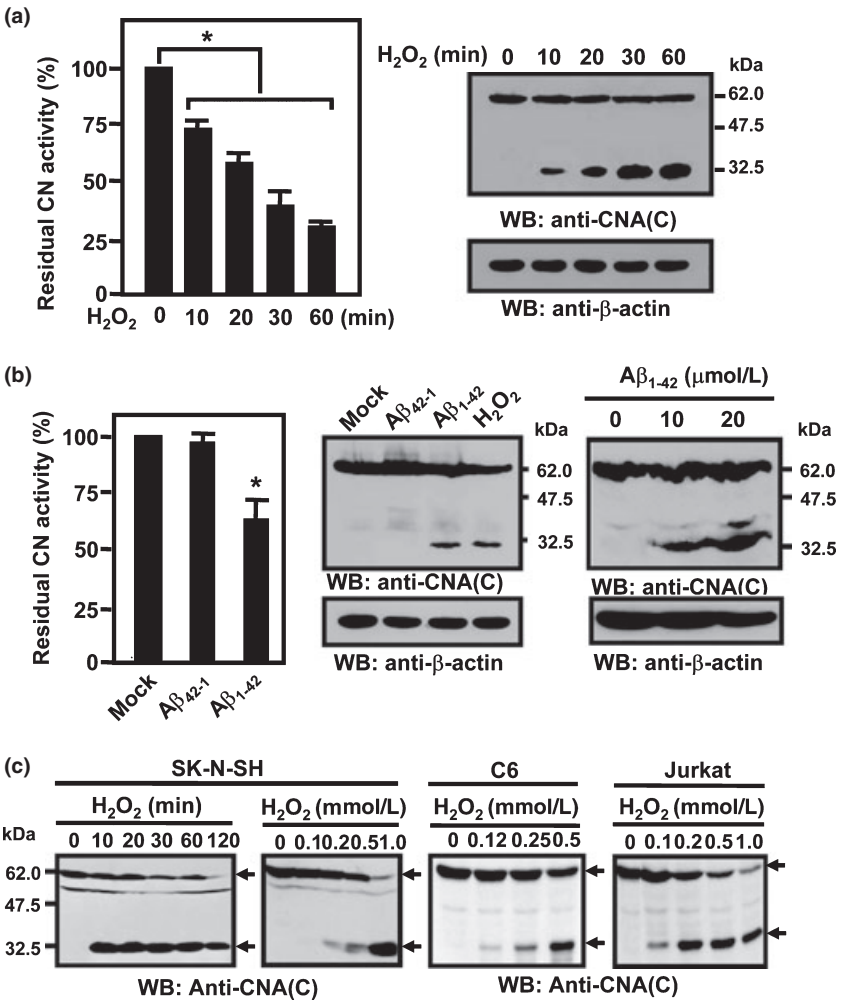

Fig. 1 Inactivation of calcineurin (CN) by H_2O_2-dependent cleavage. (a) H_2O_2 inactivates and cleaves CN *in vivo* in a time-dependent manner. Mouse primary cortical neurons were treated with 0.25 mmol/L H_2O_2 for a given time. Data are presented as mean ± SD ($n = 3$). *, $p < 0.05$ versus control. (left), CN activities were measured using a CN assay kit (Calbiochem). (right), CN was detected using an anti-CNA(C) monoclonal antibody (Pharmingen). (b) $A\beta_{1\text{-}42}$ peptide inactivated and cleaved CN. Mouse primary cortical neurons were treated with $A\beta_{1\text{-}42}$ [(left, middle) at 10 μmol/L, Bachem; (right) at 0–20 μmol/L, Sigma] or $A\beta_{42\text{-}1}$ (10 μmol/L, Bachem) peptides for 36 h at the indicated concentration. Mean ± SD ($n = 3$) are shown. *, $p < 0.05$ versus control. (left), CN activities were measured using CN assay kits (Calbiochem). (right), CN was detected using an anti-CNA(C) monoclonal antibody (Pharmingen). (c) Hydrogen peroxide induces the cleavage of CN in several cell lines. SK-N-SH human neuroblastoma, C6 rat glioma cells, and Jurkat human T lymphocytes were treated with H_2O_2.

