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Suppression of Anti-microtubule Agent-induced Apoptosis by Nitric Oxide: Possible Mechanism of a New Drug Resistance

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The propensity of a cell to undergo apoptosis has been proposed to be a determinant of sensitivity to anti-microtubule agents. The anti-microtubule agents vincristine and paclitaxel induce key features of apoptosis, such as intranucleosomal DNA fragmentation and changes in nuclear morphology in the human neuroblastoma cell line, NB-39-nu. Nitric oxide (NO) generated from NO-releasing drugs prevented anti-microtubule agent-induced apoptosis in this cell line. The mechanism of suppression of apoptosis by NO appears to be via the inhibition of an interleukin-1β converting enzyme-like protease cascade. This finding reveals a new biological function of NO, as well as a new molecular insight into resistance to chemotherapy with anti-microtubule agents.

Key words: Nitric oxide — Apoptosis — Anti-microtubule agent — ICE protease cascade

The appearance of cellular resistance to antitumor agents prevents effective cancer chemotherapy. Microtubules are one of the major subcellular targets of antitumor agents. At least two types of resistance to microtubule-associated antitumor agents have been identified; one involving the presence of the multi-drug resistance (*MDR*) gene, which codes for membrane P-glycoprotein,[1, 2] and the other, a mutation affecting either the α- or β-subunit of tubulin.[3] Recently it has been proposed that induction of apoptosis is a determinant of cell sensitivity to the actions of anti-microtubule agents.[4–6] Thus, resistance to apoptosis induction may represent yet another mechanism of drug resistance.

Numerous studies have established that endogenously generated or exogenously supplied NO is potentially toxic in macrophages, pancreatic β-cells, chondrocytes, and thymocytes.[7–10] In contrast, it has also been reported that NO inhibits neuronal cell death induced by withdrawal of neurotrophic factors from culture medium,[11] and that NO inhibits apoptosis in human B cell lines[12] or in endothelial cells treated with tumor necrosis factor α (TNF-α).[13] Thus, the role of NO in cell death is controversial, and the precise mechanisms of its effects are unclear. In this *in vitro* study, we demonstrate a protective effect of NO against anti-microtubule agent-induced cytotoxicity in human neuroblastoma NB-39-nu cells, and discuss possible mechanisms involved.

MATERIALS AND METHODS

Chemicals Paclitaxel (Taxol) was kindly provided by Bristol-Myers Squibb (Tokyo). Vincristine sulfate, *N*-acetyl-DL-penicillamine (AP), and 7-amino-4-methylcoumarin (AMC) were purchased from Sigma (St. Louis, MO). *S*-Nitroso-*N*-acetyl-DL-penicillamine (SNAP) and (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitroso-3-hexenamide (NOR3) were purchased from Dojin Chemicals (Kumamoto). Fluorescent substrate, Ac-Asp-Glu-Val-Asp-AMC for CPP32 (Caspase-3)-like proteases and CPP32-inhibitor, Ac-Asp-Glu-Val-Asp-CHO, were purchased from the Peptide Institute (Osaka).

Cell lines and culture conditions The human neuroblastoma cell line, NB-39-nu[14] was cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY) with 10% fetal calf serum (MultiSer Cytosystems, Australia) at 37°C under 5% CO_2 in air. At 50% confluency, the culture medium was changed, and the cells were treated with anti-microtubule agents and/or NO generators 4 h later.

Cell survival Cells were stained with 0.2% trypan blue, and trypan blue-excluding cells were counted microscopically using a hemocytometer.

DNA fragmentation DNA from cells was prepared as reported previously.[15] Purified DNA was dissolved in 1 ml of TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) buffer, and a 2 μg aliquot was loaded on a 2% agarose gel and separated by electrophoresis. Separated DNA was then visualized by staining with 0.5 μg/ml ethidium bromide.

Nuclear morphology Cells were collected by cell scraping in the culture medium. They were washed with Hanks buffered saline (Gibco BRL), resuspended in 40 μl of HBS and added to 360 μl of solution containing 0.7% NP-40, 4.7% formaldehyde and 11 μg/ml H33258 in phosphate-buffered saline. Nuclei were then viewed under a fluorescence microscope (Nikon, Tokyo).

