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Specific inactivation of cysteine protease-type cathepsin by singlet oxygen generated from naphthalene endoperoxides

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Abstract

Singlet oxygen is a causal factor in light-induced skin photoaging and the cytotoxic process of tumor cells in photodynamic chemotherapy. To develop a better understanding of the functional consequences of protein modification by singlet oxygen, the effects of naphthalene endoperoxide on lysosomal protease, cathepsin, were examined. When the soluble fraction of normal human fetal skin fibroblast cells was treated with the endoperoxide, the activities of cysteine proteases, cathepsins B and L/S, were inhibited, but that of aspartate protease, cathepsin D/E, was not. The reduction of the endoperoxide-treated soluble fractions by treatment with dithiothreitol barely recovered the activities. Cathepsin B, purified from normal human liver, exhibited similar profiles to that in cytosol. These data suggest that singlet oxygen oxidatively modifies an amino acid residue essential for catalysis and consequently results in the irreversible inactivation of cysteine protease-type cathepsin.

Keywords: Cathepsin; Cysteine protease; Singlet oxygen; Endoperoxide

Reactive oxygen species (ROS) are produced under physiologic conditions. An enhanced production of ROS occurs during a variety of biological processes, such as inflammation and ultraviolet irradiation, and results in the dysfunction of cells and aging [1]. Singlet oxygen is assumed to cause skin photoaging [2] and the cytotoxic process of tumor cells during photodynamic chemotherapy [3]. Singlet oxygen is not a radical but is, nevertheless, highly reactive with respect to certain biological components. Compounds containing conjugated double bonds, such as unsaturated fatty acids, appear to be preferred targets.

A photochemical reaction in the presence of a photosensitizer produces singlet oxygen and is often used as a singlet oxygen-generating system. However, other ROS are also produced in such photochemical reactions. It is, thus, not an ideal system for elucidating the function of singlet oxygen. On the other hand, the use of endoperoxides that generate pure singlet oxygen is beneficial from this point of view [4,5].

Cell death induced by singlet oxygen in T cells [6] and HL-60 cells [7] shows the characteristic profiles of apoptosis. During this process, the cleavage of Bid occurs and cytochrome *c* is released from mitochondria. Singlet oxygen and UV mediate the mitogenic signals by activating mitogen-activated protein kinases, p38, JNK, and ERK [5,8,9]. However, the molecular basis for the reaction of singlet oxygen with amino acids in proteins is poorly understood [10,11].

We have recently shown that singlet oxygen induces a cell death that is atypical to apoptosis, despite the

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release of cytochrome *c* from mitochondria into the cytosol [12]. This is due to the suppression of the caspase cascade prior to apoptosome formation and the direct inhibition of caspase activity by singlet oxygen. Since caspases have reactive cysteine residues at the catalytic center, the modification of an essential sulfhydryl may be involved in the latter reaction.

Cathepsins are lysosomal acidic proteases that hydrolyze proteins within cells and those incorporated by endocytosis and are also involved in antigen presentation during immunological reactions. Cathepsin consists of three groups: papain-like cysteine proteases, aspartate proteases, and serine proteases. Cathepsins B and L, which belong to the first group, are abundantly found in many mammalian tissues and contain a reactive sulfhydryl residue at the catalytic center [13]. On the other hand, cathepsins D and E constitute the major component of aspartate proteases in lysosomes [14]. Cathepsins are also involved in apoptosis in certain type of cell death [15–18]. In response to apoptotic stimuli, cathepsins are released from the lysosomes into the cytosol and mediate apoptotic signaling [19].

Here we report that singlet oxygen inhibits the cysteine protease cathepsin B and L/S activities, but not the aspartate protease cathepsins D/E. Inactivation of these enzymes would result in the dysfunction of intracellular protein metabolism.

Materials and methods

Materials. Peptide substrates for cathepsins were purchased from the Peptide Institute. Cathepsin B, purified from normal human liver, was obtained from Calbiochem. SNAP was obtained from Dojindo. Hypoxanthine was obtained from Sigma and xanthine oxidase was from Roche. Biotin-conjugated iodoacetamide (BIAM) was obtained from Molecular Probes. An Oxyblot kit was obtained from Intergen.

Synthesis of endoperoxides. 1-Methylnaphthalene-4-propionate endoperoxide (MNPE) and naphthalene-1,4-dipropionate endoperoxide (NDPE) were synthesized as described previously [20]. The structure and purity of the endoperoxides were determined by NMR. The half-lives of MNPE and NDPE were about 25 and 27 min at 37 °C, respectively. The generation of singlet oxygen from endoperoxides was detected by electron spin resonance (ESR) using DRD156 as a sensitive singlet oxygen-detecting probe, which specifically reacts with singlet oxygen among the ROS [21].

