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Evidence for the Association of Cellular Iron Loss in Nitric Oxide-induced Apoptosis of HL-60 Cells: Involvement of p38 Kinase, c-Jun N-terminal Kinase, Cytochrome C Release, and Caspases Pathways

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Nitric oxide has high affinity for iron, and thus it can cause intracellular iron loss. We tested the idea that intracellular iron can be the primary target of NO toxicity by comparing the signaling mechanisms involved in cell death caused by iron depletion and that caused by NO. Treatment of HL-60 cells with a NO donor, S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), decreased the intracellular iron level rapidly as that observed with the iron chelator deferoxamine (DFO). Iron chelators such as DFO and mimosine could induce death of human leukemic HL-60 cells by a mechanism requiring activation of p38 kinase, c-Jun N-terminal kinase, caspase-3 and caspase-8. DFO and SNAP also caused release of cytochrome c from mitochondria. Inhibition of p38 kinase by a selective inhibitor, SB203580, abolished the NO and DFO-induced cell death, release of cytochrome c, and activation of caspase-3 and caspase-8, thus indicating that p38 kinase lies upstream in the cell death processes. In a parallel situation, the cells that are sensitive to NO showed similar sensitivity to DFO. Moreover, simultaneous addition of ferric citrate, an iron-containing compound, inhibited the SNAP and DFO-induced activation of caspases and also blocked the NO-mediated cell cycle arrest at G_1 phase. Collectively, our data implicate that the NO-induced cell death of tumor cells including HL-60 cells is mediated by depletion of iron and further suggest that activation of p38 kinase lies upstream of cytochrome c release and caspase activation involved in this apoptotic process.

It is now clear that nitric oxide (NO)[3] either produced by activated host cells or delivered exogenously by NO donors induces apoptosis in various cell types both *in vitro* and *in vivo*. Although NO is known to mediate many physiological functions by activation of soluble guanylate cyclase and formation of guanosine 3',5'-cyclic monophosphate, the process involved in concomitant phosphorylation of signal proteins leading to the toxic or apoptotic effect of NO is still an enigma. Possible mechanisms for the apoptotic processes include interactions of NO with iron or thiol groups contained in many of the key functional enzymes with iron-sulfur centers or protein thiols (Nathan, 1992), the NAD(H)-dependent modification of glyceraldehyde-3-phosphate dehydrogenase (Mohr et al., 1996), or direct DNA damage (Wink et al., 1991). The reactions with oxygen, superoxide, thiols, and transition metals are also implicated (Beckman et al., 1990).

Recently, Feger et al. (2001) demonstrated that the addition of NO donor to the culture medium of tumor cells caused a rapid decline in intracellular iron levels, similar to the effect caused by addition of deferoxamine (DFO), a potent chelator of iron. Because iron loss by itself is cytotoxic to tumor cells, as observed with DFO (Fukuchi et al., 1994), rapid decreases in intracellular iron levels are likely to be the principal mechanism by which NO induces apoptotic cell death (Feger et al., 2001). In good agreement with these reports, after careful survey of the literature, we found an interesting reciprocal relationship between NO and iron affecting cell survival and growth. Although the involved mechanism has not been defined, addition of

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NO inhibited iron-mediated lipid peroxidation and damage of PC12 cells (Nara et al., 1999). Similar NO-induced protection against iron-mediated cytotoxicity has been reported *in vitro* in rat hepatocyte culture (Kim et al., 2000, Sergent et al., 1997) and brain homogenate (Rauhala et al., 1996) and *in vivo* in iron-infused rat substantia nigra (Rauhala et al., 1998). Conversely, NO by itself has been demonstrated to produce cytotoxicity in some cell types like the murine macrophage cell line (RAW264.7) (Jun et al., 1999), human leukemia (HL-60) (Jun et al., 1998, Jun et al., 1997) and tumor cell lines of liver (AKN1), lung (A549), skin (HaCat), and pancreas (Capan-2) (Feger et al., 2001). In these cells, the NO-mediated cytotoxicity or apoptosis was rescued either by addition of iron or by elevation of intracellular iron levels (Feger et al., 2001, Kim et al., 2000). Taken together, these reports implied that NO and intracellular iron levels have a reciprocal interaction in regulating the survival and death of cells. Because NO is known to have strong affinity for iron or iron complexes, one could imagine that NO may activate intracellular signals that cause corresponding deprivation or lowering of intracellular iron levels. If this is the case, those cells which are sensitive to NO would be equally sensitive to iron deprivation.

It has been demonstrated that iron deprivation inhibits cyclin-dependent kinase activity, and thereby reduces the proliferative potential of human breast cancer cells (Kulp et al., 1996). In addition, HL-60 cells and proliferating T cells treated with iron chelators were demonstrated to undergo apoptosis (Fukuchi et al., 1994, Gazitt et al., 2001), and this suggested that intracellular iron levels play a significant role in regulating the programmed cell death. Given the potential physiological relevance of NO with the iron deprivation-mediated consequence, it may be essential to verify the signaling pathways involved in the loss of intracellular iron by comparing them with the signals mediated by NO at a fundamental level.

As a part of NO research in apoptosis, an interesting relationship between NO and caspases activity has emerged (Yabuki et al., 1997). Caspases are a family of cysteine proteases that play an essential role in the signaling cascade leading to apoptosis. Previously, we and other groups reported that the NO-induced cell death involves the activation of capase-3 both in HL-60 and RAW264.7 cells (Jun et al., 1999, Yabuki et al., 1997). In addition to caspase-3, activation of caspase-8 following the release of cytochrome c has also been reported to occur in human neoplastic lymphoid cells during NO-induced apoptosis (Chlichlia et al., 1998). Although the processes involved in NO-dependent activation of caspases are not defined yet, results of a recent study suggested a potential role of non-heme iron content in the NO-mediated activation of caspases and apoptosis (Kim et al., 2000). In our previous study dealing with the NO-induced apoptosis, we found that, in addition to the activation of caspase, the activation of p38 kinase belonging to the mitogen- activated protein kinases (MAPK) plays a crucial role as well (Jun et al., 1999). However, the c-Jun N- terminal protein kinase (JNK) and extracellular signal regulated kinase (ERK) did not appear to be significantly involved in the NO-induced apoptotic process (Jun et al., 1999).