Hydrogen peroxide mediates the proteolytic cleavage of calcineurin

To determine whether CN cleavage by H_2O_2 occurs directly or via a protease(s) altered by H_2O_2, we first purified recombinant hexa-histidine tagged recombinant $(His)_6$-CNAα (Fig. 4a). We then incubated purified $(His)_6$-CNAα with 1 mmol/L H_2O_2 and detected CN using anti-$(His)_6$ antibody (Fig. 4b). H_2O_2 treatment alone could not trigger CN cleavage, whereas the CNAα fragment was detected at the expected molecular size (*ca*. 32 kD) when purified $(His)_6$-CNAα was incubated with SK-N-SH cell lysates (Fig. 4b). In addition, pretreatment with increasing concentrations of a protease inhibitor cocktail eliminated CN cleavage in SK-N-SH cell lysates (Fig. 4c), indicating that CN cleavage by H_2O_2 treatment occurred via protease(s).

Calpain and caspases have been reported to cleave catalytically active CN and not the inactive form (Mukerjee *et al.* 2000; Wu *et al.* 2004; Burkard *et al.* 2005; Liu *et al.* 2005). Thus, we investigated whether inhibitors of both proteases block CN cleavage upon H_2O_2 treatment. However, these inhibitors did not block proteolytic cleavage to form inactive CN (Fig. 4d). A family of secretases has been reported to cleave Amyloid Precursor Protein (APP) in the Alzheimer's Disease (AD) brain (Sisodia and St George-Hyslop 2002), but when inhibitors of β- or γ-secretase were pretreated with primary cortical neurons, they failed to inhibit CN cleavage. We next examined whether MG132, a proteasome inhibitor, blocks CN cleavage by H_2O_2, and we found that pretreated MG132 retarded degradation of the CN fragment in a dose-dependent manner (Fig. 4e). However, it did not block the initial cleavage of CN by H_2O_2.

Lysosomal proteases have been reported to be highly activated in ROS-associated diseases like AD (Nixon *et al.* 2001). In fact, chloroquine (a lysosome inhibitor) pretreatment reduced CN cleavage (Fig. 4f). Cathepsin family members are major lysosomal proteases, and thus, cells lysates of mouse primary neurons were pretreated with cathepsin inhibitor I (a cysteine proteases inhibitor) and cathepsin G inhibitor I (a serine protease inhibitor), but neither blocked CN cleavage (Fig. 4g). Based on these

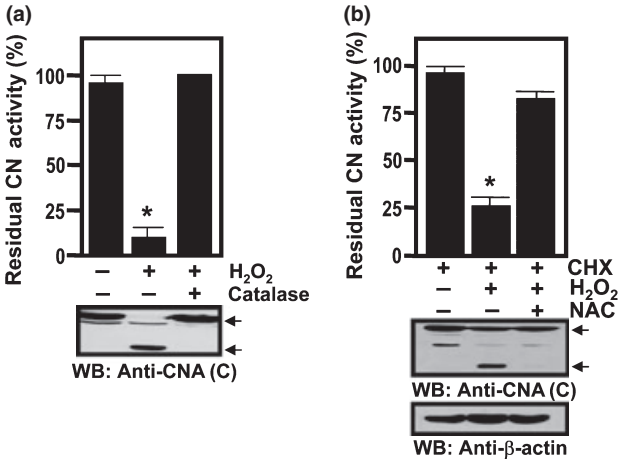

Fig. 2 Antioxidants blocked H_2O_2-mediated cleavage and the inactivation of calcineurin (CN). (a) Catalase protected CN from cleavage and activity loss by H_2O_2. Catalase (1000 U/mL) was pretreated for 30 min, and then exposed to 0.5 mmol/L H_2O_2 for 2 h. Mean ± SD ($n = 3$) are shown. *, $p < 0.05$ versus control. (b) N-acetylcysteine (NAC) protects CN for inactivation after H_2O_2 treatment. Mouse primary cortical neurons were pretreated with cycloheximide (50 μg/mL) for 2 h followed by N-acetylcysteine (NAC, 30 mmol/L) for 30 min, before being challenged with H_2O_2 (0.25 mmol/L, 30 min). Mean ± SD ($n = 3$) are shown. *, $p < 0.05$ versus control.

results, it appeared that H_2O_2 activates an unidentified lysosomal protease by either perturbing the integrity of the lysosomal membrane or by triggering other signaling pathways.

