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Redox Regulation of Caspase-3(-like) Protease Activity: Regulatory Roles of Thioredoxin and Cytochrome c

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Oxidative stress induces a variety of cellular responses, including apoptosis, and caspase family proteases are known to be involved in apoptosis. Caspase-3(-like) protease activity was examined in Jurkat T cells to investigate the mechanism of apoptosis induced by a thiol oxidant, diamide. Caspase-3 was activated when cells were cultured with 200 μM diamide that induced apoptosis, whereas no caspase-3 activation was detected with 500 μM diamide that induced necrosis. When apoptosis was induced in cells with exposure to 200 μM diamide, the intracellular thioredoxin (TRX) levels were maintained and the intracellular generation of reactive oxygen intermediates was marginal. The cytosolic fractions of cytochrome c were increased earlier than the activation of caspase-3. In contrast, when cells were exposed to 500 μM diamide, intracellular reactive oxygen intermediate generation was increased and processing of caspase-3 was not detected despite cytochrome c release, resulting in necrosis. Caspase-3 activity in cell lysate precultured with anti-Fas Ab was suppressed dose dependently by diamide and restored by thiol-reducing agents, DTT or TRX. When cells were precultured with 5 mM of buthionine sulfoximine, an inhibitor of glutathione synthesis, intracellular TRX levels were maintained, and as low as 20 μM diamide could induce apoptosis associated with the increase of cytosolic cytochrome c and the activation of caspase-3. These results indicate that the activation of caspase-3 in diamide-induced apoptosis is mediated, at least partly, by cytochrome c release from mitochondria, and the cellular reducing environment maintained by TRX, as well as glutathione, is required for caspase-3 activity to induce apoptosis. The Journal of Immunology, 1998, 161: 6689–6695.

Cellular redox state is regulated by cellular thiols, including glutathione (GSH) and thioredoxin (TRX). TRX is a small ubiquitous multifunctional protein having a redox-active disulfide/dithiol within its active site sequence, Cys-Gly-Pro-Cys-, and operates together with NADPH and TRX reductase as a protein disulfide-reducing system ([11] 1). There is a collecting evidence that the TRX system is as important as the GSH system in the cellular redox regulation against oxidative stress. Human TRX was originally cloned as adult T cell leukemia-derived factor (ADF) produced by human T cell leukemia virus type I-transformed T cell (12). Mammalian TRX has been shown to be a stress-inducible protein (1, 13–15) and secreted from cells (16–18). Intracellular TRX plays an important role in the regulation of protein-nucleic acids interactions through the redox regulation of its cysteine residues (19, 20). TRX also shows a cytokine-like extracellular activity to promote cell growth (21–23) and protect cells against oxidative stress (13, 24–26).

In this work, we examined the regulatory mechanism of caspase-3(-like) protease in Jurkat cells by oxidative stress elicited by diamide. Our results indicate critical roles of intracellular reducing factors, including TRX, and of the release of cytochrome c from mitochondria into cytosol in the activation of caspase-3(-like) protease.

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3 Abbreviations used in this paper: GSH, glutathione; Ac-DEVD-MCA, acetyl-l-aspartyl-l-glutamyl-l-valyl-l-aspartic acid α-(4-methyl coumaryl-7-amide); ADF, adult T cell leukemia-derived factor; AMC, 7-amino-4-methylcoumarin; ASK, apoptosis signal-regulating kinase; BSO, DL-buthionine-[S,R]-sulfoximine; MAF, mitogen-activated protein; NAC, N-acetyl-l-cysteine; ROI, reactive oxygen intermediates; TRX, thioredoxin.
Materials and Methods

Reagents

Agonistic anti-human Fas mAb (CH-11) (27) was purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-cytochrome c mAb and anti-caspase-3 polyclonal Ab were purchased from PharMingen (San Diego, CA). Anti-human TRX mAb (ADF-11 mAb, mouse IgG1) was provided by Fujirebio (Tokyo, Japan). A fluorogenic substrate for caspase-3 (CPP32) protease, acetyl-IETD-val-IETD-methylcoumarin (AMC) was monitored by a spectrofluorometer (Hitachi F-2000) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit was defined as the amount of enzyme required to release 5.2 pmol AMC/1 min/1 mg lysate protein at 37°C.

