Effects of Glutathione-related Compounds on Increased Caspase-3 and Caspase-6-like Activities in Ricin-treated U937 Cells

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Both caspase-3 and -6-like activities increased in the cytosolic extract from ricin-treated U937 cells that were inhibited by glutathione disulfide (GSSG) in a dose-dependent manner, but reduced glutathione (GSH) had no effect. Interestingly, caspase-6-like activity was more sensitive to GSSG than caspase-3-like activity. The IC₅₀ of GSSG against caspase-3 and caspase-6-like activities were estimated to be 2.8 mm and 0.8 mm, respectively. Cystine but not cysteine also showed similar inhibitory effect on caspase-3-like activity. The inhibitory effect of GSSG on these caspase-like activities was prevented by the addition of DTT to the assay mixture. These results suggest that an intact disulfide portion of GSSG is required for the effective inhibition of caspase activity.

Key words: ricin; apoptosis; glutathione; caspases

The cytotoxic plant lectin, ricin, inhibits protein synthesis in eukaryotic cells following receptor-mediated endocytosis through its B chain. After intracellular vesicle trafficking, the enzymatically active component (A chain) is eventually translocated into the cytosol to reach its target, i.e., the 28S RNA of the 60S ribosomal subunit. Recent studies have demonstrated that ricin induces apoptotic death in several cell lines. In the cells undergoing apoptosis, there are characteristic morphological and biochemical changes such as cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation, resulting in an oligonucleosomal ladder. Some of these characteristic apoptotic features have been observed in ricin-treated cells. It has been proposed that a unique family of cysteine proteases, which are designated caspases, play crucial roles in controlling apoptotic cell death. In fact, we have found that caspase-3 and caspase-6-like activities increased in ricin-treated U937 cells. A common catalytic property of the caspase family is the presence of a reactive cysteine in the active site, and therefore caspases may be sensitive to the redox status of the cell. The involvement of thiol groups in the caspase activity is also supported by the finding that caspases are inhibited by thiol-alkylating agents such as iodoacetamide or N-ethylmaleimide. The redox status of the cell is usually regulated by glutathione, the most abundant non-protein thiol in eukaryotic cells, ranging in concentration from 0.1–10 mm in vivo. Thus, it is possible that glutathione would be an important factor regulating caspase activity during apoptosis. Regarding the relationship between glutathione and apoptotic processes, depletion of intracellular glutathione has recently been found to occur in several different apoptotic systems, and it has been suggested that depletion of glutathione is an early event in the commitment to apoptosis. For example, thymocytes undergoing apoptosis after exposure to glucocorticoid or DNA damaging agents lose intracellular glutathione with similar kinetics to internucleosomal chromatin fragmentation. Furthermore, it has been reported that reduced glutathione was directly exported from Jurkat cells after exposure to anti-Fas/APO-1 antibody through a specific glutathione transporter rather than depleted by oxidation, and no significant increase in oxidized glutathione was detectable. Similarly we have previously reported that ricin-induced apoptosis of U937 cells is associated with glutathione depletion; glutathione is extruded during the apoptotic processes before any plasma membrane leakage. We also found that the depletion of glutathione in ricin-treated U937 cells was strongly prevented by a caspase family inhibitor, Z-Asp-CH₂-DCB. These results clearly demonstrate that the decrease in glutathione in response to ricin treatment depends on activation of certain caspases. However, the significance of glutathione depletion in apoptotic processes is still unclear. To gain insight into the involvement of glutathione in the apoptotic pathways, we examined the effects of glutathione and its related compounds on the caspase activities increased in the

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Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-VEID-CHO, acetyl-Val-Glu-Ile-Asp-aldehyde; MCA, 4-methylcoumaryl-7-amide; GSH, reduced glutathione; GSSG, glutathione disulfide; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; ICE, interleukin-1β-converting enzyme; PBS, phosphate buffered saline
cytosolic extracts from ricin-treated human myeloid leukemia U937 cells.

A human myeloid leukemia U937 cell line was maintained in RPMI-1640 medium (Gibco) with 10% fetal bovine serum in a humidified atmosphere of 5% CO2 and 95% air as described previously.10 Cells (2 × 10⁶ cell/ml) were incubated in RPMI-1640 medium containing 35 μM bovine serum albumin with ricin (10 ng/ml) for 5 h at 37°C. Then, cells were pelleted by centrifugation and resuspended in 200 μl of extraction buffer (10 μM HEPES/KOH buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 1 mM PMSF), though the usual extraction buffer contains 5 mM dithiothreitol (DTT) as described.40 After repeated freezing and thawing, cell debris were removed by centrifugation at 13000 × g at 4°C for 10 min. The supernatant (cytosolic extract) was incubated with 10 μM fluorescent substrate for caspase-3 (Ac-DEVD-MCA) or caspase-6 (Ac-VEID-MCA) in the presence or absence of indicated concentration of glutathione or glutathione-related compounds at 37°C for 10 min, and then cleavage of peptides was analyzed with excitation at 380 nm and emission at 460 nm.