Hydrogen peroxide-mediated calcineurin cleavage occurs in a calcium-independent manner

Previous reports have shown that an increase in calcium influx activates either calpain or caspase, which in turn cleaves CN in the catalytically active form (Mukerjee *et al.* 2000; Wu *et al.* 2004). As H_2O_2 can transiently increase calcium influx within cells, we attempted to determine whether calcium also induces CN cleavage using a monoclonal antibody recognizing the autoinhibitory domain of CNA (Pharmingen). However, when primary cortical neurons were treated with increasing quantities of ionomycin or glutamate, these agents did not trigger CN cleavage to the inactive form (Figs 5a and b). Moreover, in this experiment, we were not able to detect the catalytically active form of CN as previously reported (Mukerjee *et al.* 2000; Wu *et al.* 2004). This discrepancy was probably due to the different sources of anti-CN antibodies. In addition, when primary cortical neurons were pretreated with BAPTA-AM (a cell-membrane permeable calcium chelator), BAPTA-AM failed

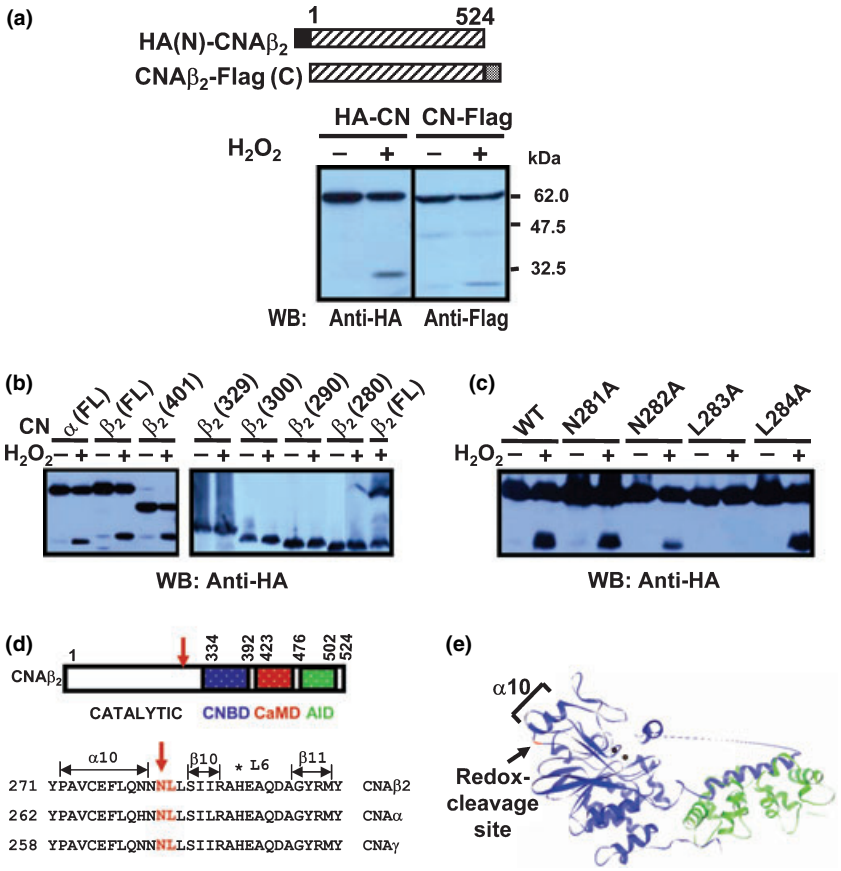

Fig. 3 Identification of the cleavage site in calcineurin (CN) by H_2O_2 treatment. (a) H_2O_2 initially cuts CN at a single point. SK-N-SH cells were transfected with either pSG-HA(N)-CNAβ2 or pcDNA-CNAβ2-Flag(C) and then treated with 0.5 mmol/L H_2O_2 for 30 min. (b) The cleavage site is located within the catalytic domain of CN. SK-N-SH cells were transiently transfected with several HA-tagged CN-deletion mutants, and then treated for 30 min with 0.5 mmol/L H_2O_2. (c) Asn282 and Leu283 in CNAβ2 are critical for H_2O_2-treated CN cleavage. SK-N-SH cells were transfected with several CN point mutants. Transfected cells were treated with 0.5 mmol/L H_2O_2. (d) The schematic structure and sequence of CN around the H_2O_2 cleavage site. His290 marked with asterisk (*) is coordinated with zinc at the active site of CN (Griffith *et al.* 1995; Kissinger *et al.* 1995). (e) Three-dimensional structure of CN (PDB entry, 1AUI) (Griffith *et al.* 1995; Kissinger *et al.* 1995). The H_2O_2 cleavage site in CN is located in the small loop between the α-10 helix and the β-10 sheet.