In this report, by exploring the signal pathways that lead to depletion of intracellular iron, we hypothesized that the loss of iron may play a crucial role in the mechanism of NO-induced cell death. To gain further insight into the relationship between NO and the loss of iron, we assessed the effects of adding exogenous NO donor and iron chelators on the survival or death of various cell types including HL-60 cells.

Materials and Methods

Materials

Antibodies against p38 kinase, JNK, ERK-1 and ERK-2, and caspase-3 and the antibodies specific to the phosphorylated forms of these proteins were purchased from Cell Signaling Technology, Inc. Cytochrome c antibody was obtained from PharMingen. S-nitorso-N-acetyl-DL-penicillamine (SNAP) was prepared as described previously (Li et al., 1997). SB203580, PD098059, propidium iodide (PI), DAPI, and the assay kits for caspase-3 and caspase-8 were purchased from Calbiochem-Behring Corp. DFO, mimosine (MIM), potassium ferricyanide (PFC), and ferric citrate (FC) were purchased from Sigma Chemical Company. Genomic DNA purification kit was obtained from Promega. All reagents used for *in situ* nick translation were obtained from Oncor.

Cell culture

HL-60 (human promyeloid leukemic cells), RAW264.7 (murine macrophage cell line), MBT-2 (murine bladder tumor cell line), immortalized HUVEC (human umbrical vascular endothelial cells), HSV-transformed human keratinocyte, and CHO-K1 (Chinese hamster ovary cells) were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) and incubated at 37℃ in a humidified atmosphere containing 5% CO_2 and 95% air. Exponentially growing cells were seeded at 5×10^5 per twelve-well plate, and used for various experimental purposes.

Cell viability and apoptosis assay

Cell viability was determined by PI incorporation (Jun et al., 1999). Cells were washed with PBS twice, resuspended in PBS containing 20 μg/mL PI, and then analyzed immediately on a FACStar (Becton-Dickinson). Cells treated with various agents were cytospined, fixed in 4% neutral buffered paraformaldehyde, and

permeabilized with PBS/0.5% Triton X-100, and their nuclei were stained for 5 min with DAPI dye. The coverslips were then washed, mounted onto slides, and viewed with a fluorescence microscope.

Quantitation assay of apoptosis

To quantitate the number of cells undergoing apoptosis, cells were fixed with 4% neutral buffered formalin. Apoptotic cells were stained using the terminal deoxynucleotidyl transferase (TdT) method (Apotag). Endogenous peroxidase was first quenched with 2% hydrogen peroxide, and the cells were permeabilized using the company-supplied equilibration buffer. The 3'OH ends of DNAs were reacted with TdT and digoxygenin-labeled ATP for 30 min. After washing with PBS, the cells on slides were reacted with an anti-digoxygenin mAb conjugated to peroxidase, washed, and developed with 3,3'-diaminobenzidine tetrahydrochloride. Stained cells were then counted using a light microscope.

Flow cytometric analysis

Flow cytometric analysis was also performed as described previously to identify the apoptotic cells (Krishan, 1975). Approximately 10^6 cells per experimental condition were harvested, washed with HBSS, and sequentially resuspended in a solution containing 0.1% Nonidet P-40 and 50 μg/mL PI. The content of DNA per cell was estimated by flow cytometry.

DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by employing agarose gel electrophoresis as described previously (Maciejewski et al., 1995). Briefly, genomic DNA was purified using a Wizard genomic DNA purification kit (Promega). After ethanol precipitation, DNA samples of 10 μg in each lane were subjected to electrophoresis on a 1.4% agarose at 50 V for 3 h and stained with ethidium bromide.

Phosphorylation assay of p38 kinase, JNK, or ERK

Cells were stimulated according to designated experimental protocols. Proteins were extracted with an extraction buffer (50 mM Tris-HCl, [pH7.4], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM 4-nitrophenyl phosphate, 10 μg/mL of leupeptin, 10 μg/mL of pepstatin A, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). Activation of MAP kinase was examined by determining its phosphorylation state using the antibodies specific to the phosphorylated forms of p38 kinase, JNK, and ERK1 and ERK2.

Western blotting analysis

Protein samples (50-100 μg) were mixed with an equal volume of 2X SDS sample buffer, boiled for 5 min, and then separated through 8-15% SDS-PAGE gels. After electrophoresis, proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with primary antibodies (diluted at 1:500-1:1000) in TBS overnight at 4℃. Primary antibody was then removed by washing the membranes four times in TBS, and labeled by incubating with 0.1 μg/mL peroxidase-labeled secondary antibodies (against mouse and rabbit) for 1 h. Following three washes in TBS, bands were visualized by ECL and exposed to X-ray film.

Assay for caspase activity

Both caspase-3- and caspases-8-like activities were measured by colorimetric assay using the peptide-based substrates Ac-DEVD-*p*NA and Ac-IETD-*p*NA, respectively, as described in the company provided manual (Calbiochem-Behring Corp). At specific times after treatment with agents, cells were collected by scraping in cold PBS, centrifuged, and lysed on ice for 10 min in the cell lysis buffer provided in the caspase-3 and caspase-8 assay kits. The extracts were then reacted with the colorimetric caspases substrates (Ac-DEVD-*p*NA or Ac-IETD-*p*NA), maintained at 37℃, and the color changes were analyzed with a 96-well microtest plate spectrophotometer (Molecular Devices) at 10 min intervals during the 3 h incubation.

