Involvement of N-Acetylcysteine-sensitive Pathways in Ricin-induced Apoptotic Cell Death in U937 Cells

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We have found that the antioxidant N-acetylcysteine (NAC) strongly inhibited ricin-induced apoptotic cell death in U937 cells (human myeloid leukemia), as judged by cytotoxicity, nuclear morphological change, and DNA fragmentation. Consistent with these observations, a significant depletion of cellular glutathione was observed in ricin-treated cells, and NAC prevented the decrease in cellular glutathione. On the other hand, among the caspase inhibitors tested, Z-Asp-CH₂-DCB, which inhibited ricin cytotoxicity, also suppressed ricin-mediated glutathione depletion, while NAC did not affect the generation of caspase-3 like activity in ricin-treated cells. These results suggest that glutathione loss takes place downstream from caspase activation during the ricin-induced apoptotic process. Treatment with a specific inhibitor of glutathione biosynthesis, buthionine sulfoximine (BSO) failed to induce apoptosis, and had no effect on the overall extent of ricin-induced apoptosis, even though the glutathione level was decreased to less than 5% of the control level. However, NAC still protected against ricin-induced apoptosis in the BSO-treated cells. We conclude that glutathione loss is one of several apoptotic changes caused by ricin, but is not a sufficient factor for the progress of apoptosis. NAC may prevent ricin-induced apoptosis through maintaining an intracellular reducing condition by acting as a thiol supplier.

Key words: ricin; apoptosis; oxidative stress; glutathione; N-acetylcysteine

Cytotoxic proteins of plants (ricin, abrin, and modeccin) and bacterial (diphteria, Pseudomonas, and Shiga toxins) inhibit protein synthesis in eukaryotic cells following receptor-mediated endocytosis.1-3) After intracellular vesicle trafficking, the enzymatically active components of these toxins are eventually translocated into the cytosol to reach their targets, i.e., the 28S RNA of the 60S ribosomal subunit for ricin, abrin, modeccin, and Shiga toxin, or elongation factor-2 for diphteria toxin and Pseudomonas toxin. Recent studies have demonstrated that ricin, modeccin, diphteria toxin, and Pseudomonas toxin induce apoptotic death in several cell lines.4-8) In the cells undergoing apoptosis, there are characteristic morphological and biochemical changes, which include cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation resulting in an oligonucleosomal ladder. In fact, these characteristic apoptotic features have been observed in ricin-treated cells,4,6,8 although the mechanism of ricin-induced apoptosis is poorly understood.

Increasing evidence has demonstrated that members of a unique family of cysteine proteases, which are designated caspases, play critical roles in controlling apoptotic cell death.9,10) The interleukin-1β-converting enzyme (ICE, i.e., caspase-1) is the first identified member of the caspase family, and is the mammalian homologue of CED-3, the product of a gene essential for programmed cell death in the nematode Caenorhabditis elegans.11) Recent study suggests that multiple caspases participate in apoptosis in mammalian cells,12,13) and caspase-3 and caspase-6 have been shown to be the major active caspases responding to different apoptotic stimuli.14)

In addition to the specific proteolytic cleavage events in the apoptotic signalling pathway, oxidative stress has also been suggested to be a common mediator of apoptosis. This hypothesis is supported by the findings that some antioxidants protected against many different forms of apoptosis.15-18)

To study the involvement of oxidative stress in ricin-induced apoptotic cell death, we examined the effect of a thiol antioxidant N-acetylcysteine (NAC) on the cell death and DNA fragmentation induced by ricin in human myeloid leukemia U937 cells. We also examined the intracellular glutathione, a major antioxidant in the cell, following exposure of cells to ricin in the presence or absence of NAC. The results show that ricin treatment leads to depletion of cellular glutathione concomitant with the onset of apoptosis, and NAC strongly inhibits ricin-induced apoptosis.