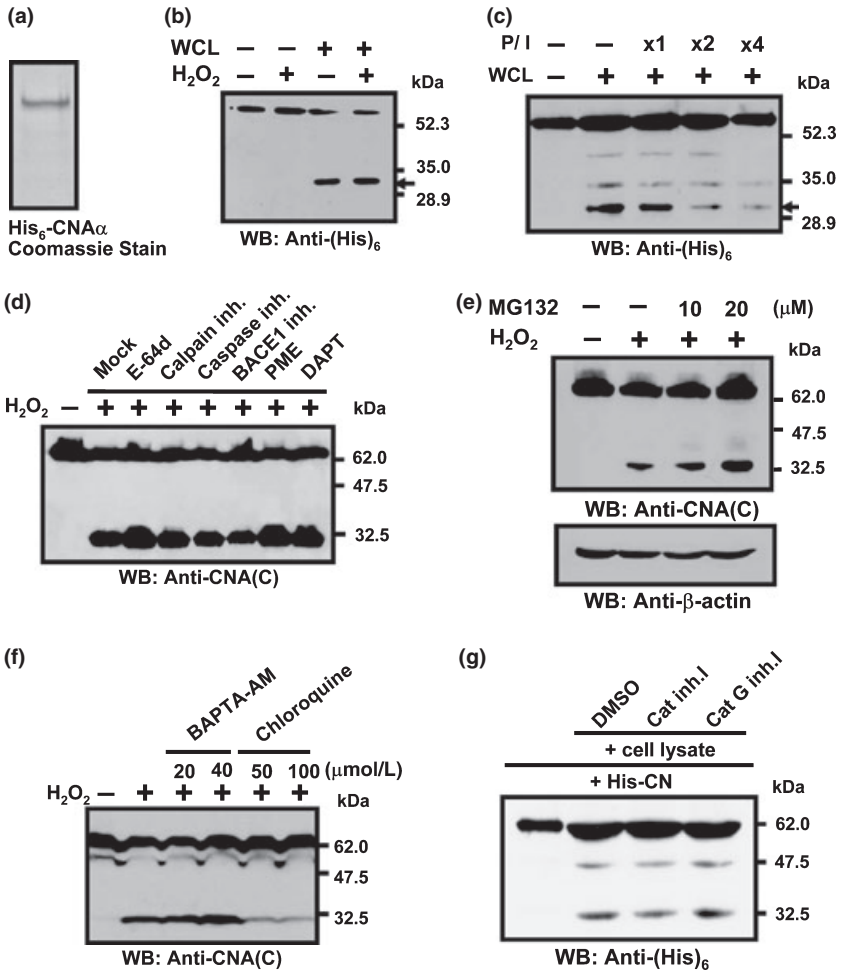

Fig. 4 H_2O_2 induces the cleavage of calcineurin (CN) via a proteolytic reaction. (a) Purification of recombinant CNα. CN purity was confirmed with Coomassie Blue staining. (b) H_2O_2 itself cannot cleave CN. Recombinant CNα (0.2 μg) was treated with either 1 mmol/L H_2O_2 or SK-N-SH whole cell lysates (WCLs) (15 μg). WCLs were prepared by lysing cells with buffer containing 20 mmol/L Tris-HCl (pH 6.0), 150 mmol/L NaCl, and 0.5% NP-40. Lysed cells were centrifuged at 15 000 *g* for 5 min and the supernatant obtained was used as WCL. After 5 min of incubation at 37°C, samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and probed with anti-$(His)_6$ antibody. (c) Gradual increases in protease inhibitor cocktail concentration progressively blocked CN cleavage by H_2O_2. Recombinant CNα (0.2 μg) was pre-incubated with increasing amounts of protease inhibitor cocktail (Roche) for 10 min, and further incubated with H_2O_2-treated SK-N-SH whole cell lysates (WCL) (15 μg) for 5 min at 37°C. (d) The effects of various protease inhibitors on CN cleavage. Mouse primary cortical neurons were pretreated with each protease inhibitor (100 μmol/L, 16 h), and then with 0.2 mmol/L H_2O_2 for 30 min. Cell lysates were subjected to 10% SDS-PAGE, and probed with anti-CNA(C) antibody recognizing the autoinhibitory domain of CN. (e) The effect of proteasome inhibitor on CN cleavage. Mouse primary cortical neurons were pretreated with the indicated concentrations of MG132 for 5 h, and then treated with 0.25 mmol/L H_2O_2 for 30 min. Cell lysates were subjected to 10% SDS-PAGE, and probed with anti-CNA(C) antibody recognizing its autoinhibitory domain. (f) Chloroquine blocked