Evaluation of cytochrome c release from mitochondria

At designated time points following exposure to experimental treatments, cells (1×10^7) were trypsinized and then washed with ice-cold buffer A (250 mM sucrose, 20 mM HEPES-KOH, 1 mM EDTA, 1 mM EGTA, 2 μg/mL leupeptin, and 1 μg/mL pepstatin, pH 7.4). Cell were resuspended in 200 μL of buffer A and carefully homogenized using a Dounce homogenizer. The homogenates were separated into cytosol (supernatant) and mitochondrial fractions (pellet) by differential centrifugation. Cytosolic (300 μg) and mitochondrial (50 μg) proteins were then subjected to immunoblot analysis using a monoclonal cytochrome c antibody as described below.

Determination of intracellular iron content

After incubation of HL-60 cells (2×10^6) treated with various reagents, the cells were washed twice with PBS and then incubated for 30 min at 4℃ in 100 μL of lysis buffer (1% Triton, 10% glycerol and 25 mM HEPES, pH 7.4 in PBS). The lysates were incubated for 20 min at 22℃ in 0.3 N HCl, TCA-precipitated and finally centrifuged at 1,200 g for 15 min. The supernatants were mixed with chromogen (0.51 mM disul--phonate bathophenantroline, 1.3 M sodium acetate, pH 4.6, 30 mM sodium pyrosulphite, and 1.83 mM p-(N-methyl)aminophenol) and the absorbance was deter-

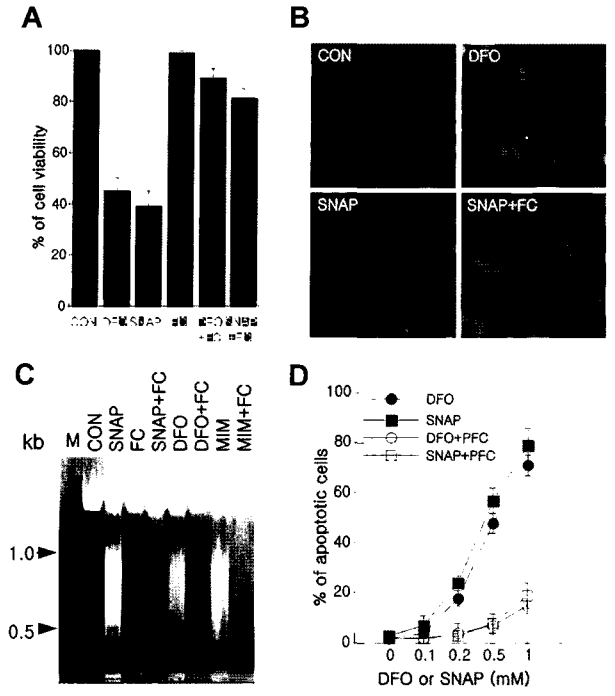

Fig. 1. Both iron chelators and NO induce cell death associated with DNA fragmentation. A-C, HL-60 cells ($0.5\text{-}1 \times 10^6$ cells/well) were treated with DFO (0.5 mM), MIM (0.5 mM) or SNAP (0.5 mM) in the presence or absence of FC (0.5 mM) for 18 h. Cell viability was measured by PI incorporation (mean ± SD., n = 3) (A). DNA fragmentation was measured by gel electrophoresis after isolation of genomic DNA (B). Indicated cells were then stained with the fluorescent DNA-binding dye, DAPI; images of DAPI fluorescence of representative microscope fields are shown (C). *D*, cells were treated with various concentrations of DFO or SNAP in the presence or absence of FC (0.5 mM). After 18 h of incubation, the cells were collected, cytospined, and subjected to *in situ* TdT apoptosis assay (mean ± SD., n = 3).

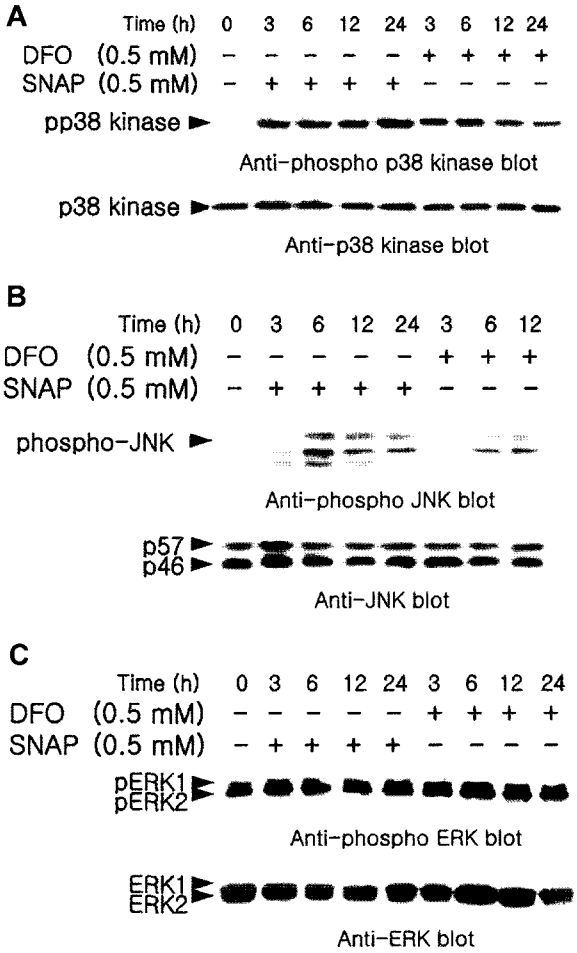

Fig. 2. Both iron chelator and NO activate p38 kinase and JNK but do not affect ERK. HL-60 cells (1×10^6 cells/well) were incubated for various duration (0-24 h) with DFO (0.5 mM) or SNAP (0.5 mM). Protein extracts were prepared at the indicated time points to assess the activation of p38 kinase (A), JNK (B), ERK1, and ERK2 (C). The levels of total (p38, JNK, ERK1 and ERK2) and phosphorylated MAP kinases (p38, JNK, ERK1 and ERK2) were determined by Western blotting using specific antibodies. The arrows indicate the position of specific immunoreactive bands corresponding to distinct MAP kinases.

mined spectrophotometrically at the wavelength of 546 nm. The iron concentration was calculated by interpolating the absorbance of each sample in the presence of iron standard curve and expressed in ng/10^6 cells, as previously described (Leardi et al., 1998).