Cell culture. A normal human fetal skin fibroblast line HFSKF-II, originally established by Drs. Isamu Ishiwata and Hiroshi Ishikawa, was obtained through the Riken BRC (Tsukuba, Japan) and maintained in HamF-12 (Gibco) containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 15% fetal bovine serum (FBS; Invitrogen). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Preparation of soluble fractions. Cells in culture dishes were washed with PBS and collected by centrifugation at 3000*g* for 5 min. After suspending the cells in extraction buffer [50 mM sodium acetate buffer, pH 5.5, 200 mM NaCl, 0.1% Triton X-100, and 1 mM dithiothreitol (DTT)], they were disrupted by sonication and centrifuged at 11,000*g* for 15 min. The supernatant was comprised the soluble fractions. Protein concentrations were determined using the BCA protein assay reagent (Pierce).

Cathepsin assay. Either soluble proteins or purified human cathepsin B, diluted in the extraction buffer, was used as an enzyme source for this assay. Cathepsin activity was assayed using the following substrates for each enzyme at acidic pH. Under these conditions, cathepsin activity was measured specifically. Cathepsin B: benzyloxycarbonyl-L-arginyl-L-arginine-4-methyl-coumaryl-7-amide in 66 mM KH_2PO_4, 9 mM Na_2HPO_4, 1 mM EDTA, and 2 mM cysteine. Cathepsin L/S: benzyloxycarbonyl-L-valyl-L-valyl-L-arginine-4-methyl-coumaryl-7-amide in 100 mM sodium acetate, pH 5.5, 1 mM EDTA, and 2 mM DTT. Cathepsin D/E: (7-methoxycoumarin-4-yl)acetyl-glycyl-L-lysyl-L-prolyl-L-isoleucyl-L-leucyl-L-phenylalanyl-L-phenylalanyl-L-arginyl-L-leucyl-N^{ε}-(2,4-dinitrophenyl)-L-lysyl-D-arginine amide in 9 mM sodium acetate, 41 mM acetic acid, and 100 mM NaCl. For cathepsin B and L/S activities, the initial rates of enzymatic hydrolysis were determined by measuring the release of 4-methyl-coumaryl-7-amide from the substrate at the emission at 460 nm upon excitation at 380 nm using a BioLumin 960 Fluorometer (Molecular Dynamics, Tokyo, Japan) equipped with a thermostated plate reader. For cathepsin D/E activity, after stopping the reaction by adding TCA to 5%, increases in the fluorescence at the emission at 393 nm upon excitation at 328 nm were measured using FP-6300 spectrofluorometer (Nihon-bunko, Tokyo, Japan).

Assay for lipid peroxidation. Thiobarbituric acid (TBA)-reactive substances (TBARS) were measured as described previously [22]. For each measurement, 1×10^7 cells were collected and washed twice with PBS. After resuspending the cell pellet in 0.3 ml of PBS, a 10 μl aliquot of the suspension was retained for use in a protein determination. The cell suspension was combined with 0.6 ml of a reagent containing 15% TCA, 0.375%(w/v) TBA, 0.25 M HCl, and 1.8 mM butylhydroxytoluene, and mixed thoroughly. The solution was heated for 15 min in boiling water, cooled in ice-cold water, and centrifuged at 10,000*g* for 10 min. The absorbance of the sample was measured at 535 nm. TBARS levels were calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Evaluation of the viability of cells by assaying lactate dehydrogenase activity. Lactate dehydrogenase (LDH) activity was measured to assess the sensitivity of the cells to singlet oxygen [23]. Twenty-four hours after treatment of cells in 24-well plates, portions of the medium were collected for measurement of LDH activity. The cells were collected and disrupted by brief sonication in PBS containing 0.1% Tween 20. Cellular extracts, free of debris, were prepared by centrifugation at 11,000*g* for 15 min. The assay for LDH activity was performed using an LDH CII kit (Wako, Osaka, Japan). The viability of the cells was calculated as the percentage of LDH activity recovered in the cellular extract against the total (cellar extract plus medium) recovered activity.

Immunocytochemistry for 4-hydroxy-2-nonenal. 1×10^3 of HFSKF-II cells were plated into an eight-well chamber slide (Nalge Nunc). Four days later, the cells were treated with 0.5 mM MNPE for 1 h, fixed in 3.7% formaldehyde solution for 10 min, and then treated with 0.1% Triton X-100 for 4 min. The cells were treated with 1% BSA for 30 min and then incubated overnight with mouse anti-4-hydroxy-2-nonenal (HNE) monoclonal antibody (HNEJ-2 at a concentration of 25 μg/ml, Nikken Foods, Shizuoka, Japan) at 4 °C. The MAX-PO complex which consists of an amino acid polymer conjugated with the Fab′ portion of a secondary antibody and peroxidase (Histofine mouse stain kit, Nichirei, Tokyo, Japan) was placed on the cells for 10 min. Specific immunolabeling was examined using 3,3′-diaminobenzidine (Nichirei, Tokyo, Japan) as the chromogen, which was placed on the cells for a few minutes. Photographs were taken with a digital camera under a light microscope (Olympus BX50, Tokyo, Japan).

SDS–PAGE and blot analysis. Protein samples were subjected to 12% SDS–PAGE and then transferred to a Hybond-P membrane (Amersham Pharmacia) under semi-dry conditions using a Transfer-blot SD Semi-dry transfer cell (Bio-Rad). The membrane was then blocked by incubation with 5% skimmed milk in TBS (150 mM NaCl and 20 mM Tris/HCl, pH 7.6) for 2 h at room temperature. The membranes were then incubated with a rabbit anti-human cathepsin B antibody (1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, USA) for 16 h at 4 °C.

