Nitric oxide (NO) is a chemical messenger implicated in neuronal damage associated with ischemia neurodegenerative disease and excitotoxicity. In the present study, we examined the biological effects of NO and its mechanisms in human malignant glioblastoma cells. Addition of a NO donor, S-nitroso-N-acetyl-penicillamine (SNAP), induced apoptosis in U87MG human glioblastoma cells, accompanied by opening mitochondrial permeability transition pores, release of cytochrome c and AIF, and subsequently by caspase activation. NO-induced apoptosis occurred concurrently with significantly increased levels of the Bak and Bim. Treatment with SNAP resulted in sustained activation of JNK and its downstream pathway, c-Jun/AP-1. The expression of dominant-negative (DN)-JNK1 and DN-c-Jun suppressed the activation of AP-1, the induction of Bak and Bim, and the SNAP-induced apoptosis. In addition, de novo protein synthesis was required for the initiation of apoptosis in that the protein synthesis inhibitor, cycloheximide (CHX), inhibited NO-induced apoptotic cell death as well as up-regulation of Bak and Bim. These results suggest that NO activates an apoptotic cascade, involving sustained JNK activation, AP-1 DNA binding activity, and subsequent Bak and Bim induction, followed by cytochrome c and AIF releases and caspases cascade activation, resulting in human malignant brain tumor cell death. J. Cell. Physiol. 206: 477–486, 2006. © 2005 Wiley-Liss, Inc.

Nitric oxide (NO) is a labile free radical that is physiologically produced through the L-arginine/NO synthase pathway. It has been reported that excessive NO generation during strokes, ischemia, and neurodegenerative diseases contributes to neuronal cell death (Coyle and Puttfarcken, 1993; Dawson and Dawson, 1996). NO can exert its cytotoxic effects in various cell types via generation of highly reactive free radicals, such as peroxynitrite, which damage DNA, proteins, and lipid by oxidation (Schulz et al., 1995; Kroncke et al., 1997; Lipton, 1999). Such damage subsequently triggers downstream signal transduction pathways, leading to cell death. However, the death pathways that are activated in nervous system in response to massive NO production are not well understood. As for brain tumors, the role of NO has not yet been fully explored (Shinoda and Whittle, 2001).

In the nervous system, JNK plays both pro- and anti-apoptotic functions during development (Kuan et al., 1999) and is also implicated in neuronal excitotoxicity (Yang et al., 1997). The main target of JNK is the transcription factor c-Jun, which is phosphorylated by JNK, thereby being activated (Kallunki et al., 1994). Transcriptionally active c-Jun is a pivotal trigger of apoptosis after neurotoxic insults, such as excitotoxicity, which occurs during ischemia and epilepsy, axotomy, and withdrawal of trophic support, a model for developmental neuronal death (Herdegen et al., 1998; Watson et al., 1998; Behrens et al., 1999; Crocker et al., 2001).

Increased mitochondrial permeability is a crucial event in many types of stress-induced apoptosis and leads to release of a number of apoptotic-related proteins (e.g., AIF, DIABLO, and cytochrome c) from mitochondrial intermembrane space. Regulation of mitochondrial permeability during apoptosis is complex, and the implementation of this apoptotic program is under the control of a family of multi-domain proteins with Bcl-2 as its founder (Kroemer and Reed, 2000; Cory and Adams, 2002). The Bcl-2 family can be divided into two classes with opposing properties: anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic (Bax, Bak, and Bok). A third family of pro-apoptotic “BH3-only” proteins (Bad, Bid, Noxa, Puma, Bim, and Bmf) mediates apoptosis in...
response to specific stimuli (Bouillet and Strasser, 2002; Cory and Adams, 2002). Studies on knockout mice indicate that the presence of at least one of the two pro-apoptotic multi-domain proteins, Bak and Bak, is necessary for the execution of apoptotic program. In the absence of these proteins, the apoptotic program downstream of BH3 protein activation is severely impaired (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001). Evidence has accumulated to show that the BH3 only proteins transduce diverse proximal apoptotic signals to the BH1-3 multi-domain proteins by a variety of post-translational modifications or transcriptional regulation.

In the present study, we have examined the apoptosis-inducing potential of NO in U87MG human glioblastoma cells which express relatively low levels of Bak and Bim proteins and dissected the NO-mediated apoptotic signaling pathway.