Electron microscopy

Jurkat cells cultured with diamide or anti-Fas mAb were collected and washed with ice-cold PBS ( ), and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA containing 50 μg digitonin. Then cells were incubated at 37°C for 10 min. Lysates were centrifuged at 15,000 rpm for 3 min, and supernatants were collected. A total of 10 μg protein was incubated with 50 μM Ac-DEVD-MCA at 37°C, and the release of 7-amino-4-methylcoumarin (AMC) was monitored by a spectrofluorometer (Hitachi F-2000) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit was defined as the amount of enzyme required to release 5.2 pmol AMC/1 min/1 mg lysate protein at 37°C.

Flow-cytometric estimation of intracellular redox state

Intracellular redox state was estimated by levels of intracellular ROI, which were measured by the flow-cytometric analysis with dihydroethidium (an oxidation product of ethidium). The activity of caspase-3(-like) protease in Jurkat cells cultured with 0, 50, 100, 150, 200, 300, and 500 μM of diamide for 6 h. Time course of caspase-3-like protease activity in Jurkat cells cultured with 150 or 200 μM diamide. This result is a representative among three separate experiments.

For cytochrome c assay, soluble cytosolic fraction was prepared as described (31). Cells were collected and washed twice with ice-cold PBS ( ), and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA containing 50 μg digitonin. After sitting on ice for 15 min, the cells were disrupted by douncing 15 times with a loose-fitting pestle (Dounce type homogenizer, Wheaton, NJ). The suspension was centrifuged twice at 1000 × g for 5 min at 4°C to remove the nuclei. The supernatant was further centrifuged at 103,000 × g for 1 h (Beckman TL-100 Ultra centrifuge). The resulting supernatant was used as soluble cytosolic fraction.

For caspase-3 immunoblotting, cells were collected and washed twice with ice-cold PBS ( ), and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA containing 50 μg digitonin. Then cells were incubated at 37°C for 10 min. Lysates were centrifuged at 15,000 rpm for 3 min, and supernatants were collected. Equal amounts of protein were subjected to electrophoresis on a 15% SDS-polyacrylamide gel, and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% BSA and 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 at 4°C overnight, the membrane was incubated with anti-TRX or anti-cytochrome c mAb, followed by horseradish peroxidase-conjugated anti-mouse Ig (Amersham, Little Chalfont, U.K.). For caspase-3 immunoblotting, the membrane was incubated with anti-caspase-3 polyclonal Ab, followed by horseradish peroxidase-conjugated anti-rabbit Ig (Amersham). Detection of chemoluminescence was performed with an ECL Western blot detection kit (Amersham), according to the supplier’s recommendation.

Results

Induction of apoptosis and activation of caspase-3(-like) protease in Jurkat cells by diamide

We previously demonstrated that diamide induces cell death and DNA fragmentation in Jurkat T cells (5). To investigate the regulatory mechanism of diamide-induced cell death, we first examined the caspase-3-like protease activity in Jurkat cells cultured with various concentrations of diamide. As shown in Fig. 1A, the
activation of caspase-3(-like) protease was observed. The activity was highest in cells cultured with 200 μM diamide, and gradually decreased in cells with higher concentration of diamide. The activity could not be detected in cells cultured with 500 μM diamide.

As shown in Fig. 1B, caspase-3(-like) protease began to be activated 4 h after the culture with 200 μM diamide, and the peak of the activity was observed after about 10 h of culture.

Transmission-electron microscopy demonstrated that nuclei of Jurkat cells showed the characteristic morphologic change of apoptosis: condensation and fragmentation of chromatin in nuclear periphery, intact nuclear membranes, and almost intact mitochondria (Fig. 2B), which is similar to those cultured with 100 ng/ml anti-Fas mAb (Fig. 2D). When the cells were examined 3 h after exposure to 200 μM diamide, the fragmentation of nuclei was not detected yet, although the condensation of chromatin was observed (data not shown). After 7 h of the culture with 200 μM diamide or anti-Fas mAb, the fragmentation of nuclei was remarkable, and some population of apoptotic cells began to lose the integrity of plasma membrane (Fig. 2, B and D).