Results

Activation of p38 kinase and JNK during iron deprivation-induced apoptosis

Cell types differ considerably in their sensitivity to the cytotoxic actions of NO, e.g. human promyeloid leukemic HL-60 cells are known to be more sensitive to the NO-induced toxicity (Jun et al., 1997). Interestingly, this cell also shows a similar degree of sensitivity to the iron deprivation-induced toxicity (Fukuchi et al., 1994). If the primary target of NO is intracellular iron, the depletion of iron induced by pharmacological chelators should produce same signals that follow after an activation by NO. As observed by addition of SNAP (a NO donor), within 9 h after exposure to DFO, a well-known iron chelator, HL-60 cells responded with apoptotic cell death, characterized by chromatin condensation and DNA laddering (Fig. 1). MIM, an iron chelator structurally distinct from DFO, also induced apoptosis. Conversely, the addition of FC (Fe^{3+}) or PFC ($Fe^{3+}CN$) significantly prevented the cell death induced either by SNAP or DFO (Fig. 1). On the contrary, the cell death induced by hydroxyurea, a known S-phase inhibitor, or actinomycin D was not prevented by the addition of PFC, implying that loss of iron has no significant effect on the cell death evoked by different mechanisms (data not shown). SNAP induced apoptosis of HL-60 cells in the concentration ranges of 0.2-0.5 mM, and this was comparable with the apoptosis induced by 0.2-1 mM of DFO, as verified by DAPI staining and *in situ* TdT-apoptosis assay (Fig. 1).

To determine whether the activation of MAP kinase is involved in the DFO- or SNAP-induced apoptosis, HL-60 cells were incubated for various durations (0-24

h) with DFO or SNAP. Then, the cell lysates were prepared and phosphotyrosine-containing bands were visualized by immunoblot analysis. As shown in Fig. 2A, treatment of cells with either DFO or SNAP significantly increased the level of phosphorylated p38 kinase rapidly without affecting the total amounts of p38 kinase protein. JNK was also activated, but at a late stage of incubation (~6 h) (Fig. 2B). Actually, we reported previously that NO delivered by sodium nitroprusside (SNP) has no effect on the JNK activity in HL-60 cells up to 12 h (Jun et al., 1999). It has been noted that the rate of NO release from SNP is relatively slower than that from SNAP and the decomposition products of SNP are known to include CN^-, Fe^{2+} and $[Fe(CN)_6]^4$ (Terwel et al., 2000). The released metal ion, Fe^{2+}, is likely to be responsible for delay or the prevention of JNK activation at early times in HL-60 cells. Thus, the observed discrepancy may have been caused by the use of different NO donor. As previously observed with the NO-treated HL-60 cells, DFO treatment did not induce the phosphorylation of ERK-1 and 2 or the alteration of total amount of ERK protein (Fig. 2C).

Since p38 kinase was shown earlier to contribute to the induction of apoptosis by NO in HL-60 cells (Jun et al., 1999), we considered the possibility that p38 kinase activation is also responsible for the cytotoxic action of DFO. Thus we determined whether the highly specific p38 kinase inhibitor, pyridinylimidazole compound SB203580 (Schwenger et al., 1997), could rescue HL-60 cells from the DFO-mediated cytotoxicity. We found that SB203580 (10-50 μM), which by itself had no effect on cell morphology or viability, significantly reduced the cytotoxic action of DFO (0.5 μM), while PD098059 (10-50 μM), a selective inhibitor of MEK1 kinase (Bitangcol et al., 1998), had no effect (Fig. 3, A and B). These results suggested that, in parallel with the NO-mediated apoptosis, the activation of p38 kinase by iron depletion is involved in the induction of apoptotic cell death in HL-60 cells.

Activation of caspase-3 and caspase-8 and release of cytochrome c during iron deprivation-induced apoptosis

Caspase-3 activity is known to increase in NO-mediated apoptotic cells (Yabuki et al., 1997). Thus, we determined if the caspase-3 activity is also increased in HL-60 cells undergoing apoptosis induced by DFO-derived iron deprivation. Fig. 4A shows that the extracts prepared from HL-60 cells exposed to either DFO (0.5 mM) or SNAP (0.5 mM) contained strong activity of caspase-3 to cleave Ac-DEVD-*p*NA, an exogenous caspase-3 substrate, up to 12 h after the DFO or SNAP treatments. Along with the observations on caspase-3 activity, disappearance of the procaspase-3, an inactive form of caspase-3, was observed in both the DFO- and SNAP-treated cells (Fig. 4B).