MATERIALS AND METHODS

Cell culture and reagents

Human glioblastoma cancer cell line U87MG was cultured in DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA). Bovine serum (penicillin-streptomycin solution (SNAP) was purchased from Calbiochem (San Diego, CA). 1-galactosidase reduced ethyl ester (GSH) and cycloheximide (CHX) from Sigma-Aldrich (St. Louis, MO), and SP600125 from Biosource International (Camarillo, CA). Antibodies against AIF, caspase 9, p-JNK, JNK1, and c-Jun were from Santa Cruz (Santa Cruz, CA), p-c-Jun (serine 63) and p-c-Jun (serine 73) from Cell Signaling Technology (Beverly, MA), and Bim from Stressgen Biotechnology (San Diego, CA).

Nitrite and kinase assay

Nitrite was measured by the Griess reagent, as described previously (Ing et al., 1999), and calculated from sodium nitrite (NaNO₂) standards. Concentrations of total proteins were measured, and nitrite level was expressed as nmol per μg of protein.

For measurement of the kinase activity of JNK, cells were lysed in a lysis buffer (20 mM Tris-HCl (pH 7.4), 1.25 mM β-glycerophosphate, 137 mM NaCl, 1 mM EDTA, 2 mM NaF, 1% NP-40, 1 mM sodium orthovanadate, and protease cocktail), immunoprecipitated using the anti-JNK1 antibody and protein A/G plus agarose, and washed twice in lysis buffer and twice in kinase assay buffer (50 mM Tris-HCl (pH 7.4), 137 mM NaCl, 1 mM MgCl₂, 1 mM sodium orthovanadate, 2.5 mM β-glycerophosphate, 2 mM EDTA, and 5 μM ATP). Kinase reactions were carried out by the addition of 50 μl of complete kinase buffer in the presence of [γ-32P]ATP and GST-c-Jun to precipitated immunocomplexes and incubation at 30°C for 30 min.

Overexpression, luciferase reporter assay, and EMSA

The retrovirus of dominant negative JNK1 (DN-JNK1), a catalytically inactive mutant of JNK1 with a N-terminal flag tag (Gupta et al., 1999), and plasmid of DN-c-Jun, a mutant (A63S and A73S) of c-Jun, were provided by Dr. Lee (Lee et al., 1999), and Dr. Papavassiliou (Papavassiliou et al., 1995), respectively. Transfection was carried out using Lipofectamine 2004 (Invitrogen) and Dr. Bohmann (Papavassiliou et al., 1995), respectively. Transfection reagent (Invitrogen). For the AP-1 reporter assay, cells were transfected with empty vector control or DN-JNK1 along with pAP-1-Luc (luciferase reporter construct) and pCMV-β-gal encoding β-galactosidase. Luciferase activity was measured by luminometer (Promega, Madison, WI). Data are presented as the fold increase in activity relative to control.

For electrophoretic mobility shift assay (EMSA), 5 μg of nuclear protein extracts were preincubated with binding buffer for 20 min at 25°C, and [32P]labeled oligonucleotides (AP-1, 5'-CGGCTTGATGATCGGGCGAA-3') were added, and the reaction mixtures were incubated for 20 min at 25°C.

RT-PCR analysis

Two micrograms of total RNA isolated using TRI REAGENT (Molecular Research Center, Cincinnati, OH) was transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). Amplifications were performed using Tag DNA polymerase (Promega) and specific primers for Bim (5'-CAGCTGGCGGAAGGCTC-3' and 5'-TGCGCTGCTGCTTGTC-3'), Bak (5'-GAGTCGACAGCAGACCT and 5'-GAGACGCTTACGAGCCC-3'), or β-actin (5'-GGATTCCTATGTGGGCGACAG-3' and 5'-CGGTCGGTGGAGATCTTCTAG-3').

Statistical analysis

Data are presented as mean ± SD. Comparisons between groups were used to the paired Student's t-test. Asterisk (**P < 0.001, *P < 0.01, *P < 0.05) was considered to be statistically significant.