In contrast to the culture with 200 μM diamide, Jurkat cells cultured with 500 μM diamide (Fig. 2C) revealed typical features of necrosis, as expected from our previous observation (5). In cells cultured with 500 μM diamide for 3 h, the vacuole formation and the swelling of mitochondria were seen, whereas the integrity of nuclear membrane was still maintained (data not shown). After 7 h of the culture with 500 μM diamide, the vacuole formation became remarkable, and the integrity of nuclear and plasma membrane as well as the structure of mitochondria such as cristae were destroyed (Fig. 2C).

The morphologic changes induced by diamide correlate with the caspase-3(-like) protease activity (Figs. 1A and 2, B and C) as well as the DNA fragmentation, as previously reported (5).

**FIGURE 3.** Flow-cytometric analysis of intracellular redox state estimated by the levels of intracellular ROI. Jurkat cells were cultured with the indicated concentration of diamide or anti-Fas mAb (CH-11) for 3 h, and 2 μM dihydroethidium was added for the last 20 min at 37°C. The fluorescence intensity was measured by flow cytometry. Dark histograms indicate the fluorescence in treated Jurkat cells, and clear histograms indicate the fluorescence in untreated Jurkat cells. On the right side of each graph, mean fluorescence intensity values in treated Jurkat cells are shown.

A, Untreated Jurkat cells. B, Jurkat cells cultured with 200 μM diamide for 3 h. C, Jurkat cells cultured with 500 μM diamide for 3 h. D, Jurkat cells cultured with 100 ng/ml anti-Fas mAb (CH-11) for 3 h.

**Generation of ROI and intracellular redox state estimated by flow cytometry**

Diamide oxidizes the intracellular thiols, including GSH and TRX. We previously showed the transient decrease of cellular GSH and the oxidation of TRX during diamide-induced apoptosis (5). It is supposed that the decrease of these reducing factors causes the generation of intracellular ROI. The generation of intracellular ROI in Jurkat cells was estimated by flow-cytometric analysis with dihydroethidium. As shown in Fig. 3, Jurkat cells cultured with 200 μM diamide for 3 h displayed only a slight increase, indicating that generated ROI might be buffered by intracellular reducing factors. In contrast, Jurkat cells cultured with 500 μM diamide for 3 h displayed a considerable increase in the intracellular ROI.

**FIGURE 2.** Transmission-electron microscopy (×1500). A, Untreated Jurkat cells. B, Jurkat cells cultured with 200 μM diamide for 7 h. C, Jurkat cells cultured with 500 μM diamide for 7 h. D, Jurkat cells cultured with 100 ng/ml anti-Fas Ab (CH-11) for 7 h.
used as a positive control. The cell lysates (10 µg protein/lane) were electrophoresed and subjected to immunoblot analysis with anti-cytochrome c mAb. Cytochrome c (10 ng) from bovine heart, purchased from Sigma, was used as a positive control.

**Immunoblot analysis for the release of cytochrome c into cytosol**

As shown in Fig. 4A, immunoblot analysis demonstrated that the exposure of cells to 200 µM diamide in culture medium resulted in the release of cytochrome c from mitochondria into cytosol as early as 1 h. When cells were cultured with 500 µM diamide, the release of cytochrome c was also detected as early as 1 h (Fig. 4B).

**Immunoblot analysis for processing of caspase-3**

As shown in Fig. 5, the processed large subunits of caspase-3 (32, 33) began to be detected after 3 h of culture with 200 µM diamide as well as anti-Fas mAb. This processing of caspase-3 correlates with the time course of the caspase-3(-like) protease activity (Fig. 1B). However, when cells were cultured with 500 µM diamide, the processing was not detected.

**Influences of the caspase-3(-like) protease activity by various redox-active reagents**

To test the possibility that the activity of caspase is regulated by the redox state of thiol in its active site cysteine, we examined the effect of various redox-active reagents on the activity of caspase-3(-like) protease in the lysate of Jurkat cells cultured with anti-Fas mAb. Reducing agents such as 2-ME, human tTRX, or GSH in cell lysate per se only marginally increased the activity (Fig. 6, A–C).

On the contrary, caspase-3(-like) protease activity was dose dependently decreased by the addition of diamide to the lysate of Fas-activated cells (Fig. 6D). The decreased enzymatic activity was recovered by the addition of thiol-reducing agents such as DTT or TRX (Fig. 6, E and F). Less than 500 µM of diamide itself did not decrease the fluorescence of AMC nor affected the assay (data not shown).