CN cleavage by H_2O_2. Mouse primary cortical neurons were pretreated with chloroquine (50, 100 μmol/L, for 1 h) or BAPTA-AM (20, 40 μmol/L, for 30 min) and then treated with 0.2 mmol/L H_2O_2 for 10 min. (g) The effect of cathepsin inhibitors on CN cleavage. Cell lysates of mouse primary cortical neurons (30 μg) were pretreated with 400 μmol/L of cathepsin inhibitor I (Cat inh.I) or 60 μmol/L of cathepsin G inhibitor I (Cat G inh.I) for 30 min, and then incubated with purified recombinant CNα (0.2 μg) for 10 min at 37°C. Samples were subjected to 10% SDS-PAGE, and probed with anti-$(His)_6$ antibody. WCL was prepared by lysing cells with a buffer containing 20 mmol/L Tris-HCl (pH 6.0), 150 mmol/L NaCl, and 0.5% NP-40. Lysed cells were centrifuged at 15 000 *g* for 5 min and supernatants were used as WCLs.

to block H_2O_2-mediated CN cleavage and to restore CN enzymatic activity. These findings indicate that H_2O_2 cleaves CN and inactivates its enzymatic activity in a calcium-independent fashion (Fig. 5c).

Hydrogen peroxide-induced cleaved form of calcineurin is catalytically inactive

To examine whether the cleaved form of CNAβ2 by H_2O_2 is active, we transfected cells with expression vectors contain-

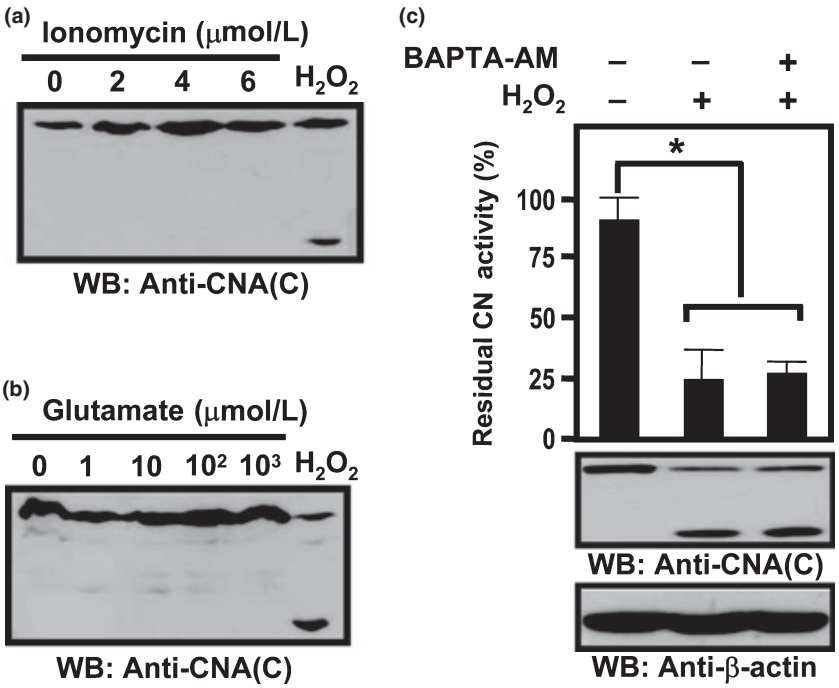

Fig. 5 Hydrogen peroxide induces the cleavage of calcineurin (CN) in a calcium-independent manner. (a) and (b), Calcium influx did not induce CN cleavage. Primary cortical neurons were treated with ionomycin or glutamate for 90 min. (c) The calcium chelator BAPTA-AM could not block H_2O_2-induced CN cleavage and its enzymatic activity. Primary cortical neurons were pre-treated with BAPTA-AM (30 μmol/L) for 30 min and then treated with 0.25 mmol/L H_2O_2.