Another cellular event associated with cell death is

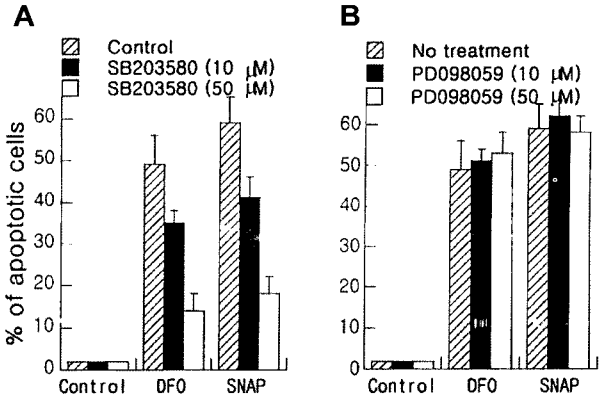

Fig. 3. Induction of apoptosis by DFO or SNAP and their inhibition by SB203580. HL-60 cells were incubated for 18 h with DFO (0.5 mM) or SNAP (0.5 mM) in the presence or absence of SB203580 (A) or PD098059 (B). For the combination treatment, cells were incubated with MAP kinase inhibitors for 1 h before the addition of DFO or SNAP. Quantitative analysis of apoptosis was performed by the *in situ* TdT-apoptosis assay method (mean + SD., n = 4).

the release of cytochrome c from mitochondria (Yang et al., 1997). Therefore, we next examined whether mitochondrial cytochrome c was released into cytosol using Western blot analysis. Treatment with both DFO or SNAP rapidly resulted in cytochrome c release into cytosol (Fig. 4C). These data indicate that the cell death caused by iron deprivation is most likely dependent on caspase-3 activation and cytochrome c release in HL-60 cells. In the meantime, both the caspase-3 activation (Fig. 4D) and cytochrome c release (data not shown) by NO were significantly reduced in the presence of PFC (0.5 mM), thereby implicating that the direct interaction of iron and NO in the process of apoptotic cell death.

Caspase-8 (also known as FLICE, MACH, and Mch5) activation has also been implicated in NO-mediated, CD95 (APO-1/Fas)-dependent and independent apoptosis in human neoplastic lymphoid cells (18). Since caspase-8 has been implicated as the apical signaling protease in Fas-induced apoptosis, we questioned whether caspase-8 is also involved in the iron deprivation-mediated apoptotic process in HL-60 cells. As shown in Fig. 4E, extracts prepared from the cells exposed for 12 h to DFO (0.5 mM) or SNAP (0.5 mM) contained strong caspase-8 activity to cleave Ac-IETD-*p*NA, an exogenous caspase-8 substrate. The activation of caspase-8 was greatly diminished when the cells were supplemented with PFC (0.5 mM) (Fig. 4E).

Activation of p38 kinase lies upstream of mitochondrial membrane potential loss and caspases cascade

We next questioned whether the activation of p38 kinase is associated with the release of mitochondrial cytochrome c and the activation of caspases. To clarify this, HL-60 cells were treated with a selective p38 kinase inhibitor, SB203580 (50 μM), and the cell lysates