After washing with TBS containing 0.1% Tween 20, the membrane was incubated with 1:1000 diluted peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Detection of protein carbonyls was performed by an Oxyblot kit (Intergen) according to the manufacturer's instruction. After washing, the peroxidase activity on the membranes was detected by a chemiluminescence method using an ECL Plus kit (Amersham Pharmacia Biotech) and exposed to X-ray films (Kodak, Rochester, USA).

Detection of sulfhydryl group in proteins by BIAM. After incubation with 1 mM MNPE at 37 °C for 1 h, purified human cathepsin B (0.46 μg) was incubated with 20 μM BIAM in 100 mM NaCl and 50 mM Mes, pH 6.5, for 30 min at 25 °C. The labeling reaction was terminated by the addition of DTT to a final concentration of 4 mM. A portion of the labeled cathepsin B was subjected to SDS–PAGE and transferred to a Hybond-P membrane. Proteins labeled with BIAM were detected by treatment with HRP-conjugated streptavidin followed by an ECL plus kit.

Statistics. Data are presented as means ± SD of triplicate experiments. Student's *t* test was used to compare the significance of differences between the data. A value of $P < 0.05$ was considered significant.

Results

Effects of MNPE on cells

Although photosensitization reactions are frequently used as a singlet oxygen-generating system, the reaction also produces other ROS and results in complex oxidative modification. To evaluate the effects of only singlet oxygen in reactions with cellular components, we synthesized and used naphthalene endoperoxides, compounds that exclusively generate singlet oxygen [20]. A normal human fibroblast-derived line, HFSKF-II, was used because many cancer cell lines are resistant to photodynamic therapy and may interfere in an evaluation of the action of singlet oxygen. To evaluate the efficacy of the inhibition in cells, the dose-dependent effects of MNPE on cathepsin activities in cultivated cells were examined (Fig. 1A). MNPE inhibited cathepsin B and L/S, but had no substantial effect on cathepsin D/E, activities, under the culture conditions employed herein. When cellular viability was measured 24 h after MNPE treatment, the dose-dependent damage corresponded to the inhibition of cathepsins (Fig. 1B).

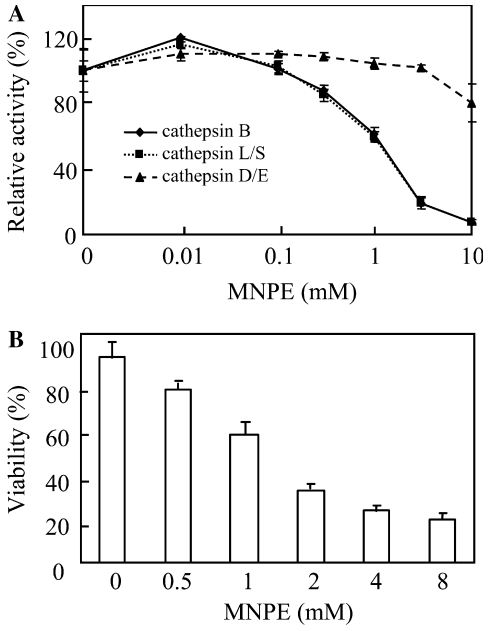

Fig. 1. Dose-dependent effects of MNPE on cultured HFSKF-II cells. HFSKF-II cells were incubated with various concentrations of MNPE for 2 h. Cathepsin activities were measured in soluble fractions prepared from the cells (A). At 22 h after changing to fresh media, cell viability was assessed by measuring LDH activity (B). The means ± SD of triplicate assays are shown.

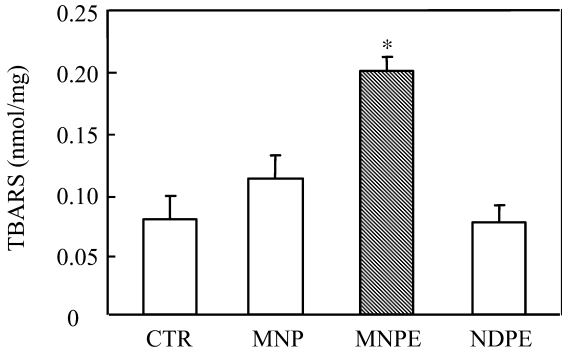

Fig. 2. TBARS levels in cells treated with MNPE, NDPE, or MNP. HFSKF-II cells were incubated with 2 mM MNPE, NDPE, or MNP for 2 h. Thiobarbituric acid-reactive substances (TBARS) in a whole cell extract were measured. CTR: control. The means ± SD of triplicate assays are shown. *$P < 0.005$ versus control.

Effect of singlet oxygen on lipid peroxidation in cells

Two endoperoxides MNPE and NDPE were used in a previous study, which showed that only MNPE effectively induced cell damage [12]. Since singlet oxygen preferentially peroxidizes conjugated double bond in lipids, we determined the levels of TBARS in cells that had been treated with NDPE, MNPE, or MNP (Fig. 2). The results show that only MNPE significantly peroxidized lipids in cells, which supports the view concerning the membrane permeability of MNPE.