Measurement of CPP32-like activity NB-39-nu cells were treated with or without vincristine (1 μg/ml), SNAP (0.5 m*M*), and/or AP (0.5 m*M*) for 6, 12, 18, 24 and 30 h at 37°C. Cytosolic extracts were then prepared by repeated freezing and thawing of cells in 100 μl of extraction buffer (50 m*M* PIPES-NaOH, pH 7.0, 50 m*M* KCl, 5

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m*M* EGTA, 2 m*M* $MgCl_2$, 1 m*M* DTT, 1 m*M* PMSF, 1 μg/ml leupeptin) as described previously.[16] Five microliters of cell lysate (25 μg protein) was diluted with 45 μl of interleukin-1β converting enzyme (ICE) standard buffer (100 m*M* HEPES-KOH, pH 7.5, 10% sucrose, 0.1% 3-(cyclohexylamino)-1-propanesulfonic acid, 10 m*M* DTT and 0.1 μg/ml ovalbumin), and incubated at 30°C for 30 min with 100 μM fluorescent substrate, Ac-Asp-Glu-Val-Asp-AMC. The fluorescence of the cleaved substrate was measured using a fluorometer set at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The amount of AMC was calculated from a standard curve obtained with known amounts of AMC. The protein concentration was measured by the Bradford method with an assay kit (Bio-Rad, Richmond, CA). One unit corresponds to the activity that cleaves 1 pmol of the fluorescent substrate at 30°C in 30 min.

Western blot analysis Cells were harvested and washed with phosphate-buffered saline three times. Crude cell extracts were prepared by sonication in extraction buffer (0.6 *M* NaCl, 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 0.5 m*M* DTT, 10 m*M* $NaHSO_3$, 1 m*M* PMSF, 1 m*M* leupeptin, and 7.5 m*M* 3-aminobenzamide) as described previously.[17] The protein concentration was determined by the Bradford method with an assay kit (Bio-Rad). Twenty micrograms of the crude cell extract was separated on 8% sodium dodecyl sulfate polyacrylamide gel, and transferred to an Immobilon membrane (Millipore). Human poly(ADP-ribose) polymerase (PARP) protein was detected by an anti-human PARP-specific guinea pig antibody, and visualized by reaction of the avidin-biotin complex with 3,3′-diaminobenzidine as a chromophore.

RESULTS

Suppression of anti-microtubule agent-induced cytotoxicity by NO To determine the effect of NO on vincristine cytotoxicity, NB-39-nu cells were co-treated with vincristine (1 μg/ml) and various concentrations of SNAP, a chemical NO-generator, for 48 h. Cell survival was determined by use of the trypan blue dye-exclusion assay. As shown in Fig. 1A, vincristine-induced cell death was reversed by SNAP in a dose-dependent manner. As with vincristine (shown in Fig. 1A), treatment with paclitaxel (10 μ*M*) also decreased the number of viable cells to less than 20% of the initial cell number plated, and SNAP treatment protected cells from death induced by paclitaxel, resulting in approximately 80% cell survival at 48 h (Fig. 1B). The protective effect of NO was evident even after prolonged treatment with anti-microtubule agents. Treatment with vincristine or paclitaxel alone decreased the number of viable cells to less than 10% of the initial cell number plated. However, SNAP treatment protected cells from death induced by vincristine or paclitaxel, resulting in approximately 50% cell survival at 72 h. A similar protective effect of NO on vincristine-induced cell death was also observed in human hepatocellular carcinoma cells (HepG2) and a human T cell line (Jurkat) (data not shown).