ing GFP-fused NFAT and several HA-CNAβ2 mutant variants (Fig. 6a). We verified that full-length CN drives GFP-NFAT into the nucleus upon treatment with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and also that the overexpression of constitutively active CNAβ2 (1-401) is sufficient to trigger the complete nuclear translocation of GFP-NFAT4 and NFAT dephosphorylation, even in the absence of PMA and ionomycin (Figs 6a and b). Mutation at H160 in CNAβ2 has been reported to result in loss of CN activity (Shibasaki *et al.* 1996), and in the present study, CNAβ2 (1-401/H160Q) mutant did not translocate NFAT to the nucleus or dephosphorylate NFAT (Figs 6a and b). Like CNAβ2 (1-401/H160Q) mutant, CNAβ2 (1-280) mutant did not translocate or dephosphorylate NFAT, indicating that this CNAβ2 cleavage form is inactive (Figs 6a and b). Because CNAβ2 (1-401/L283A) mutant exhibited H_2O_2 resistance, it was expected to function as an H_2O_2-resistant, catalytically active species. However, it did not manifest enzymatic activity, suggesting that the α-10 helix is probably structurally important for the maintenance of CN activity.

To reconfirm these results, we examined NFAT-promoter-driven reporter activity (Fig. 6c). Neither CNAβ2 (1-280) nor CNAβ2 (1-401/L283A) activated NFAT transcription activity, like CNAβ2 (1-401/H160A) mutant. In addition, neither of these two species harbored residual enzymatic activity *in vivo* (Fig. 6d). To confirm that H_2O_2 reduced CN activity, we first expressed a constitutively active mutant CNAβ2 (1-401/WT) that drives NFAT into the nucleus. After 2 h of H_2O_2 treatment, we observed relocalization of NFAT from nucleus to cytosol, indicating that H_2O_2 inactivates CN at the intracellular level (Fig. 6e).

Discussion

Calcineurin activity has been shown to be sensitive to oxidative stress, and may also be modulated by intracellular redox potential (Furuke *et al.* 1993; Wang *et al.* 1996; Carballo *et al.* 1999; Sommer *et al.* 2000). Here, we propose a different mechanism for the inactivation of CN under oxidative conditions. In addition to the previously proposed mechanism that H_2O_2 oxidizes the metal center of CN, we found that H_2O_2 inactivates CN via the cleavage of a specific sequence within its catalytic domain. Moreover, this cleavage form of CN was found to be catalytically inactive. We also found that H_2O_2 inactivates CN at the intracellular level. However, because H_2O_2 is a strong oxidant and converts ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in metalloproteins, we cannot exclude the possibility that H_2O_2 modulates the metal center of CN at the intracellular level.

In this study, we observed that purified recombinant CN treated with H_2O_2 shows no protein cleavage, indicating that H_2O_2 itself does not have ability to cleave CN. Instead, incubation of purified CN with cell lysates, even from H_2O_2-non-treated cells, induced CN cleavage, indicating that an as-yet-unidentified protease is probably involved in CN cleavage under oxidative conditions.

H_2O_2 is known to activate cdk5 by inducing an increase in calcium influx (Kusakawa *et al.* 2000; Lee *et al.* 2000). Unlike the conversion of p35 to p25 by the H_2O_2-mediated influx of calcium, CN was not found to be significantly cleaved by calcium signaling in this study (Fig. 5). Actually, this appears to be feasible in view of the basic notion that CN is a well-known calcium/calmodulin-dependent phosphatase