We, then, attempted to detect protein adducts with HNE, a lipid peroxidation marker in the cells. Some intracellular components were positively stained with this antibody even under control conditions. The detection of HNE-modified proteins by an immunocytological method showed an enhanced staining in the cytoplasm, strongly at perinuclear region, of MNPE-treated cells (Fig. 3), although detailed analysis is required to define them. Thus, the increase in HNE-modified proteins inside the cells indicates that MNPE actually entered the cells and produced singlet oxygen, resulting in lipid peroxidation. The data also supported the view that contribution of singlet oxygen generated outside a cell to cytotoxicity was minimal.

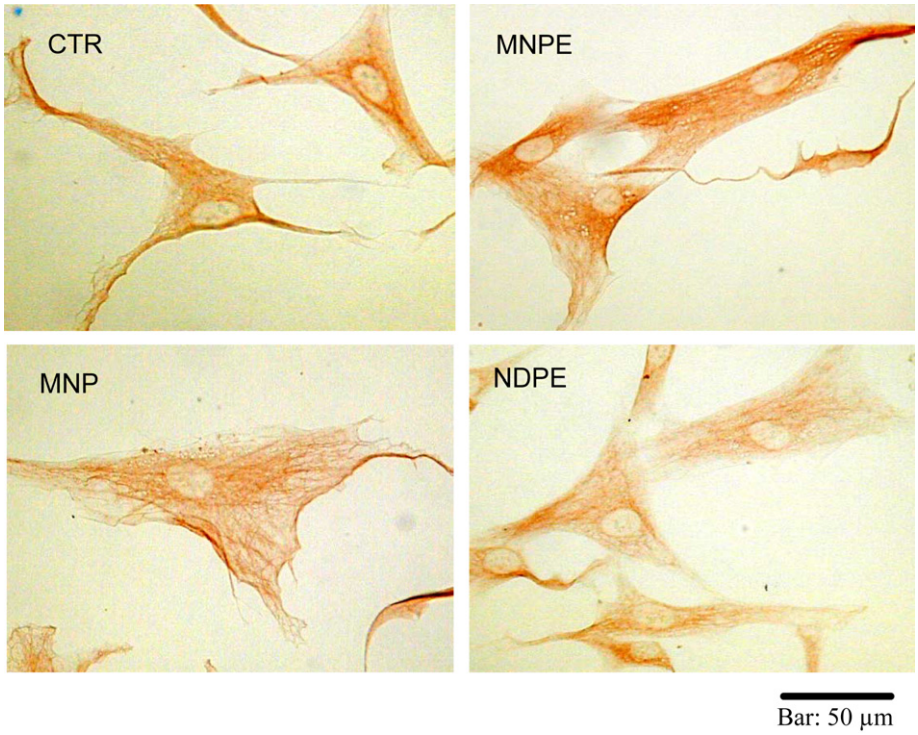

Fig. 3. Immunocytochemistry for HNE on MNPE-treated HFSKF-II cells. HFSKF-II cells were incubated with 0.5 mM MNPE, NDPE, MNP, or CTR (solvent: 1 mM NaOH) for 1 h.

Inhibition of cathepsin activity in soluble fraction by endoperoxides

When the soluble fraction of the cells was incubated with various concentrations of MNPE for 1 h, cathepsin B and L/S activities were inhibited in a dose-dependent manner by MNPE to a similar extent, while cathepsin D/E activities remained unaffected under these conditions (Fig. 4A). The time course of the inhibition was consistent with the generation of singlet oxygen from MNPE whose half-life is about 25 min under these conditions (Fig. 4B). Since cathepsins B, L, and S are cysteine proteases, but cathepsins D and E are aspartate proteases, this suggests that singlet oxygen specifically inhibited cathepsin in the cysteine protease. The effective concentrations of MNPE on cultured cells were much higher than those on the soluble fraction, with a half-maximal inhibition of 1 mM and 10 μM for cells and cytosol, respectively. The difference in values can be partly explained by the amount of MNPE that actually entered the cells.

Comparison of inhibitory effects of singlet oxygen with other ROS and reactive nitrogen oxide species

The sulfhydryl group at the catalytic center of cysteine proteases are highly reactive and, hence, are targets of various oxidative modifications by ROS and reactive nitrogen oxide species (RNOS). We compared the effects