To exclude the possibility of chemical inactivation of vincristine or paclitaxel by SNAP, vincristine (1 μg/ml) or paclitaxel (10 μ*M*) was incubated with or without SNAP (0.5 m*M*) for 48 h at 37°C in cell-free culture medium, and then the drug-containing medium was assayed for cytotoxicity. Since the half-life of SNAP in an aqueous solu-

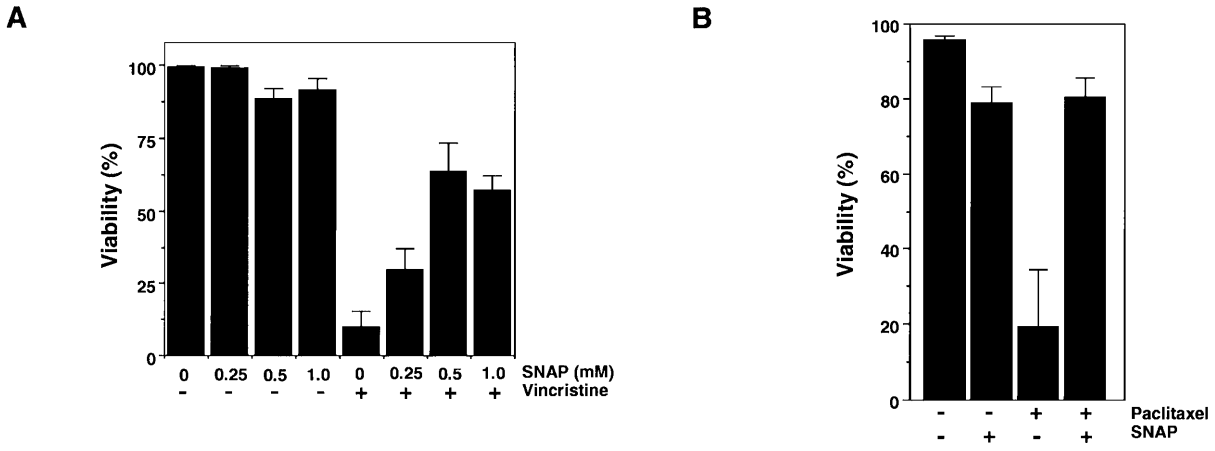

Fig. 1. Suppression of anti-microtubule agent-induced cytotoxicity in human neuroblastoma NB-39-nu cells by SNAP. A, Cells were treated with vincristine (1 μg/ml) and/or SNAP (0. 25, 0.5 or 1 m*M*) for 48 h. B, Cells were treated with paclitaxel (10 μ*M*), and/or SNAP (0.5 m*M*) for 48 h. Cell viability was determined by trypan blue-exclusion assay. Viability is expressed as the percent of initial cell number surviving. Data are mean values of 3 independent experiments.

tion is about 8 h, and NO has a half-life of several seconds in aqueous solution, no pharmacologically active concentration of SNAP or NO would have been present at 48 h. Upon culture of cells in the treated medium, no decrease in the antitumor activity of vincristine or paclitaxel by NO was observed (data not shown).

The activation of soluble guanylyl cyclase is one of the best-characterized biochemical consequences of NO treatment of cells.[18] Recently it has been demonstrated that NO delays the death of neurotrophic factor-depleted neuronal cell cultures via a cGMP-mediated mechanism.[11] We thus examined whether activation of soluble guanylyl cyclase (sGC) by NO is necessary for inhibition of anti-microtubule agent-induced cell death. When 1 μM LY83583 or 5 mM methylene blue (sGC inhibitors) was added to cells treated with vincristine (1 μg/ml) and SNAP (0.5 mM), suppression of vincristine-induced cell death by SNAP was not affected (data not shown). These results indicate that suppression of vincristine-induced apoptosis by NO is independent of sGC activation.