Materials and Methods

Materials. Ricin was obtained from Sigma Chemical Co. (St. Luis, MO). Ricin was also isolated from small

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Abbreviations: NAC, N-acetylcysteine; DTNB, 5,5'-dithio-bis (2-nitrobenzoic acid); BSA, bovine serum albumin; LDH, lactate dehydrogenase; Z-Asp-CH₂-DCB, carbobenzoxy-Asp-1-yl-[2-6-dichlorobenzoyl]oxy]methane; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-VEID-CHO, acetyl-Val-Glu-Ile-Asp-aldehyde; MCA, 4-methyl-coumaryl-7-amide; GSH, glutathione; GSSG, glutathione disulfide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; ICE, interleukin-1β-converting enzyme; PBS, phosphate buffered saline; BSO, D, L-buthionine-S, R-sulfoxime; TNF, tumor necrosis factor
castor beans as described by Mise et al.\textsuperscript{19} The fluorescent tetrapeptide substrate of caspase-3 (Ac-DEVD-MCA) and caspase inhibitors (Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-VEID-CHO, and Z-Asp-CH\textsubscript{2}-DCB) were obtained from the Peptide Institute, Inc., Osaka, Japan. N-acetylcysteine (NAC) and bisbenzimide H33258 (Hoechst 33258) were purchased from Wako Chem. Co. Japan. 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) was purchased from Nacalai tesque, Inc. Kyoto, Japan. \textsuperscript{[3]}H]Leucine (60 Ci/mmol) was obtained from NEN Research Products (Boston, MA).

**Cell culture.** A human myeloid leukemia U937 cell line was obtained from the Riken Cell Bank, Tsukuba, Japan. Cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air.

**Cytotoxicity assay.** Cytotoxicity of toxins was measured by Alamar blue assay.\textsuperscript{20} In brief, 5 × 10\textsuperscript{4} cells/well in a 96-well plate in RPMI-1640 medium containing 35 μM bovine serum albumin (BSA) were treated with NAC or not, for 1 h at 37°C. After a 24-h treatment with ricin in the presence or absence of NAC, cell viability was assessed as described.\textsuperscript{20} Cytotoxicity of ricin was also measured by lactate dehydrogenase (LDH) release assay in which LDH released from lysed cells was measured by the measurement of 2-(p-iiodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) reduction as described previously.\textsuperscript{7}

**Measurement of protein synthesis inhibition.** Cells in 48-well plates (5 × 10\textsuperscript{5} cells/well) in RPMI-1640 medium containing 35 μM BSA were treated with NAC or not, for 1 h at 37°C. After 3 h of incubation with varying concentrations of ricin in the presence or absence of NAC, the medium was replaced by leucine-free medium containing 1 μCi/ml \textsuperscript{[3]}H]leucine by centrifugation, and cells were incubated for 45 min at 37°C. The incorporation of \textsuperscript{[3]}H]leucine into perchloric acid/phosphotungstic acid-insoluble materials was measured as described previously.\textsuperscript{20} The results were expressed as the percentage of incorporation in control cells incubated without ricin but otherwise treated in the same way.

**DNA fragmentation assay.** Cells (5 × 10\textsuperscript{4} cells/ml) in RPMI-1640 medium containing 35 μM BSA were treated with NAC or not, for 1 h at 37°C. Cells were incubated with the indicated concentrations of ricin in the presence or absence of NAC for 9 h at 37°C. After removal of the medium, cells were washed once with phosphate-buffered saline (PBS) and lysed in 1 ml of lysis buffer (0.2 M NaCl, 10 mM EDTA, 20 mM Tris, 1% sodium dodecyl sulfate, pH 8.0) as described previously.\textsuperscript{7} The lysate was incubated with 250 μg/ml of protease K for 10–15 h at 37°C. The solution was then extracted with phenol/chloroform and precipitated with 2.5 volumes of ethanol for 20 min at −83°C, and the precipitate was dried by evaporative centrifugation. The DNA was then dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 100 μg/ml RNase (DNase free) and incubated for 1 h at 37°C. The DNA of each sample was then analyzed by electrophoresis in 2.0% agarose gel, stained with ethidium bromide, and then photographed on a UV illuminator.