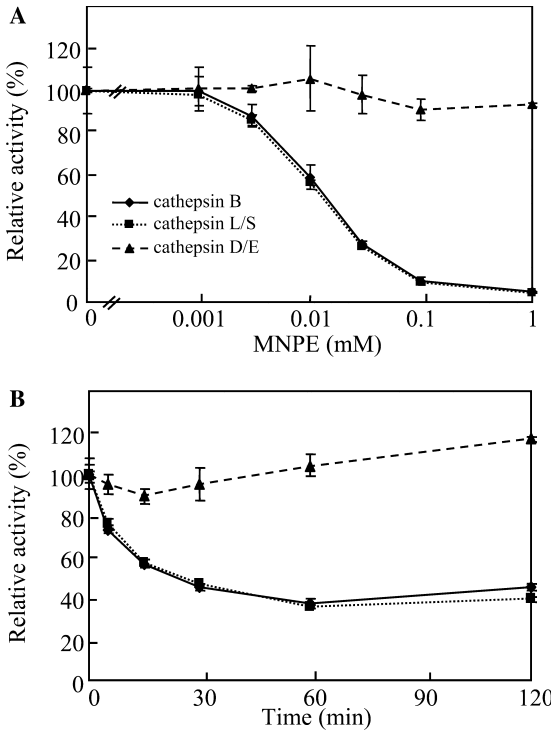

Fig. 4. Inhibition of cathepsin B and L/S activities by MNPE. The soluble fraction was prepared from HFSKF-II cells and incubated with varying concentrations of MNPE for 1 h (A) or with 0.03 mM MNPE for various periods at 37 °C (B). Activities of cathepsin B, L/S, and D/E were measured using specific synthetic, fluorescent substrates. The means ± SD of triplicate assays are shown.

of SNAP, hydrogen peroxide, and hypoxanthine/xanthine oxidase, as well as MNPE, on cathepsin activity (Fig. 5). The concentrations of all compounds were equivalent to 0.1 mM ROS/RNOS. Among the ROS and RNOS examined, MNPE inhibited cathepsin B and L/S activities the most strongly under these conditions.

Characterization of cathepsin inhibition by singlet oxygen

We further characterized the modification of cathepsins by singlet oxygen in two ways. If Cys is the target of singlet oxygen, the presence of large excess of thiol compounds would be expected to protect a cathepsin from inactivation. The results show that Cys was the most effective in protecting the enzymes from inactivation by singlet oxygen (Fig. 6). Although His and azide are well-known quenching agents of singlet oxygen, their protective effects were less than those of Cys under these conditions.

To investigate the reversibility of the inhibition, the effects of reduction by DTT on the cathepsin activities of singlet oxygen-treated cell extracts were examined (Fig. 7). The activity of cathepsins B and L/S that had been inactivated by SNAP treatment was partially recovered by treatment with 5 mM DTT. However, DTT failed to recover the activity of cathepsins B and L/S that had been treated with MNPE. Thus, the inac-

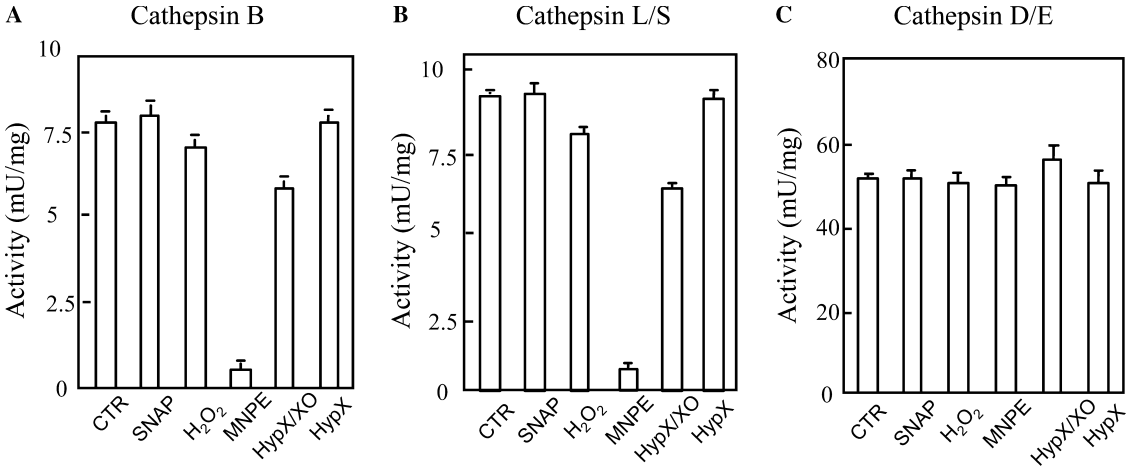

Fig. 5. Comparison of the effects of selected oxidants on cathepsin activities. Soluble fractions were incubated without (CTR) or with 0.1 mM SNAP, 0.1 mM hydrogen peroxide (H_2O_2), 0.1 mM MNPE, 0.05 mM hypoxanthine/xanthine oxidase (HypX/XO), or 0.05 mM hypoxanthine (HypX) for 1 h. Cathepsin B (A), L/S (B), and D/E (C) activities in the oxidant-treated soluble fractions were then measured. The means ± SD of triplicate assays are shown.