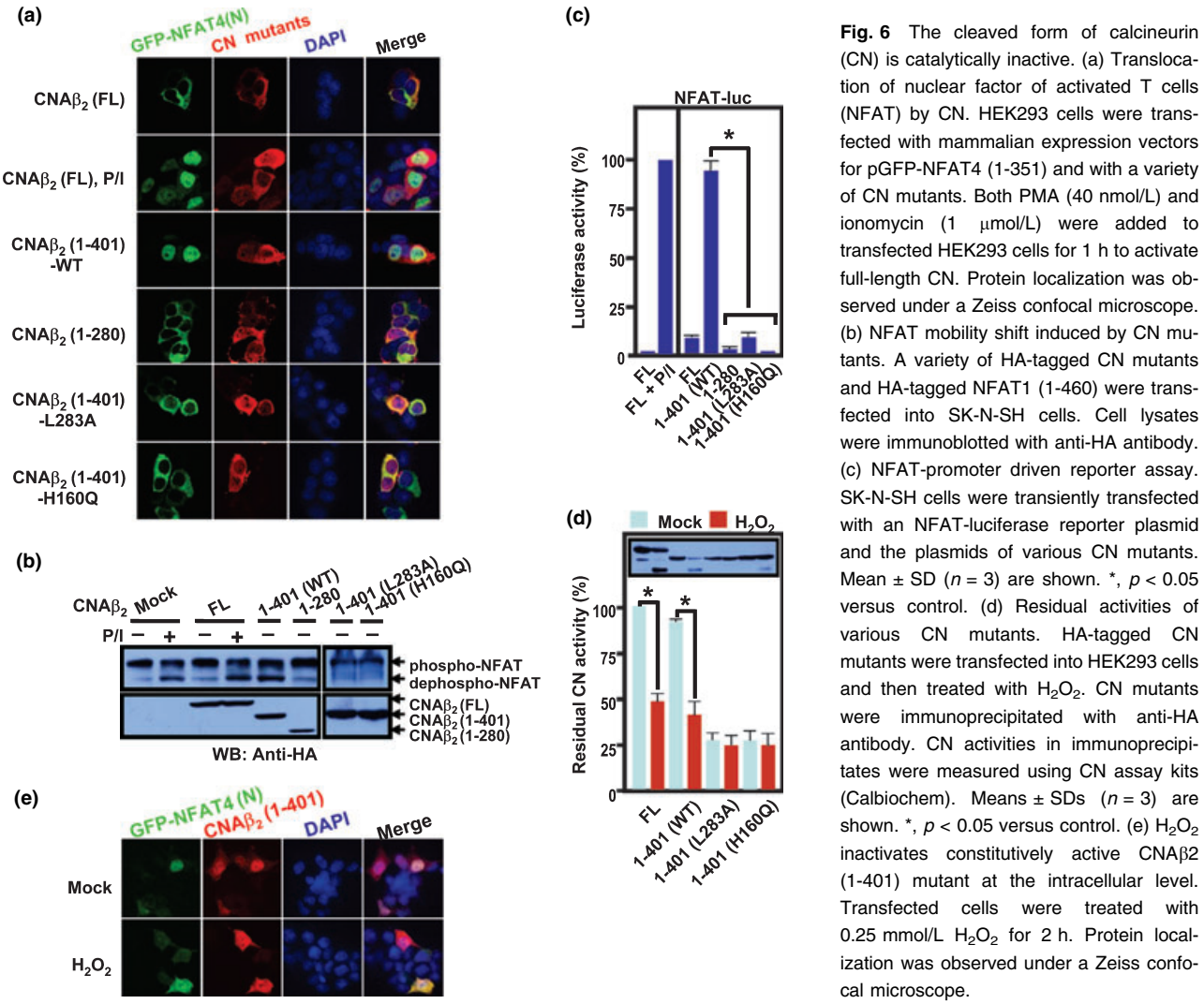

Fig. 6 The cleaved form of calcineurin (CN) is catalytically inactive. (a) Translocation of nuclear factor of activated T cells (NFAT) by CN. HEK293 cells were transfected with mammalian expression vectors for pGFP-NFAT4 (1-351) and with a variety of CN mutants. Both PMA (40 nmol/L) and ionomycin (1 μmol/L) were added to transfected HEK293 cells for 1 h to activate full-length CN. Protein localization was observed under a Zeiss confocal microscope. (b) NFAT mobility shift induced by CN mutants. A variety of HA-tagged CN mutants and HA-tagged NFAT1 (1-460) were transfected into SK-N-SH cells. Cell lysates were immunoblotted with anti-HA antibody. (c) NFAT-promoter driven reporter assay. SK-N-SH cells were transiently transfected with an NFAT-luciferase reporter plasmid and the plasmids of various CN mutants. Mean ± SD ($n = 3$) are shown. *, $p < 0.05$ versus control. (d) Residual activities of various CN mutants. HA-tagged CN mutants were transfected into HEK293 cells and then treated with H_2O_2. CN mutants were immunoprecipitated with anti-HA antibody. CN activities in immunoprecipitates were measured using CN assay kits (Calbiochem). Means ± SDs ($n = 3$) are shown. *, $p < 0.05$ versus control. (e) H_2O_2 inactivates constitutively active CNAβ2 (1-401) mutant at the intracellular level. Transfected cells were treated with 0.25 mmol/L H_2O_2 for 2 h. Protein localization was observed under a Zeiss confocal microscope.