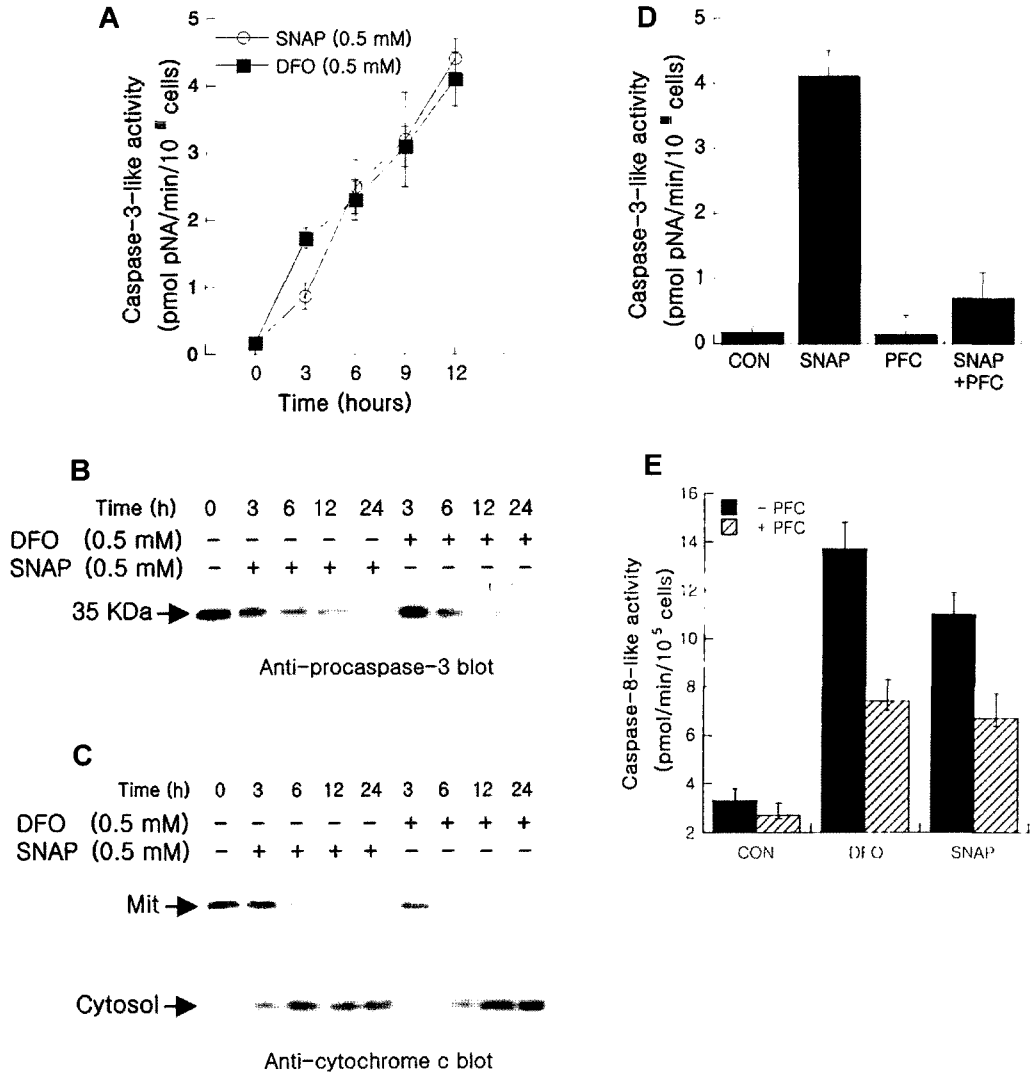

Fig. 4. Both the iron chelator and NO induce caspases-3 and caspases-8 activation and cytochrome c release. *A*, caspase-3-like activity. HL-60 cells (2×10^6 cells/well) were treated with DFO (0.5 mM) or SNAP (0.5 mM). At various time points (0-12 h), the cells were extracted and caspase-3-like activity was measured as indicated in the *Materials and Methods* (mean ± SD., n = 3). *B and C*, procaspase-3 cleavage and cytochrome c release. After treatment with DFO or SNAP for various duration (0-24 h), whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. Procaspase-3 was visualized by Western blot analysis (B). The cell lysates were also further separated to mitochondrial and cytosolic fractions, and then subjected to Western blot analysis using an antibody against cytochrome c (C). *D*, HL-60 cells (2×10^6 cells/well) were treated for 12 h with SNAP (0.5 mM), PFC (0.5 mM), or SNAP in combination with PFC. Caspase-3-like activity was measured as described in A (mean ± SD., n = 3). *E*, caspase-8-like activity. HL-60 cells (2×10^6 cells/well) were treated for 12 h with DFO (0.5 mM) or SNAP (0.5 mM) in the presence or absence of PFC (0.5 mM). After incubation, the cells were extracted and caspase-8-like activity was measured as indicated in *Materials and Methods* (mean ± SD., n = 3).

were then assessed for the release of cytochrome c and the activation of caspase-3 and caspase-8. As shown in Fig. 5, treatment with SB203580 significantly down-regulated not only the activities of caspase-3 and caspase-8 but also the release of cytochrome c induced by DFO. Similar results were also observed in SNAP-treated cells (data not shown except for Fig. 5C). These data clearly indicate that p38 kinase is associated with signaling pathways mediated by caspases cascade and acts as a key factor in transferring signals for mitochondrial release of cytochrome c in the iron depletion-induced apoptosis of HL-60 cells. These results further corroborate the fact that both NO and iron deprivation share almost identical signals in the process of apoptotic death of HL-60 cells.

Iron deprivation by NO, and its relationship with apoptosis

Finally, we asked whether those cells, which are sensitive to NO show similar sensitivity to the iron loss induced by DFO. We compared the effects of SNAP and DFO on the survival of various cell lines available

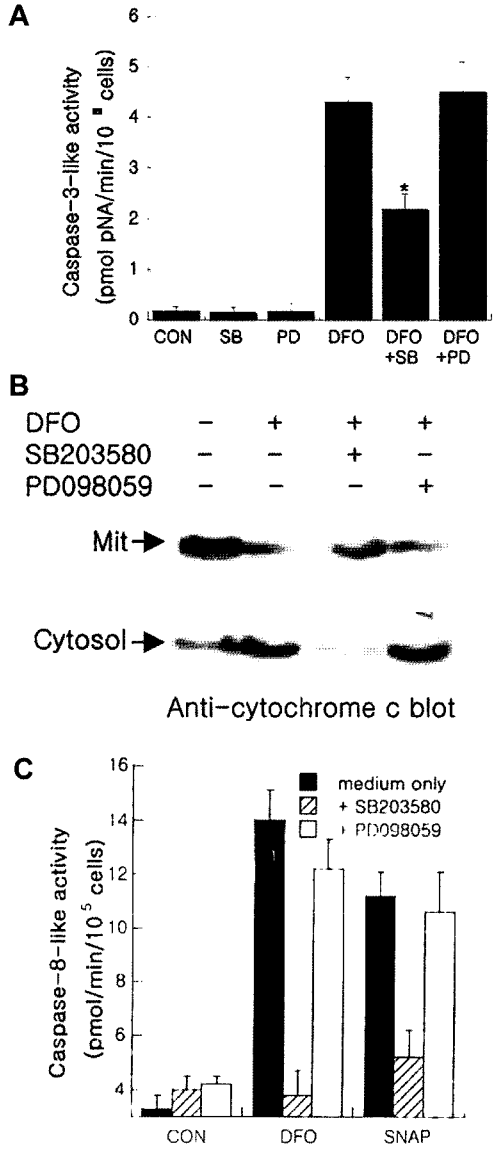

Fig. 5. Inhibition of p38 kinase prevents DFO- or SNAP-induced release of cytochrome c and caspase-3 as well as caspase-8 activation in HL-60 cells. A and B, HL-60 cells were pretreated with the medium alone, SB203580 (50 μM), or PD098059 (50 μM) for 1 h, and then exposed to DFO (0.5 mM) or SNAP (0.5 mM; for the experiment in C) for 12 h. Cells were harvested and lysed. Caspase-3-like activity (mean ± SD., n = 3) (A), cytochrome c release (B), and caspase-8-like activity (C, mean ± SD., n = 4) were measured using the same methods as in Fig. 4.

in our laboratory, and found that these cells show very similar sensitivities to both SNAP and DFO (Table I). SNAP breakdown and NO generation did not vary in any of the cell lines, as measured by the Griess method (data not shown). Combination of SNAP and DFO had an additive apoptotic effect on HL-60 cells (Fig. 6A). In parallel with the previous work (Feger et al., 2001), treatment of HL-60 cells with various concentrations of SNAP (0.1-0.5 mM) significantly decreased intracellular iron levels, a comparable effect to that observed with DFO (0.2-0.5 mM) (Fig. 6B). The effects

Table 1. Effects of DFO and SNAP on the survival of various cell lines

Cell lines	Cell viability (% of control)[a]	
	DFO	SNAP
HL-60	42 ± 5	39 ± 4
RAW264.7	34 ± 6	31 ± 3
CHO-K1	84 ± 5	87 ± 3
MBT-2	69 ± 3	77 ± 7
HUVEC	81 ± 5	75 ± 4
Keratinocyte	60 ± 6	58 ± 4

[a] Various lines of cells ($0.1\text{-}1 \times 10^6$/well) were incubated for 18 h in the presence of either DFO (0.5 mM) or SNAP (0.5 mM). Then the cells were collected and centrifuged, and their viabilities were examined by PI incorporation (mean ± SD., n = 3).

of adding FC on the SNAP-induced alteration of cell cycle distribution were assessed to further confirm the direct association of NO with iron. As shown in Fig.