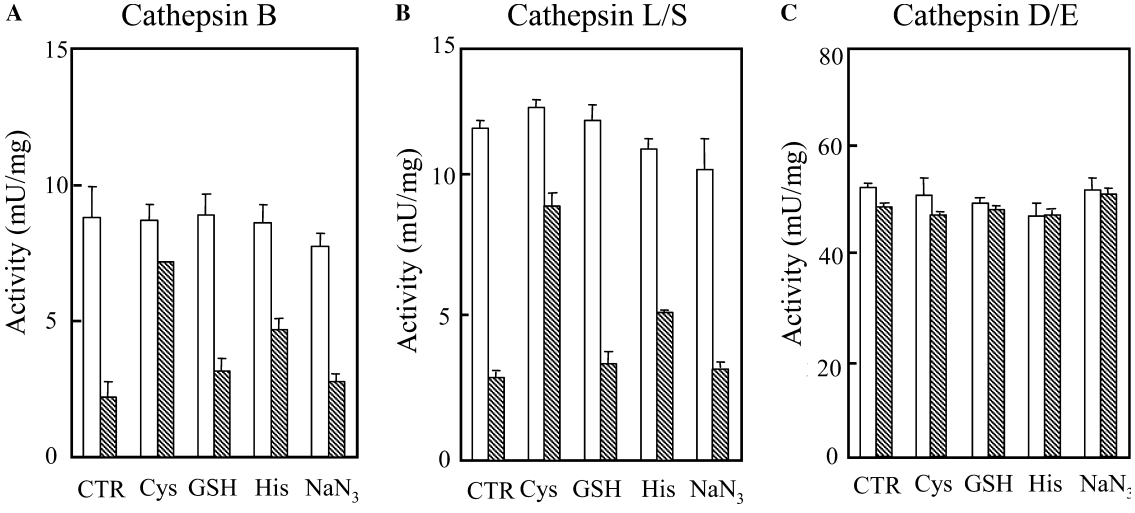

Fig. 6. Comparison of the effects of singlet oxygen-quenching agents on MNPE-induced cathepsin inhibition. Soluble fractions were incubated without (open columns) or with (striped columns) 0.03 mM MNPE for 1 h. During the incubation, 10 mM of Cys, GSH, His, or NaN_3 was included. Cathepsin B (A), L/S (B), and D/E (C) activities in the treated soluble fractions were then measured. The means ± SD of triplicate assays are shown.

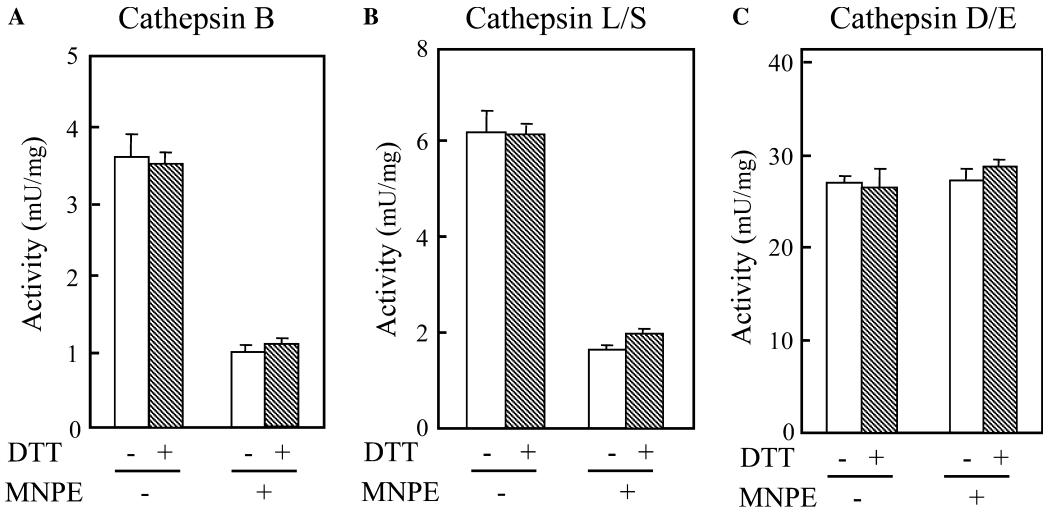

Fig. 7. Effects of reduction with DTT on MNPE-inhibited cathepsin activities. After treatment of soluble fractions with or without MNPE at 37 °C for 1 h, they were further incubated with or without 5 mM DTT at 37 °C for another 1 h. Then, cathepsin B (A), L/S (B), and D/E (C) activities in the treated soluble fractions were then measured. The means ± SD of triplicate assays are shown.

tivation of enzymes by oxidative modification by singlet oxygen generated from MNPE was essentially irreversible.

Effects of singlet oxygen on purified cathepsin B

We also examined the effects of MNPE on cathepsin B purified from normal human liver. The dose–response inhibition by MNPE and the time course for the inhibition roughly corresponded to those for the soluble fraction (Figs. 8A and B). The effects of oxidants, including MNPE and following reduction by DTT, on the activity were also similar to those for soluble proteins (Fig. 8C). Since the data regarding the inhibitory action of MNPE on purified cathepsin B were quite similar to the values obtained, when the soluble fraction was used, we attribute the above observations to the direct action of singlet oxygen on the cathepsins.