in the calcium-activating signaling pathway (Aramburu *et al.* 2000; Crabtree 2001). In addition, calcium signaling activates CN via another mechanism, i.e., calpain activates CN by removing the COOH-terminal CaM-binding domain and the autoinhibitory domain from the catalytic domain (Wu *et al.* 2004; Burkard *et al.* 2005).

In this study, we could not detect any cleaved form of CN under calcium-activating (PMA and ionomycin, glutamate) conditions (Fig. 5). On the contrary, after H_2O_2 treatment, CN was detected in its cleaved inactive form, and this CN cleavage was not blocked by a calpain inhibitor or by a calcium blocker (Figs 4d and 5c).

It was unclear why we did not detect a catalytically active CN fragment resulting from calpain-mediated cleavage. This discrepancy may be due to different antibody specificities. In the present study, we used a CN antibody preferentially recognizing the inactive fragment of CN. Moreover, when both of ectopically expressed CN [HA(N)-CNAβ2 and CNAβ2-Flag(C)] in SK-N-SH cells were treated with H_2O_2, both anti-Flag and anti-HA monoclonal antibodies also preferentially recognized the inactive form of CN (Fig. 3a).

H_2O_2 can increase calcium influx into cells. However, H_2O_2 can affect cells in different ways, e.g., it can destabilize lysosomal membranes and cause leakage of lysosomal proteases into the cytosol. Therefore, H_2O_2 can affect CN activity in opposing ways, i.e., (i) by activating CN via calcium influx, or (ii) by inactivating CN by proteolytic cleavage, and it remains to be determined how these two contradictory phenomena are reconciled. Possibly, transient treatment with H_2O_2 increases CN activity by converting resting CN to catalytically active CN via a CaM-mediated conformational change or calpain-mediated cleavage. However, the long-term effect of H_2O_2 on CN activity may differ from short-term effect. Practically, when constitutively active CN mutant was incubated with H_2O_2, its activity was

reduced (Figs 6d and e), indicating that chronic H_2O_2 treatment preferentially inactivates CN.

Intracellular conditions in AD are likely to involve mixed chronic excitations because of calcium and ROS signaling and other signal types. In fact, many proteases are known to be activated in the AD brain (Brunk *et al.* 1995; Nixon *et al.* 2001; Sisodia and St George-Hyslop 2002). In the present study, we confirmed that β-secretase or γ-secretase cannot cleave CN, and that chloroquine inhibits H_2O_2-mediated CN cleavage, indicating that the protease involved in CN cleavage could be localized in lysosome. As it is known that ROS alters lysosomal proteins in Alzheimer's disease (Nixon *et al.* 2001) and that even non-lethal concentrations of H_2O_2 can destabilize the lysosomal membrane (Brunk *et al.* 1995), we speculate that H_2O_2 could cause the leakage of the protease(s) concerned from lysosome.

In this study, we also found that H_2O_2 initially cleaved CN at a specific sequence around 282Asn and 283Leu (Fig. 3), and we confirmed that the cleavage product of CN by H_2O_2 is catalytically inactive (Fig. 6). In particular, H_2O_2-treatment inactivated CN at the intracellular level (Fig. 6e). Thus, proteolytic cleavage appears to be the mechanism underlying the inactivation of CN under oxidative conditions. By protease inhibitor testing, we found that calpain, caspase, and cathepsins (proteases involved in CN cleavage) do not cleave CN to its inactive form. Only chloroquine was found to partially block the H_2O_2-mediated cleavage of CN, indicating that an as yet unidentified lysosomal protease directly cleaves CN to an inactivate form under oxidative conditions. Our findings may provide a novel insight into the role of CN inactivation in pathogenic mechanism of ROS-related diseases.

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