As shown in Fig. 1, the Ac-DEVD-MCA cleavage activity in the cytosolic extract from ricin-treated U937 cells was inhibited by GSSG in a dose-dependent manner with an IC₅₀ of approximately 2.8 mM. However, no significant inhibitory effect of reduced glutathione (GSH) on the Ac-DEVD-MCA cleavage activity was observed at concentrations ranging from 1 to 20 mM (Fig. 1). If GSSG inhibits the caspase-3 activity through acting on the catalytic site of the enzyme, then it would most likely also influence other caspases because they all contain a conserved pentapeptide (QACRG) which includes the catalytic cysteine,21 although their substrate recognition sites are different. To investigate this point, we examined the effects of GSSG on Ac-VEID-MCA cleavage activity (caspase-6-like activity), which is also increased in the cytosolic extracts from ricin-treated U937 cells.10 GSSG inhibited caspase-6 like activity in a dose-dependent manner with an ID₅₀ of 0.8 mM, which is 3.5-times lower than that for caspase-3 like activity as described above (Fig. 1). The different sensitivity to GSSG between caspase-3 and caspase-6 like activities may be due to the different reactivity toward GSSG of their respective target thiols. Similar to caspase-3 like activity, GSH had no significant effect on caspase-6 like activity (Fig. 1). The inhibitory effect of GSSG on caspase-3 and caspase-6 like activities was almost completely prevented by the addition of DTT to the assay mixtures (Fig. 2), indicating that an intact disulfide was required. To examine the specificity for GSSG inhibition of these caspase like activities, we tested the effects of glutathione-related compounds on the enzyme activities. As shown in Table 1, glutamic acid, glycine,
of γ-glutamyl-cysteine, cysteine, cysteinyl-glycine, s- methyglutathione. Interestingly, cystine had a week but significant inhibitory effect on caspase-3 like activity, while it had no effect on caspase-6 like activity. These results further support the notion that the disulfide portion is responsible for the inhibitory property of GSSG against caspase-like activities. Consistent with the recent report of nitric oxide inhibition of caspase-3, NO donors such as S-nitrosoglutathione and S-nitroso-N-acetyl-DL-penicillamine also had inhibitory effect on caspase-3 and caspase-6-like activities, and their effects were abolished by the addition of DTT to the reaction mixtures, confirming the presence of catalytic thiol in the caspases as the target for protein thiol modification by S-nitrosylation.

In agreement with our results, it has been reported that dithiocarbamate disulfide (disulfiram), a thiol modulating agent, strongly inhibited purified caspase-1 through a thiol-disulfide exchange reaction between inhibitor and target. In that study, protein mixed disulfide formation between disulfiram and caspase-1 was directly demonstrated using 35S-labeled disulfiram. 22 Contrary to our results, GSH has recently been shown to inhibit caspase-3 and caspase-8 activities in human neutrophils treated with anti-Fas antibody. 23 GSH has been also reported to block the ability of granzyme B to cleave poly(ADP-ribose) polymerase, a caspase-3 dependent process. 24 Although the reason for the discrepancy between our results and these findings was uncertain, the activity of caspases is usually optimal under reducing conditions, and millimolar DTT is routinely included in experimental assay buffers to prevent spontaneous thiol oxidation. 14 Therefore, it is more plausible that GSH would keep caspases functional by maintaining them in a reduced state rather than inhibiting their enzymatic activities. This notion is also supported by the finding that caspase-3 like activity in the cystolic extracts from anti-Fas/APO-1 antibody treated Jurkat cells was inhibited by GSSG, but GSH even slightly increased the enzyme activity over the control level. 22

The intracellular redox state is generally considered to be coupled to the oxidation state of cysteine residues in proteins 25 by complex thiol/disulfide exchange mechanisms through which redox status influences the activity of a variety of enzymes. 26 Thus, it is possible that the apoptotic signaling pathway is modulated by redox regulation of specific proteins including caspases via their thiol groups. Although the physiological significance of caspase inhibition by GSSG remains to be clarified, a dose-dependent switch from apoptosis to necrosis with oxidants and redox-active compounds is known. 27 Thus, one can speculate that inhibition of caspase function by GSSG may be responsible for the choice between apoptotic and necrotic cell death. In addition, the prooxidant status of some tumor cell lines suggests that their insensitivity to apoptosis may result from inhibition of caspase function by increased intracellular GSSG under oxidizing conditions. 28

There are other examples of redox-regulated proteins, such as NF-kB and AP-1, which appear to be involved in responding to changes in intracellular redox status. 29 Since oxidative stress has been reported to inhibit apoptosis, 30 our results together with other previous findings suggest the possibility of physiological regulation of caspases by the redox state of glutathione. Probably, change in the glutathione redox state could influence caspase activity, and thereby apoptosis.

### References

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