**Effect of preculture with BSO**

We previously showed that apoptosis is induced by much lower concentration of diamide in BSO-precultured Jurkat cells as compared with BSO-untreated cells (5). The preculture with BSO itself caused neither the induction of apoptosis nor the activation of caspase-3(-like) protease in Jurkat cells (5, data not shown). Intracellular total GSH decreased to about 1.4% after the culture with 5 mM BSO for 24 h (data not shown). As shown in Fig. 7A, GSH-depleted cells precultured with 5 mM BSO for 24 h, caspase-3(-like) protease was activated in cells cultured with 5–50 µM diamide, whereas the enzymatic activity could not be detected in cells cultured with more than 100 µM of diamide. As compared with Fig. 1A, lower concentration of diamide activated caspase-3(-like) protease in GSH-depleted cells than BSO-untreated cells. Morphologic observation also showed that GSH-depleted cells with 20 µM diamide were apoptotic, and those with 200 µM diamide were necrotic (data not shown).

As shown in Fig. 7B, GSH-depleted Jurkat cells cultured with 20 µM diamide for 3 h displayed a slight increase in the intracellular ROI, estimated by flow-cytometric analysis with dihydroethidium. In contrast, GSH-depleted cells cultured with 200 µM diamide for 3 h displayed a considerable increase in the intracellular ROI.

Immunoblot analysis demonstrated that a marginal release of cytochrome c into cytosol occurred when cells were cultured with 5 mM BSO for 24 h, and that the release was markedly facilitated by the following culture with 20 µM diamide (Fig. 7C).

**Immunoblot analysis for TRX protein**

Immunoblot analysis demonstrated that intracellular TRX protein levels were conserved in Jurkat cells cultured with apoptosis-inducible concentration of diamide (Fig. 8). When cells were precultured with BSO, intracellular protein levels of TRX were also preserved in cells cultured with 20 µM diamide.

**Discussion**

We have shown that 150–200 µM of diamide induces apoptosis in Jurkat T cells, while at the concentration over 400 µM, diamide induces necrosis (5). In this study, we show that caspase-3(-like) protease is activated in Jurkat T cells cultured with 200 µM diamide, while the protease activity is not detected in the cells cultured with 500 µM diamide (Fig. 1A). Recently, Hampton et al.
have shown that 50 μM hydrogen peroxide induces apoptosis in Jurkat cells with caspase activation, and higher concentration of hydrogen peroxide causes necrosis without caspase activity. Flow-cytometric analysis indicates the possibility that oxidative stress induces apoptosis as long as intracellular reducing factors can buffer the generated ROI, while it induces necrosis when a physiologic intracellular reducing environment collapses under the excessively generated ROI (Figs. 3 and 7B). The disruption of enzymatic caspase activity induced by excessive oxidation may cause the shift from apoptosis to necrosis (35). Our results suggest the importance of an intracellular reducing environment for the activity of caspases.

Caspases have cysteine residues in the catalytic domains (9, 10). It has been reported that the activity of caspase-1 is lost when the active site cysteine is replaced by other amino acid (10). In this study, we show that caspase-3(-like) protease activity is regulated by redox condition of cysteine by use of a thiol-oxidizing agent, diamide (D). The protease activity was measured by the detected fluorescence, as described in Materials and Methods. Next, the sample once treated with diamide was dialyzed with Column PD-10 containing Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) to remove excess diamide. After incubation with the indicated concentration of reducing agents, i.e., 2-ME (A), human rTRX (B), or GSH (C), or with thiol-oxidizing agent, diamide (D), the protease activity was measured by the detected fluorescence, as described in Materials and Methods. The data are mean ± SD of three samples.