**Nuclear staining.** Cells (5 × 10\textsuperscript{6} cells/ml) in RPMI-1640 medium containing 35 μM BSA were pretreated with NAC or not, for 1 h at 37°C. Cells were further incubated with ricin for the indicated periods of time at 37°C in the presence or absence of NAC. Cells were washed with PBS, then fixed with 1% glutaraldehyde for 30 min at room temperature. Then the cells were stained with Hoechst 33258 (40 μM) for 5 min, and observed by fluorescence microscopy (Olympus IMT-2).

**Measurement of intracellular glutathione and non-protein thiols.** The DTNB-GSSG reductase recycling assay was used to measure cellular glutathione as described.\textsuperscript{21} In brief, cells (2 × 10\textsuperscript{6} cells/ml) treated with NAC or not, for 1 h at 37°C in RPMI-1640 medium containing 35 μM BSA were incubated with ricin for the indicated periods of time at 37°C in the presence or absence of NAC. Cells were washed with PBS by centrifugation, and 100 μl of 5% trichloroacetic acid (TCA) containing 5 mM EDTA was added to the cell pellet, and the cells were sonicated for 1 min. After cell debris were removed by centrifugation at 13000 × g at 4°C for 20 min, 40 μl of the supernatant was assayed for total glutathione (GSH + GSSG, in GSH equivalents). GSSG was also measured by this method after treating GSH with 2-vinylpyridine as previously described.\textsuperscript{21} Fifty μl of the supernatant was directly mixed with DTNB and the non-protein thiols content was photometrically measured at 412 nm as described.\textsuperscript{21}

**Peptide cleavage assay.** Cells (5 × 10\textsuperscript{6} cell/ml) were incubated in RPMI-1640 medium containing 35 μM BSA with 20 mM NAC or not, for 1 h at 37°C, followed by the addition of ricin (10 ng/ml). After 9 h of incubation at 37°C in the presence or absence of ricin and/or NAC, cells were washed twice with PBS by centrifugation and resuspended in 200 μl of extraction buffer (10 mM HEPES/KOH buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF) as described.\textsuperscript{22} After repeated freezing and thawing, cell debris were removed by centrifugation at 13000 × g at 4°C for 20 min. The supernatant was incubated with 10 μM fluorescent substrate (Ac-DEVD-MCA) at 37°C for 10 min, and then cleavage of peptides was analyzed with excitation at 380 nm and emission at 460 nm. The specific inhibitor for caspase-3 (Ac-DEVD-CHO) was added to the reaction mixture at a concentration of 4 μM. Specific caspase-3 like activity was measured by subtracting the value obtained in the presence of the inhibitor.

**Results**

**Effects of N-acetylcysteine on the cytotoxicity of ricin in U937 cells**

To test the effects of N-acetylcysteine (NAC) on the cytotoxicity of ricin in U937 cells, cells treated with NAC at 5–20 mM for 1 h were incubated with varying
concentrations of ricin in the presence or absence of NAC for an additional 24 h. Then, the cell viability was assessed by the Alamar blue assay.\(^{20}\) As shown in Fig. 1A, NAC inhibited the cytotoxicity of ricin in a dose-dependent manner, and NAC at 20 mM strongly inhibited ricin cytotoxicity. NAC alone at 20 mM had no significant toxic effect on U937 cells. Thus, this concentration of NAC was used in later experiments. The protective effect of NAC (20 mM) against ricin cytotoxicity was also confirmed by an LDH release assay (Fig. 1B).

Effects of NAC on the ricin-induced DNA fragmentation and nuclear morphological change

One of the most characteristic features of apoptosis is a nuclear change concomitant with DNA degradation and the formation of a DNA ladder due to internucleosomal cleavage of chromosomal DNA. Analysis of DNA extracted from U937 cells treated with ricin by agarose gel electrophoresis showed that ricin induced the degradation of DNA and the formation of a ladder as previously reported.\(^{4,6,8}\) As shown in Fig. 2, NAC suppressed the DNA fragmentation induced by ricin. Furthermore, ricin-induced apoptotic nuclear morphological change was also prevented by NAC as examined by Hoechst 33258 staining (Fig. 3B).