We then tried to characterize modification of cathepsin B (Fig. 8D). Cathepsin B is present in two forms: a single-band form with M_r 29 kDa and two-band form with M_r 25 and 5.5 kDa. Both forms have the same enzymatic activity, and catalytic Cys-29 is located at the 29 and 5.5-kDa polypeptides [24]. An immunoblot analysis of the purified cathepsin B indicated that MNPE treatment did not cause massive structural change, such as aggregation or cleavage, to both forms of cathepsin B, although the 5.5-kDa band ran at dye front and was not detectable. The reactivity of sulfhydryl residues in cathepsin B was examined via the use of BIAM, which reacts to a sulfhydryl with a low pK_a at pH 6.5, but to most sulfhydryls at pH 8.5 [25]. BIAM bound to sulfhydryls only in the 29-kDa form at pH 6.5, but no difference was observed between MNPE-treated and -untreated cathepsin B. Since 29-kDa band was the only form that was labeled with BIAM, the bound Cys residue appeared to be the one constituting the catalytic center. We also used an Oxyblot kit to detect carbonyls in cathepsin B. Both in MNPE-treated and -untreated cathepsin B showed about the same band intensity, suggesting that even slight modification by singlet oxygen affected enzymatic activity of cathepsin B.

Discussion

Although the cytotoxic and signaling effects of singlet oxygen have been investigated in cells, oxidative modification of responsible proteins is largely unknown [4,26]. The findings herein, using naphthalene endoperoxides as a singlet oxygen donor, show that cysteine proteases, cathepsins B and L/S, are preferential targets of singlet oxygen in cells. The detrimental effects of singlet oxygen were confirmed using purified human cathepsin B.

When we examined the photochemical reaction using Rose Bengal or methylene blue as a photosensitizer, protein damage including aggregation was observed, while MNPE showed a much lower level of side effects (data not shown). However, involvement of other ROS than singlet oxygen is not eliminated in the photochemical reaction. MNPE generates singlet oxygen exclusively among ROS as judged by NMR and ESR studies [21]. Thus, the use of an endoperoxide was found to be adequate for investigating the reactions of singlet oxygen in a biological system.

Even though the two naphthalene endoperoxides, MNPE and NDPE, produce singlet oxygen with similar half-lives, their cytotoxic effects were quite different [12]. Since the hydrophobic MNPE was cytotoxic, but the less hydrophobic NDPE was not, we hypothesize that