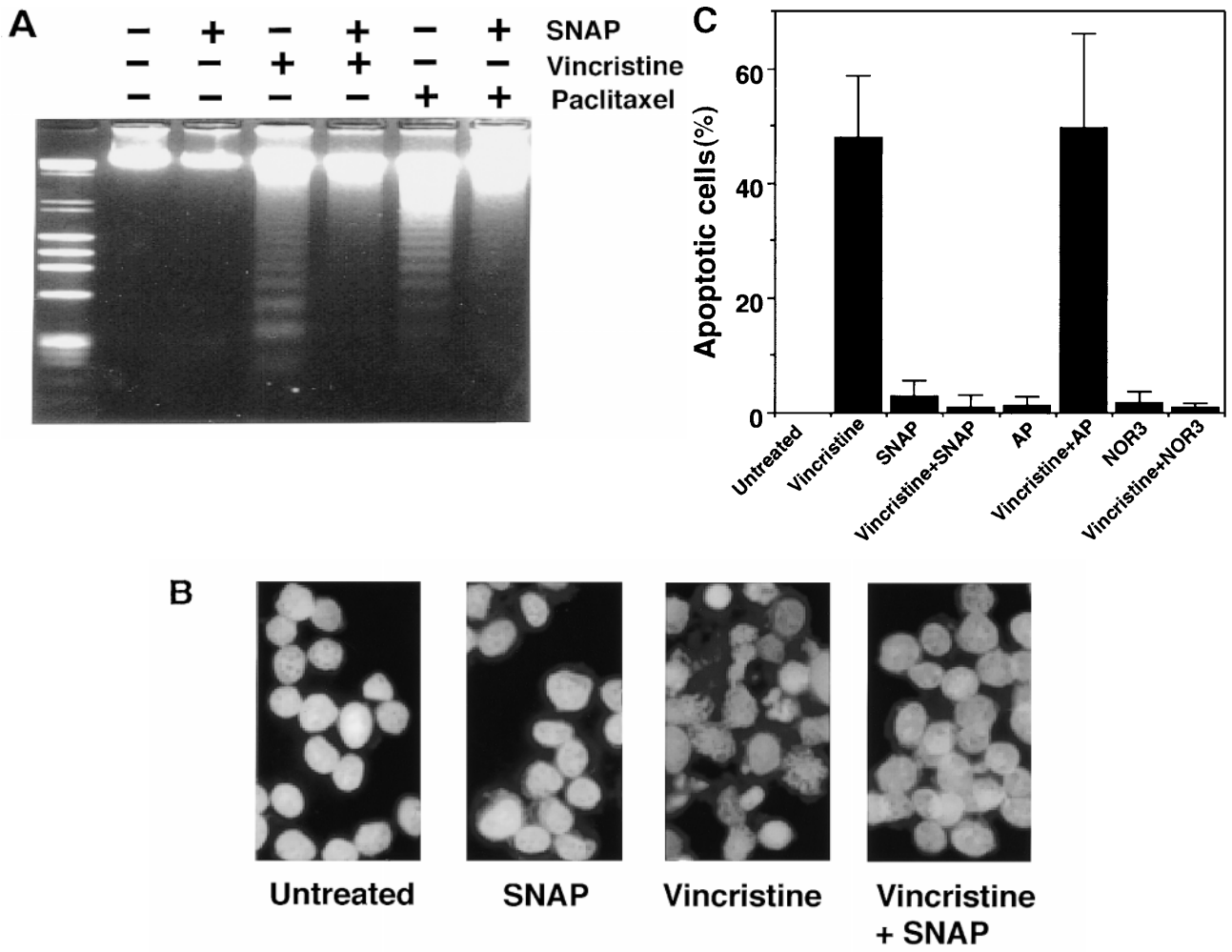

Fig. 2. Suppression of anti-microtubule agent-induced apoptosis by NO. A, Suppression of DNA ladder formation by SNAP in anti-microtubule agent-treated cells. Cells were treated with vincristine (1 μg/ml) or paclitaxel (10 μM) with or without SNAP (0.5 mM) for 36 h. DNA (2 μg) was analyzed on a 2% agarose gel. A ϕ×174 DNA digested with *Hae*III was used as a size marker. B, Suppression of nuclear morphological changes by SNAP in vincristine-treated cells. Cells were treated with vincristine (1 μg/ml) and/or SNAP (0.5 mM) for 24 h. C, Suppression of vincristine-induced apoptosis by NO. Cells were treated with SNAP, AP, or NOR3 (each at 0.5 mM) with or without vincristine (1 μg/ml) for 24 h. Apoptotic cells were observed after staining with DNA-binding dye H33258 under a fluorescence microscope. Apoptotic cells are expressed as percent of fragmented nuclei per total nuclei. Data are mean values of 3 independent experiments.