Effects of NAC on the protein synthesis inhibitory activity of ricin

To test the effects of NAC on the protein synthesis inhibitory activity of ricin in U937 cells, cells treated with 20 mM NAC or not, for 1 h were incubated with varying concentrations of ricin in the presence or absence of NAC for an additional 3 h. After removal of the medium by centrifugation, the cells were labeled with \(^{3}H\)leucine for 45 min in leucine-free medium, and the amount of \(^{3}H\)leucine incorporation into proteins was measured. As shown in Fig. 4, NAC had no effect on the activity of ricin, suggesting that the protective effect of NAC against ricin-induced apoptosis is not due to the inactivation of ricin. Although the exact mechanism by which ricin induces apoptosis is not clear, these results also suggest that ricin-induced apoptosis may not be a direct consequence of its ability to inhibit protein synthesis.

Effects of NAC on the intracellular glutathione and non-protein thiols in ricin-treated and untreated U937 cells

Since NAC is a well-known antioxidant, it is reasonable to suggest that ricin causes apoptotic death of U937 cells via induction of oxidative stress or modification of the cellular redox status. Therefore, we measured the in-

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**Fig. 1.** Dose-dependent Inhibition of Ricin Cytotoxicity by NAC in U937 Cells.

Cells in 96-well plates (5 x 10^4 cells/well) were incubated with 0 (●), 5 (▲), 10 (▲), 15 (▲), or 20 mM (○) of NAC (in RPMI-1640 medium containing 35 μM BSA for 1 h at 37°C, followed by the addition of varying concentrations of ricin. Cells were incubated for another 24 h at 37°C in the presence or absence of NAC, and cell viability was assessed by an Alamar blue assay (A) or LDH release assay (B) as described under Materials and Methods. Each point represents the mean of duplicate measurements.

**Fig. 2.** The Effects of NAC on the Ricin-induced DNA Fragmentation in U937 Cells.

Cells were incubated in the presence (lane 4) or absence (lane 3) of 20 mM NAC in RPMI-1640 medium containing 35 μM BSA for 1 h at 37°C, followed by the addition of ricin (10 ng/ml). Cells were incubated for another 9 h at 37°C, and DNA was extracted and analyzed on 2.0% agarose gel as described under Materials and Methods. Lane 1; molecular weight standards (kbp), lane 2; control untreated cells.
Fig. 3. The Effects of NAC on the Ricin-induced Nuclear Morphological Changes in U937 Cells.

(A) Cells (5 x 10⁶ cell/ml) were incubated with ricin (10 ng/ml) for the indicated periods of time (0, 3, 6, 9 h) in RPMI-1640 medium containing 35 μM BSA at 37°C. (B) Cells (5 x 10⁶ cell/ml) were preincubated in the presence or absence of 5 mM BSO in RPMI-1640 medium containing 35 μM BSA for 24 h at 37°C. After further incubation with or without 20 mM NAC for 1 h, ricin (10 ng/ml) was added, and the incubation was continued for 9 h at 37°C with or without BSO and/or NAC. Cells were fixed and stained with 40 μM of Hoechst 33258, and observed under a fluorescence microscope.

Tracellular glutathione level by the DTNB-GSSG reductase recycling assay. In this assay method, total glutathione including both reduced (GSH) and oxidized (GSSG) forms can be measured simultaneously. In the absence of GSSG reductase, DTNB-reacting substances in TCA-soluble extracts were considered as non-protein thiols. Both total glutathione and non-protein thiols contents of U937 cells were found to decrease after exposure to ricin (Fig. 5). More than 95% of the glutathione detected in the cells was in the reduced form and no significant increase in GSSG level was observed during ricin treatment (data not shown). On the other hand, leakage of cytosolic lactate dehydrogenase into the culture medium was not observed, at least during 9 h of ricin treatment (data not shown). The course of glutathione decrease in ricin-treated cells was consistent with the appearance of apoptotic cells, which were detected by Hoechst 33258 staining (Fig. 3A). In the
presence of NAC, the cellular non-protein thiols increased in both ricin-treated and untreated cells. This may be due to the efficient incorporation of NAC into the cells. Interestingly, NAC significantly prevented ricin-induced glutathione depletion (Fig. 5). Regarding the GSH transport system, it has been shown that the hepatocyte GSH transporter is inhibited by thiol-related compounds such as L-cystine or methionine. Thus, one can speculate that NAC might inhibit GSH efflux by modulating a specific GSH transporter, which may be involved in glutathione depletion caused by ricin. Preliminary study indicates that ricin induces selective efflux of GSH from the basolateral membrane of polarized MDCK cells (T. Oda, N. Komatsu, T. Muramatsu, unpublished data). This finding supports the notion that ricin-induced depletion of GSH may occur through a specific GSH transporter.