Diamide has exclusively inhibitory, but not enhancing effect on the caspase activity when added to the cell lysate (Fig. 6D). Caspase-3(-like) protease, however, was markedly activated when Jurkat cells were cultured with a limited range of the concentration of diamide (Fig. 1). Our results indicate that diamide activates caspase-3(-like) protease not directly, but via some cellular event induced by diamide. One of the possibilities involved in the activation of caspase-3(-like) protease by diamide is the release of cytochrome c from mitochondria into cytosol (31, 36–39). It has been reported that diamide induces apoptosis in mouse thymocyte with mitochondrial membrane potential disruption (40, 41). There might be a redox-sensitive molecule in the outer membrane of mitochondria, which is responsible for the release of mitochondrial cytochrome c, because cytochrome c resides in the space between the outer and inner membranes of mitochondria. TRX2 (42), which was recently cloned and localized in mitochondria, may modulate apoptosis-inducing signal via mitochondria in cooperation with other thiol molecules. Although the release of cytochrome c was detected as early as 1 h (Fig. 4A), it took 4 h for caspase-3(-like) protease to be activated in cells cultured with 200 μM diamide (Fig. 1B), suggesting that caspase-3(-like) protease activity is regulated by several factors, including the intracellular redox status in addition to the release of cytochrome c from mitochondria into cytosol. When cells were cultured with 500 μM diamide, the processing of caspase-3 was not detected in spite of the release of mitochondrial cytochrome c (Figs. 4B and 5). The processing of caspase-3 seems to be suppressed under a thiol-oxidized state (43). These results indicate that diamide also decreases the activity of caspase-9, which is also cysteine protease, or interferes with the cytochrome c/Apaf-1/caspase-9 complex, the likely activator of caspase-3.
were electrophoresed and subjected to immunoblot analysis with anti-TRX mAb. This result is a representative among three separate experiments. Other possibilities involved in the activation of caspase-3(-like) protease by diamide is c-Jun N-terminal kinase (JNK) or p38 MAP kinase (44, 45). Quite recently, it has been reported that reduced protease by diamide is c-Jun N-terminal kinase (JNK) or p38 MAP kinase (44, 45). When TRX is oxidized by oxidative stress, ASK 1 is dissociated from oxidized TRX and activated to induce apoptosis signal. Indeed, the existence of an intracellular reducing environment by TRX is quite important for the activation of caspase-3(-like) protease. Cisplatin, which is one of the most widely used anti-cancer drugs and which cytotoxicity is attributed to induce apoptosis in response to sulfhydryl oxidation (5).

GSH system is one of the intracellular thiol-reducing factors to antagonize the excessive oxidative oxidation. When Jurkat cells were cultured with 150 or 200 μM diamide, intracellular total GSH level transiently decreased slightly, but maintained during the process of apoptosis (5, S.U., unpublished observations). We comparatively studied the effect of GSH depletion in Jurkat cells cultured with BSO, which inhibits GSH synthesis and depletes intracellular reduced form of GSH. In GSH-depleted cells, lower concentration of diamide can induce the activation of caspase-3(-like) protease and the release of cytochrome c as well as apoptosis than BSO-un-treated cells (Fig. 7). Our results indicate that GSH-depleted cells are more sensitive to oxidative stress. Interestingly, the intracellular levels of TRX in untreated Jurkat cells were maintained even when cells were treated with BSO, suggesting that the maintenance of an intracellular reducing environment by TRX is quite important for the activation of caspase-3(-like) protease. Cisplatin, which is one of the most widely used anti-cancer drugs and which cytotoxicity is attributed to induce apoptosis as well as necrosis in cancer cells, but to induce mainly necrosis in BSO-treated cells (48). When Jurkat cells were cultured with N-acetyl-l-cysteine (NAC), apoptosis induced by 200 μM diamide was only slightly inhibited (S.U., unpublished observations). It has been reported that Fas-mediated apoptosis in T cell as well as NK cell is inhibited by the preculture with NAC and enhanced by the preculture with BSO (49–51), and that Fas expression is down-regulated by the culture with NAC (50). Taken together, it is likely that cells containing much thiols, including GSH and TRX, tend to survive from a variety of stress or death signal.

In conclusion, the activation of caspase-3(-like) protease is mediated, at least partly, by the release of cytochrome c from mitochondria into cytosol, which is sensitive to oxidative stress, and endogenous reducing factors, including TRX, are required for caspase activity to induce apoptosis. We thank Drs. Keizo Furuke, Norihito Sato, Takashi Inamoto, and Akira Yamauchi for helpful discussions; Drs. Shigeru Tsuyuki and Hirotaka Kazama for technical advice; Dr. Makio Fujiioka for help with electron microscopy; and Ms. Yoko Kanekiyo for secretarial help.