Suppression of anti-microtubule agent-induced apoptosis by NO Anti-microtubule agents are known to induce apoptosis in a variety of cell types.[4–6, 19–21] We therefore examined whether protection from anti-microtubule agent-induced cell death by SNAP is due to suppression of the induction of apoptosis by NO. Two characteristic parameters were analyzed: 1) DNA fragmentation and 2) the appearance of condensed and fragmented chromatin in cell nuclei. For detection of DNA fragmentation, NB-39-nu cells were treated with vincristine (1 μg/ml) or paclitaxel (10 μM) with or without SNAP (0.5 mM) for 24 h, and then the pattern of DNA fragmentation was analyzed. As shown in Fig. 2A, fragmented DNA was not detected in control or SNAP-treated cells. However, pronounced DNA ladder formation was observed in vincristine- or paclitaxel-treated cells. In addition, DNA ladder formation by both anti-microtubule agents was effectively inhibited by SNAP. Condensed chromatin and fragmented nuclear morphology were also detected in cells upon staining with the DNA-binding dye H33258. As shown in Fig. 2B, the nuclei of NB-39-nu cells were homogenous and round in untreated cells, with nuclear morphological changes being observed in less than 1% of SNAP-treated cells. Conversely, more than 50% of nuclei in cells treated with vincristine alone showed severe morphological changes, displaying condensed and fragmented chromatin. Nuclei of cells treated with both vincristine and SNAP showed no morphological changes. To determine whether NO itself is the molecular entity that confers protection from vincristine-induced apoptosis, NOR3 (0.5 mM), another chemical NO-generator, and AP (0.5 mM), a non-NO-generating analogue of SNAP, were investigated. As shown in Fig. 2C, no morphological changes were observed in cell nuclei treated with NOR3 or AP. Like SNAP, NOR3 inhibited vincristine-induced nuclear morphological changes, and >90% of cell nuclei were found to be intact. However, AP treatment did not inhibit the morphological changes of nuclei induced by treatment with vincristine.