**Effects of inhibition of glutathione synthesis by BSO on ricin-induced apoptosis**

To further examine the potential role of glutathione in ricin-induced apoptosis, glutathione synthesis was inhibited by a specific inhibitor of γ-glutamylcysteine synthetase, D, L-buthionine-S, R-sulfoximine (BSO). As shown in Fig. 6A, the glutathione level was decreased to less than 5% of the control after 24 h of incubation with 5 mM BSO. However, the depletion of glutathione by BSO treatment failed to increase the sensitivity of U937 cells to ricin cytotoxicity (Fig. 6B). U937 cells depleted of glutathione by BSO were viable at least for the next 24 h, indicating that glutathione depletion itself does not lead cells to apoptosis. Furthermore, NAC was still capable of inhibiting ricin cytotoxicity and ricin-induced nuclear morphological change in BSO-treated cells (Fig. 3B and Fig. 6B). Therefore, it is likely that the protective effect of NAC against ricin-induced apoptosis is mediated through a direct effect of the NAC molecule itself as a thiol compound. In fact, the level of non-protein thiols in BSO-treated cells was increased by the addition of NAC, while the level of glutathione remained very low (Table 1).

**Effects of caspase family inhibitors on ricin-induced glutathione depletion**

Recent studies have suggested that caspase family proteases are important in many cells undergoing apoptotic death. To study the involvement of caspases in ricin-induced glutathione depletion, U937 cells were incubated with several caspase inhibitors. Among the inhibitors tested, Z-Asp-CH$_2$-DCB, an aspartate-based caspase inhibitor, which was found to inhibit ricin cytotoxicity (Fig. 7A), prevented glutathione depletion caused by ricin (Fig. 7B). Similar to the recent finding that caspase-3 was activated in the cells undergoing apoptosis, an increase in caspase-3-like activity was observed in ricin-treated U937 cells. As shown in Table 2, NAC did not affect the generation of caspase-3-like activity. At present, we do not know the role of increased caspase-3-like activity in ricin-induced apoptosis, since the caspase-3 specific inhibitor, Ac-DEVD-CHO, failed to protect cells against ricin cytotoxicity. Other Z-Asp-CH$_2$-
DCB-sensitive caspases may be more important in ricin-induced apoptosis. Taken together, these results suggest that glutathione depletion take place downstream from caspase activation in ricin-treated cells.
Discussion

Oxidative stress has recently been suggested to act as a common mediator of several systems of apoptosis. In fact, exposure to low doses of H₂O₂ induces apoptosis in a variety of cell types; treatments or agents that induce apoptosis, such as chemotherapeutic agents, γ-irradiation, TNF, and glucocorticoids are also known to involve an oxidative stress. In addition, inhibitory or delaying effects of several antioxidants have been reported in many different forms of apoptosis.

In this study we have found that a thiol antioxidant, NAC inhibited the ricin-induced apoptotic death of U937 cells, as suggested by inhibition of cell death (Fig. 1), DNA fragmentation (Fig. 2), and nuclear morphological change (Fig. 3). Since NAC did not affect the protein synthesis inhibitory activity of ricin, the suppression of ricin-induced apoptosis is not due to the inactivation of ricin itself by NAC (Fig. 4). This also indicates that ricin-induced apoptosis is not a simple or straightforward consequence of the inhibition of protein synthesis. Probably, a ricin-specific pathway leading to apoptosis may exist beyond the inhibition of protein synthesis. This notion is supported by the recent finding that macrophage adherence prevents ricin-induced apoptosis but has no effect on the inhibition of protein synthesis by ricin.