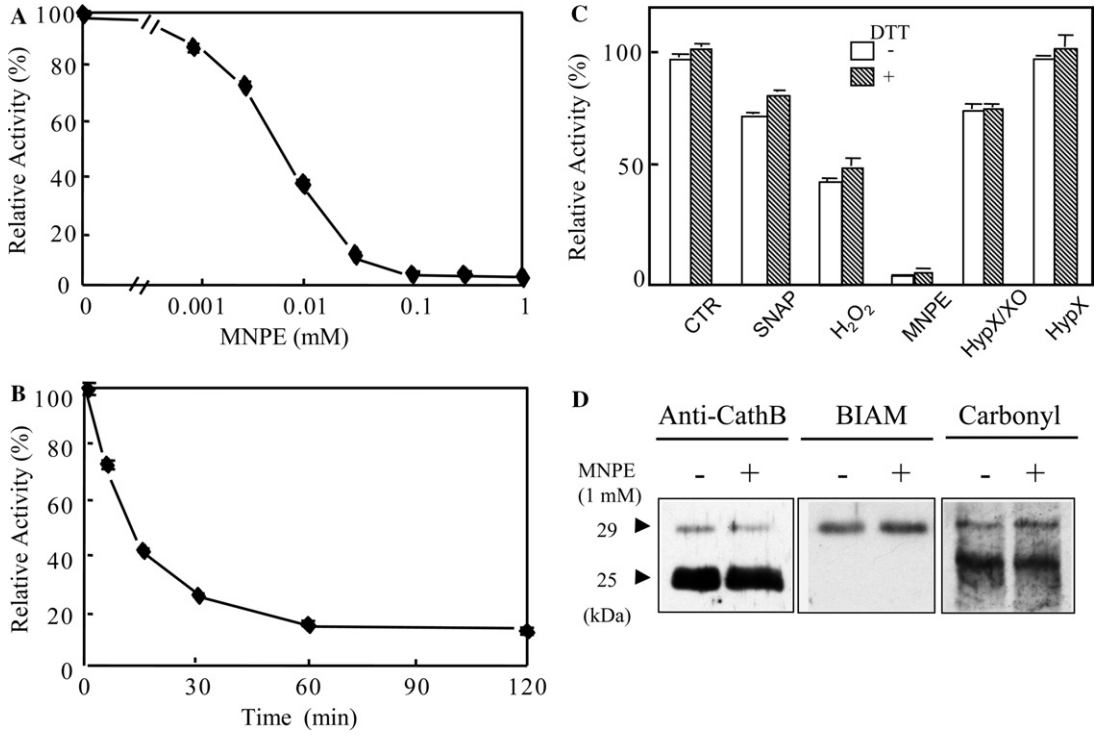

Fig. 8. Effects of MNPE on purified cathepsin B from human liver. (A,B) Purified cathepsin B from human liver was diluted with extraction buffer containing 1 mg/ml BSA and incubated with varying concentrations of MNPE for 1 h (A) or with 0.03 mM MNPE for the indicated periods at 37 °C (B). (C) After treatment of the purified cathepsin B with 0.1 mM SNAP, 0.1 mM H_2O_2, 0.1 mM MNPE, 0.05 mM hypoxanthine/xanthine oxidase (HypX/XO) or 0.05 mM hypoxanthine for 1 h at 37 °C, the enzyme was further incubated with or without 5 mM DTT at 37 °C for another 1 h. The means ± SD of triplicate assays are shown. (D) Purified cathepsin B was first incubated without or with 1 mM MNPE for 1 h. An aliquot (0.46 μg) was subjected to an immunoblot analysis using an anti-human cathepsin B antibody followed by HRP-conjugated anti-rabbit Ig (anti-CathB) or was reacted with 20 μM BIAM in 100 mM NaCl and 50 mM Mes, pH 6.5 (BIAM), for 30 min at 25 °C followed by HRP-conjugated streptavidin. Detection of carbonyls was performed by an Oxyblot kit according to the manufacturer's instruction (carbonyl). Positions of single-band form (29 kDa) and heavy chain of the two-band form (25 kDa) are shown.

the difference was due to their ability to permeate the membrane. When TBARS and a protein adduct with a lipid peroxidation product, HNE, were assayed, treatment with MNPE, but not with NDPE, elevated their levels (Figs. 2 and 3). This supports the view that MNPE actually entered the cells and released singlet oxygen, resulting in detrimental effects on intracellular components. Quantification of degradation products in cells indicated that MNPE was actually incorporated seven times more than NDPE [12]. The levels of MNPE reaching the cytoplasm of cells, however, would be much lower than those in the culture media because about two-orders higher concentrations were required to obtain a half-maximal inhibition of cathepsins in cells (Figs. 1 and 4). The hydrophobic nature of MNPE may permit it to anchor to a large portion of membrane components.

Conjugated double bonds, such as those of unsaturated fatty acids, are well-known targets of singlet oxygen [1]. The data presented here demonstrate that cathepsins in the cysteine protease group were also highly sensitive to singlet oxygen-induced oxidative modification. Although we examined only cathepsin B and L/S activities, in addition to caspases in a previous study [12], it would reasonably be expected that other papain-like cysteine proteases are also sensitive targets. Since members of the cathepsin family have multiple roles, that include the hydrolytic break down of internalized proteins by endocytosis, antigen presentation, and apoptosis [13], their inhibition may impair pathogenic conditions.

ROS and RNOS are known to inhibit cysteine proteases [27]. Cathepsin K, another cysteine protease in the family, is inhibited by nitric oxide donors [28]. The inhibition is due to the formation of mixed disulfides and a sulfenic acid and, hence, can be reversed by reduction with DTT. The oxidative modification of cathepsins by singlet oxygen, however, appears to be neither simple disulfide formation nor oxidation to sulfenic acid because DTT treatment had a negligible effect on recovering the activities (Figs. 7 and 8B). Although another sulfhydryl near the reactive cysteine is required for disulfide bond formation, no candidate sulfhydryl residue is present nearby the catalytic center in three-dimensional structure of cathepsin B [13,29]. This was supported by the capability of labeling with BIAM (Fig. 8D).

It has been shown that some of the products formed on the reaction of singlet oxygen with amino acids and peptides can have potent inhibitory effects on caspase

activity [30]. Since we observed essentially the same inhibitory action of MNPE on purified cathepsin B (Fig. 8), this could be caused by direct action of singlet oxygen on cathepsin B. The oxidative modification of a reactive sulfhydryl with singlet oxygen has been reported for thioredoxin [31] and xanthine oxidase [32]. Using the sulfhydryl-detecting agent BIAM, however, we did not obtain data that suggested modification of sulfhydryls on purified human cathepsin B by treatment with MNPE (Fig. 8). Cys29 and His199 form a catalytic center in human cathepsin B, as commonly observed in papain-like cysteine proteases. Hence, an alternative explanation is that inactivation may be induced by oxidative modification of His199 or any other essential residue for the activity. A precise protein chemical analysis will be required to define the modification by singlet oxygen.

The inhibitory action of singlet oxygen was effectively quenched by sulfhydryl compounds, with Cys being the most effective (Fig. 6). The quenching efficiencies of various thiols have been precisely examined and are generally lower than that of His or NaN_3 [33,34]. Thus, the quenching effect by Cys observed here may not merely be due to the direct scavenging of singlet oxygen but to protection of the catalytic center of the enzyme by an alternate mechanism. This hypothesis is supported by data that GSH, which has high redox potential, was much less protective than Cys. Since GSH is acidic and about three times larger than Cys, ionic and/or steric effects may cause the difference in the protective function.

The activity of papain-like cysteine proteases, cathepsins in addition to caspases [12], is prone to modification by singlet oxygen. Thus, singlet oxygen may have a more divergent function than is generally accepted. Since cathepsins, as well as caspases, are involved in apoptosis [18,19], exposure to a certain level of singlet oxygen would shut down the apoptotic machinery, resulting in necrosis-like cell death. This mechanism may deteriorate inflammatory conditions during exposure to UV light.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.03.146.

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