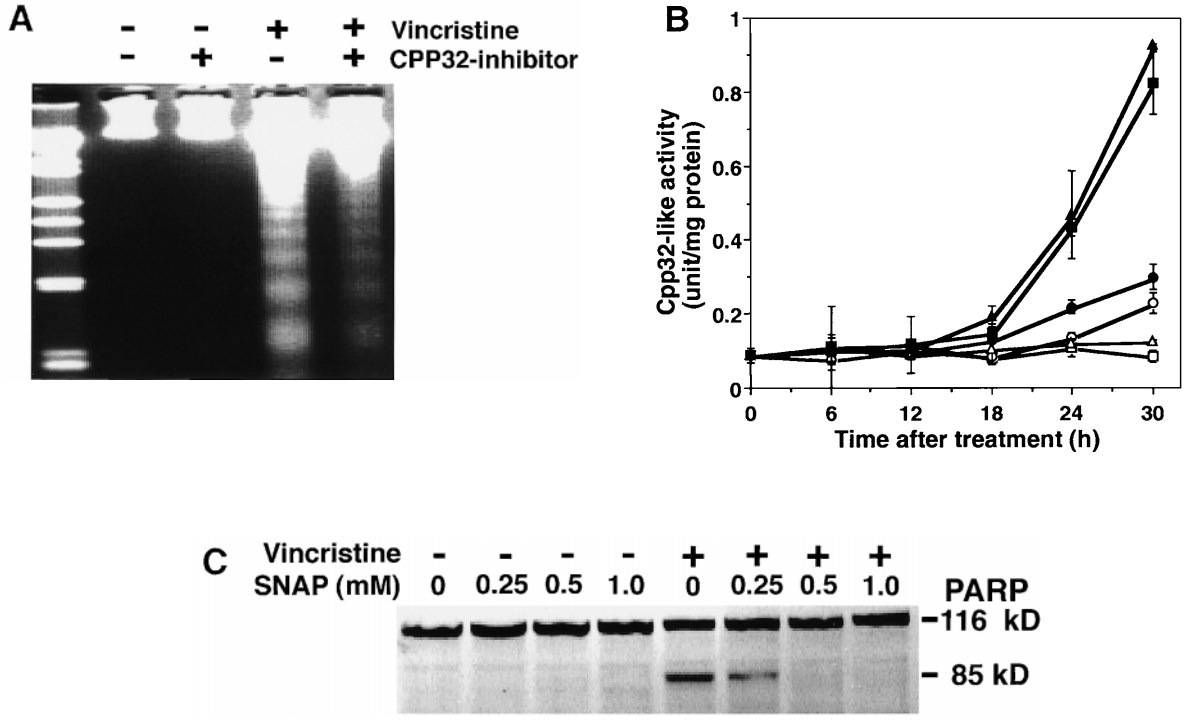

Fig. 3. Involvement of activation of CPP32-like protease in vincristine-induced apoptosis, and suppression of its activation by SNAP. A, Suppression of DNA ladder formation by CPP32-inhibitor in vincristine-treated cells. Cells were treated with vincristine (1 μg/ml) with or without or CPP32-inhibitor (200 μM) for 36 h. DNA (2 μg) was analyzed on a 2% agarose gel. A ϕ×174 DNA digested with *Hae*III was used as a size marker. B, Suppression of activation of CPP32-like proteases by SNAP in vincristine-induced apoptosis. Vincristine (1 μg/ml) was added to NB-39-nu cells with or without SNAP (0.5 mM) or AP (0.5 mM) for the times indicated. CPP32-like activity was measured as described in "Materials and Methods." Results were similiar in three independent experiments. △ untreated, ▲ vincristine, ○ SNAP, ● vincristine and SNAP, □ AP, ■ vincristine and AP. C, Dose-dependent suppression by SNAP of the specific cleavage of PARP by CPP32-like proteases. Cells were treated with vincristine (1 μg/ml) with or without various concentrations of SNAP for 36 h. PARP protein was detected by western blotting as described in "Materials and Methods."

Suppression of ICE protease cascade in vincristine-induced apoptosis by NO Regulation of the activation of the ICE protease cascade is thought to be critical for apoptosis.[22] To elucidate the molecular mechanism of the inhibition of anti-microtubule agent-induced apoptosis by NO, we examined whether activation of the ICE protease cascade is involved in vincristine-induced apoptosis. In addition, we examined whether NO inhibits the activation of the ICE protease cascade. Among members of the ICE family, CPP32-like proteases are thought to be the final proteases in the ICE protease cascade.[22] We first determined whether the activation of CPP32-like proteases is involved in vincristine-induced apoptosis. A CPP32-inhibitor (Ac-Asp-Glu-Val-Asp-CHO, 200 μM) partly inhibited DNA fragmentation in vincristine-treated cells (Fig. 3A). NB-39-nu cells were treated for 6, 12, 18, 24, and 30 h with vincristine and the CPP32-like activity in the cytosolic extracts was then determined by using the fluorescent substrate, Ac-Asp-Glu-Val-Asp-MCA. As shown in Fig. 3B, CPP32-like activity was detectable at 18 h, and a high level of activity was observed at 30 h. Moreover, these results suggest that the activation of CPP32-like proteases may have a role in the process of vincristine-induced apoptosis. The effect of SNAP on the activation of CPP32-like proteases in vincristine-induced apoptosis is shown in Fig. 3B. When compared to vincristine alone, an approximately 70% decrease in CPP32-like activity was observed in cells co-treated with vincristine and SNAP. At 30 h, the activity in SNAP plus vincristine-treated cells was similar to that in cells treated with SNAP alone. No significant increase in CPP32-like activity was observed in untreated cells at any time point. Moreover, AP was without effect on CPP32-like activity in vincristine-treated cells, further indicating that NO protection is essential for the inhibitory effect.