The intracellular glutathione content of U937 cells was found to decrease during ricin treatment and depletion of glutathione was well correlated with the appearance of apoptotic cells which were judged by nuclear morphological criteria (Fig. 3A and Fig. 5A). It is unlikely that glutathione depletion is a consequence of intracellular oxidation of reduced glutathione (GSH), since an increase in oxidized glutathione (GSSG) was not observed in ricin-treated cells. In addition, leakage of cytosolic lactate dehydrogenase into the culture medium was not observed at least during 9 h of ricin treatment (data not shown). Thus, it seems that glutathione loss is due to a specific extrusion rather than the result from membrane damage. This notion is consistent with the recent findings that GSH was directly exported from JURKAT cells undergoing apoptosis after exposure to anti-Fas/APO-1 antibody via activation of a specific transmembrane channel rather than depleted by oxidation.

When ricin-induced cell death was inhibited by NAC, depletion of glutathione was suppressed, indicating that glutathione depletion is correlated with the progress of ricin-induced apoptosis (Fig. 5). Although further biochemical analysis is necessary to discover the mechanism of glutathione depletion during ricin-mediated apoptosis, our results suggest that caspase family proteases play a regulatory role in this event (Fig. 7). Among the caspase inhibitors tested, Z-Asp-CH₂-DCB inhibited ricin-induced glutathione depletion, and protected cells from ricin cytotoxicity, but other acetyl-type tetrapeptide inhibitors failed to show such inhibitory effects. At the present, we could not rule out the possibility that inability of these acetyl-type inhibitors to prevent the ricin-induced glutathione depletion is due to their poor membrane permeability. Further studies are required to clarify this point. Consistent with our results, van den Dobbelsteen et al. have reported that efflux of GSH from JURKAT cells exposed to anti-Fas/APO-1 antibody was inhibited by Z-Val-Ala-Asp-chloromethylketone, a inhibitor of the ICE family proteases.

To further examine the potential role of glutathione as a mediator of the NAC-dependent effects on ricin-induced apoptosis, glutathione synthesis was inhibited by BSO, a selective inhibitor of γ-glutamylcysteine synthetase. Treatment with 5 mM BSO had no effect on ricin cytotoxicity (Fig. 6), even though the glutathione level was decreased to less than 5%. Furthermore, significant protection by NAC against ricin cytotoxicity was still observed in BSO-treated cells in which intracellular GSH was kept at a very low level. These results suggest that protective effect of NAC against ricin-induced apoptosis is mediated through affecting the cellular thiol/disulfide status via acting as a thiol supplier. In agreement with these results, it has been reported that BSO pretreatment did not influence the overall extent of apoptosis of U937 cells induced by puromycin or etoposide, while these reagents also led to extrusion of glutathione concomitant with the onset of apoptosis. Therefore, glutathione depletion may be one of several cellular events associated with apoptotic processes caused by ricin or other apoptotic agents, and GSH depletion alone is insufficient for induction of apoptosis.

The intracellular redox state is generally considered to be coupled to the oxidation state of cysteine residues in proteins by complex thiol/disulfide exchange mechanisms through which redox status influences the activity of a variety of enzymes. Thus, it is possible that the apoptotic signalling pathway is modulated by redox regulation of specific proteins via their thiol groups. For example, protein-tyrosine-phosphatases have a reactive cysteine residue in their active site. Thus, decreases in GSH, a major intracellular thiol, may lead to alterations in the activity of redox-sensitive enzymes, including protein-tyrosine kinases and protein-tyrosine phosphatases. There are other examples of redox-regulated proteins, such as NF-κB and AP-1, which appear to be involved in responding to changes in intracellular redox status. There is no direct evidence for a general requirement of these proteins in apoptosis, but efficient activation of NF-κB-dependent genes by TNF requires that a cell is in an oxidized redox state, suggesting that TNF action may exert only a limited response if the cell is not in an appropriate redox equilibrium. AP-1, which has recently been characterized as an antioxidant-responsive factor, may also contribute to regulation of apoptosis. Identification of the critical redox-active component(s) that interact with NAC is needed to understand the complex apoptotic signalling pathway induced by ricin.

References