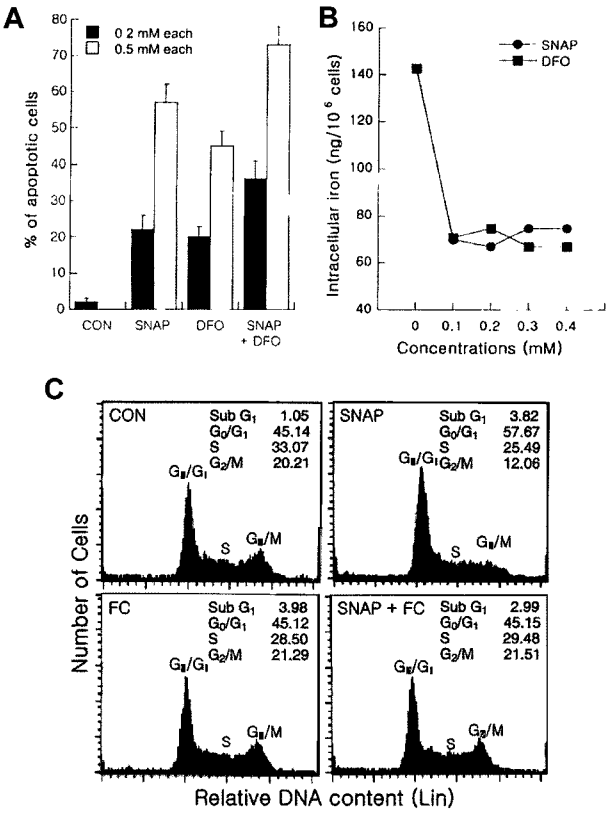

Fig. 6. SNAP mimics the effect of DFO to decrease the intracellular iron level and arrest the cell cycle at G_1 phase. A, The additive effect between SNAP and DFO for induction of apoptosis in HL-60 cells. HL-60 cells (0.5×10^6 cells/well) were treated for 18 h with DFO (0.5 mM), SNAP (0.5 mM), or DFO in combination with SNAP. After the incubation, quantitative analysis of apoptosis was performed using the *in situ* TdT-apoptosis assay method (mean ± SD., n = 3). B, Decreased intracellular iron levels after HL-60 cell incubation with various concentrations of DFO or SNAP. HL-60 cells were incubated for 3 h with DFO (0-0.4 mM) or SNAP (0-0.4 mM). The amounts of intracellular iron were measured as described in *Materials and Methods*. C, SNAP-induced cell cycle arrest at G1 phase was recovered by the treatment with FC in HL-60 cells. HL-60 cells (0.5×10^6 cells/well) were treated with SNAP (0.5 mM) in the presence or absence of FC (0.5 mM) and were then incubated for 9 h. After the incubation, the cells were stained with PI and their DNA contents were analyzed by flow cytometry. The experiment was repeated four times and representative histograms are presented.

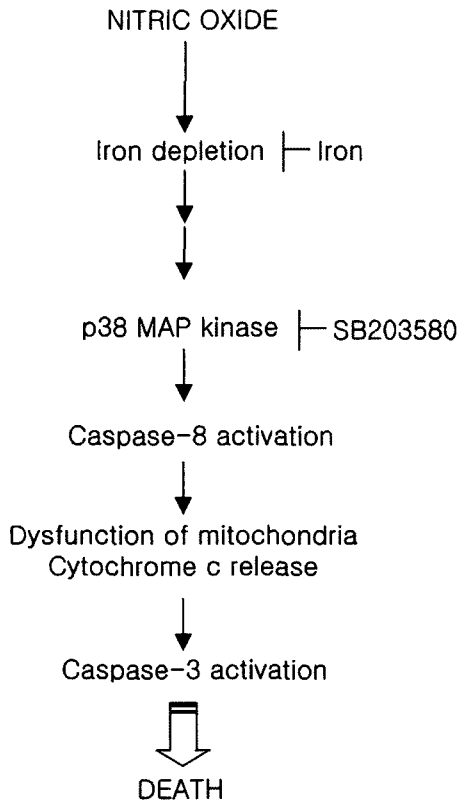

Fig. 7. A model showing pathways that mediate NO-induced apoptosis of HL-60 cells. NO is toxic, probably because it directly binds intracellular iron. Iron deprivation may give a signal to phosphorylate p38 kinase, and the activation of p38 kinase may induce activation of caspase-8, the mitochondrial membrane alterations resulting in the release of cytochrome c, and activation of caspase-3.

6C, treatment with FC completely abolished the SNAP-induced cell cycle arrest at G_1 phase, thereby appearing almost comparable to the control cells.

Discussion

The primary target of NO to initiate apoptotic cell signaling is not well understood. Here, we expanded the idea of Feger et al. (Feger et al., 2001) in which the authors implied that direct interaction of NO with intracellular iron, and the resulting loss of iron might be a principal mechanism of the NO-mediated anti-tumor activity. The interesting reciprocal relationship between NO and intracellular levels of iron reported in the literature also urged us to explore the signaling events which occur during the iron deprivation-mediated cell death. The intracellular iron level was significantly decreased after HL-60 cell incubation with SNAP, a well known NO donor. Furthermore, the cells that are sensitive to NO showed comparable sensitivity to DFO, a pharmacological chelator of iron. We found that both NO and iron deprivation induce activation of the MAP kinase subfamilies, p38 kinase and JNK, in HL-60 cells. In addition, SB203580, a highly specific p38 kinase inhibitor, which by itself had no effect on cell morphology or viability, significantly reduced the cytotoxic action of DFO, while PD098059, a selective inhibitor of MEK1 kinase, had no effect on the DFO inducible apoptosis. In parallel with NO action, iron deprivation-mediated signals were also coupled with disruption of mitochondrial integrity and activation of caspases. Interestingly, SB203580 also decreased DFO-induced release of cytochrome c and activation of caspase-3 and caspase-8, indicating that activation of p38 kinase lies at the upstream of disruption of mitochondrial transmembrane potential and activation of caspases.