PARP is proteolyzed during apoptotic processes and may serve as a natural substrate for CPP32-like proteases.[23, 24] Therefore we determined whether NO inhibits the specific proteolytic cleavage of PARP, using western blot analysis. NB-39-nu cells were treated with SNAP (0, 0.25, 0.5 or 1 mM) and/or vincristine (1 μg/ml) for 36 h. As shown in Fig. 3C, SNAP alone did not cause the specific proteolytic cleavage of PARP. However, vincristine treatment caused PARP cleavage, resulting in the production of 85 kD and 31 kD protein fragments, and this cleavage was effectively inhibited by SNAP treatment. The dose-dependent inhibition of PARP cleavage by SNAP was consistent with the dose-dependent suppression of cell death by SNAP (Figs. 1A and 3C)

DISCUSSION

Pre-clinical and clinical studies of anti-microtubule agents have shown a wide range of anti-tumor activity against several types of tumor.[25–27] Resistance to anti-microtubule agents has been reported to involve the presence of the *MDR* gene and also a mutation affecting the gene for either the α- or β-subunit of tubulin.[1–3] Recently it has been indicated that the induction of apoptosis is a determinant of cell sensitivity to the actions of various anti-tumor agents including anti-microtubule agents.[4–6] In the present study, we demonstrated the suppression of anti-microtubule agent-induced apoptosis by SNAP, and showed that NO itself is responsible for the inhibition of anti-microtubule agent-induced apoptosis. Interestingly, the cells treated with SNAP and vincristine, but not with vincristine alone, for 48 h started to grow upon withdrawal of those agents from the culture medium (unpublished data). From a clinical perspective, NO is endogenously produced in some tumors or in infiltrating cells surrounding tumors. In addition, anti-microtubule agents induce inducible NO synthase expression in some tumor cells (unpublished data). Thus, under conditions where NO is produced in the body, tumor cells may more easily acquire resistance to anti-microtubule agents.

Apoptosis occurs in response to specific cell-death signals activated by stimulation of Fas or tumor necrosis factor receptors, DNA damage or growth factor withdrawal.[28, 29] The regulation of the ICE protease cascade is thought to be critical for apoptosis.[22] Our results suggest the suppression of anti-microtubule agent-induced apoptosis by NO. Our data clearly show that CPP32-like protease activation is involved in vincristine-induced apoptosis, and NO inhibits this activation. Thus, inhibition of the ICE protease cascade by NO is involved in the suppression of anti-microtubule agent-induced apoptosis.

Recent research has demonstrated that ICE-like proteases are inhibited by viral proteins (CrmA or p35), and by truncated products of their own gene.[22] In the present study, we have obtained evidence indicating that endogenously produced NO also has a role as a cellular inhibitory factor of the ICE-like protease cascade in apoptosis.

It has been proposed that thiol- and metal-containing proteins serve as major targets for NO, and *S*-nitrosylation of thiol residues modulates protein function.[30] ICE proteases are likely to be intracellular targets of NO since they have a cysteine residue at the catalytic site.[22] Recently it has been reported that NO directly inhibits TNF-α-induced ICE protease activity via *S*-nitrosylation of the cysteine group at the catalytic site.[13] We have also demonstrated that CPP32-like enzyme activity could be inhibited reversibly by SNAP when a free sulfhydryl agent was omitted from the enzymatic reaction mixture.[31] The present data show that NO treatment decreased intracellular levels of active CPP32-like proteases. It is not clear at present whether NO acts directly on the enzyme to cause this decrease in CPP32-like activity. However direct inactivation of the enzyme by NO seems unlikely, since the CPP32-like activity was unchanged in the presence

of dithiothreitol, which can reactivate the NO-inhibited activity.

Overexpression of Bcl-2 protein is also reported to overcome apoptotic cell death by anti-microtubule agents.[32, 33] Therefore, overexpression of Bcl-2 by NO might be another possible mechanism of inhibition of anti-microtubule agent-induced apoptosis. Recently it has been demonstrated that cytochrome *c* release from mitochondoria triggers a variety of features of apoptosis.[34-36] NO may also modulate the biological function of holocytochrome *c* in apoptosis. We are currently investigating these possibilities.

In the present study, we have demonstrated that NO suppresses anti-microtubule agent-induced apoptosis. This novel finding will facilitate the identification of the molecular mechanisms of NO action under physiological or pathophysiological conditions *in vivo*, and also offer new insights at the molecular level into resistance to chemotherapy by anti-microtubule agents.

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