The potential mechanisms involved in the NO-induced cell death were partially attributed to the inhibition of respiratory chain complexes, probably by nitrosylating or oxidizing protein thiols and by removing iron from enzymes with iron-sulphur centers (Beckman et al., 1990, Beckman and Crow, 1993, Cheng et al., 2001, Dawson et al., 1991, Xia et al., 1996). However, since NO has high affinity for iron, direct interaction between NO and iron and the resulting depletion of iron was suggested by other group (Feger et al., 2001), to be a critical mechanism responsible for the proapoptotic effects of NO. In fact, many lines of evidence suggested that NO counteracts the effects of added iron. Further, iron chelators could in some aspects mimic the mode of NO action on the proliferation and survival of various cell types (Feger et al., 2001, Fukuchi et al., 1994, Leardi et al., 1998). For example, apoptosis induced by glutathione depletion and oxidative stress in embryonic cortical neuronal cultures could be prevented by chelating the cellular iron with either DFO or MIM (Zaman et al., 1999). On the other hand, DFO could induce apoptosis by itself (Fukuchi et al., 1994) and augment the apoptosis induced by ara-C in HL-60 cells (Leardi et al., 1998).

It was interesting to compare the signaling pathways induced by NO and iron deprivation, because the signal transducers involved in NO-mediated cell death are now partially understood. As we demonstrate in this paper, the activation of both p38 kinase and caspases played pivotal roles both in the NO-induced or iron deprivation-mediated apoptotic processes in HL-60 cells. These signal pathways dealing with NO-induced apoptosis were partially verified previously (Cheng et al., 2001, Jun et al., 1999), and better confirmed in the current study. As shown in the Results section, the signal pathways evoked by the two agents (SNAP and DFO) were almost identical. However, this observation does not indicate whether iron is a primary factor in NO-mediated cell death because the following three possible mechanisms or explanations can all be applied. First, it can be speculated that both MAP kinases and caspases are commonly involved in the process of all different kinds of apoptotic stimulation. Actually, reports have demonstrated that both JNK and p38 kinase (Han et al.,

1994, Lee et al., 1994, Rouse et al., 1994) are strongly and commonly activated by a variety of stimulation such as UV irradiation, osmotic stress, and the inflammatory cytokines TNF and IL-1. Second, it is also possible that SNAP and DFO may have different primary targets, and the signals transduced by different initial molecules can be joined before or at p38 kinase to activate the downstream enzyme cascades. Third, as we have already suggested, the direct affinity of NO to iron, and the resulting iron loss can serve as the principal mechanism for p38 kinase activation, and disruption of mitochrondrial integrity and caspase activation all at the same time. More precise experiments such as 1) direct measurement of intracellular iron content of HL-60 cells after NO treatment, 2) comparison of cellular sensitivity to NO or iron chelator, 3) determination of protective effects of iron-containing compounds in NO-mediated cell death, and 4) determination of additive apoptotic effect observed by combination of SNAP with DFO may indicate that the third mechanism is more plausible.

It was particularly interesting to find that caspase-8 can be activated by the DFO treatment in HL-60 cells. Because caspase-8 has been known as an initiator of apoptosis in the death receptor-mediated system CD95 (APO-1/Fas), activation of caspase-8 by DFO indicates that iron deprivation induces cell death by activating the death receptor-mediated system cascades, as previously shown for the NO-mediated apoptosis with human neoplastic lymphoid cells (18). NO has been shown to induce greater apoptotic cell death in APO-S (high expression of CD95) than in APO-R (lacking expression of CD95) jurkat cells (18). Thus, it might be interesting to compare the intracellular iron content in both Jurkat cells. In fact, Kim et al. (8) demonstrated that hepatocytes (cells with high non-heme iron content) are highly resistant to NO, while RAW264.7 cells (a cell type with low non-heme iron levels) are not.

Iron has been shown to favor neoplastic cell growth and to display carcinogenic activity (Toyokuni, 1996, Weinberg, 1989). Authors have clearly shown that iron accumulation in many organs is correlated with the process of carcinogenesis. Accordingly, multiple studies have indicated that cell iron efflux could contribute to prevention and management of cancer (Amoroso et al., 2000, Toyokuni, 1996, Weinberg, 1999). As the host may endeavor to withdraw the iron from cancer cells via the synthesis of NO (Bastian et al., 1994, Drapier et al., 1991), elucidation of the relationship between NO and intracellular iron level may help to develop new therapeutic approaches to reduce or prevent neoplastic tumor cell growth.

Despite the extensive research in the field of NO biology, the mechanism by which NO inhibits cell death by blocking caspase activity in some cells and induces cell death through activation of caspases in others, is still an unexplained paradox. Here, we suggest that cell types sensitive to iron loss may undergo apoptotic cell death upon NO exposure by operating the signal pathways of the cellular execution machinery. In conclusion, the data obtained in the present study are compatible with the schematic representation in Fig. 7. NO either produced endogenously by NO synthase or delivered exogenously by NO donors, is toxic probably because it directly binds intracellular iron. The iron deprivation may give a signal to phosphorylate p38 kinase, and the activated p38 kinase may then transduce the signal for further activation of apoptotic enzyme cascades that include the release of cytochrome c and activation of caspases. The model system developed from this study will be used in future studies to evaluate the clinical approaches dealing with NO-derived immunotherapy or chemotherapy of cancer cells.

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