RESULTS

SNAP induces apoptosis in U87MG glioblastoma cells

To examine whether NO was capable of inducing apoptosis, we treated U87MG glioblastoma cells with a NO donor SNAP. Upon exposure to SNAP, significant apoptotic cell death and accumulation of NO were detected in dose- and time-dependent manner (Fig. 1A,B). In general, the extents of cytotoxicity increased significantly at higher concentrations of SNAP and longer exposure time. To assess whether SNAP-induced apoptosis of U87MG glioblastoma cells involved generation of cellular oxidative stress, the apoptotic cell death was tested in the presence of cell-permeable free radical scavengers, such as GSH (Nakajima et al., 2002), NAC (Takahashi et al., 1998), and DTT (Ing et al., 1999). As expected, when the cells were pre-incubated with free radical scavengers, the amount of NO and the percentage of apoptotic cells decreased significantly (Fig. 1C,D). These results suggest that NO may induce apoptosis in U87MG glioblastoma cells by causing oxidative stress.

Mitochondria dysfunction is involved in NO-induced apoptosis

Next, we assessed the changes in mitochondrial membrane potential (MMP) and release of cytochrome c and AIF from mitochondria after exposure of U87MG glioblastoma cells to SNAP. As shown in Figure 2A,B, when the cells were treated with SNAP, the loss of MMP was detected in dose- and time-dependent manners. To assess cytochrome c and AIF released from the mitochondria into the cytosol, the subcellular fractionation was performed, and each fraction was analyzed by Western blot analysis (Enomoto et al., 2004; Woo et al., 2004). We observed an increase of cytochrome c and AIF in the cytosol, accompanying activation of caspase-9, when the cells were treated with various doses of SNAP for 48 h, whereas cytochrome c and AIF in the mitochondria were decreased (Fig. 2C): cytochrome c and AIF began to be released from the mitochondria to the cytosol at 12 h and continuously released over 48 h culture period with 600 μM SNAP. Active caspase-9 was detected concomitant with cytochrome c release (Fig. 2D). In addition, co-treatment of GSH with SNAP suppressed the loss of MMP (Fig. 2E) and SNAP-induced mitochondrial molecular events (Fig. 2F). zVAD FMK, a pan-caspase inhibitor, however failed to inhibit SNAP-induced loss of MMP (Fig. 2G) and cell death (Fig. 2H), implying that SNAP-induced mitochondrial dysfunction and cell death in U87MG glioblastoma cells are caspase-independent.
Taken together, these data suggest that mitochondrial death pathway plays an important role in NO-induced apoptosis of U87MG glioblastoma cells.

SNAP induces a sustained activation of JNK which plays an important role in NO-induced apoptosis

To determine whether MAPKs signal transduction pathways are important in NO-induced apoptosis of U87MG glioblastoma cells, we explored the phosphorylation of MAPKs in the cells by Western blot analysis. SNAP induced the phosphorylation of JNK and increased expression of c-Jun, beginning at 3 h, and this was sustained until 48 h (Fig. 3A). The changes in the levels of c-Jun and phospho-c-Jun were parallel to the phosphorylated levels of JNK. However, SNAP was not able to induce the phosphorylation of p38 or ERK in these cells under the culture conditions (Fig. 3A). Next, we pre-treated the cells with SP600125, a selective inhibitor of JNK, to study the role of JNK in the NO-induced apoptosis. As seen in Figure 3B, in the presence of SP600125, the NO-induced JNK and c-Jun phosphorylation were markedly decreased whereas phosphorylation of Akt and Erk was not changed. We further examined whether SP600125 blocked NO-induced apoptosis and apoptotic mitochondrial events. As shown in Figure 3C,D, cell death, the loss of MMP, and release of cytochrome c and AIF induced by SNAP were significantly suppressed in the presence of SP600125. To further confirm the role of JNK in NO-induced apoptosis, we cultured the cells for 48 h in the presence of 600 μM SNAP, followed by infecting the glioblastoma cells with dominant-negative JNK1 (DN-JNK1) retrovirus for 24 h. DN-JNK1 infected cells markedly reduced JNK kinase activity and c-Jun phosphorylation (Fig. 3E) and apoptosis (Fig. 3F). These results indicate that JNK activation plays a pivotal role in NO-induced apoptosis,
and that mitochondrial events contribute to apoptosis downstream of JNK activation in U87MG glioblastoma cells.

**SNAP induces AP-1DNA binding activity via c-Jun phosphorylation**

c-Jun phosphorylation at serines 63 and 73 by JNK is necessary for DNA binding of AP-1 (Behrens et al., 1999). In this study, AP-1 binding activity was determined by EMSA. As illustrated in Figure 4A,B, SNAP treatment resulted in a substantial increase of AP-1 DNA binding activity in dose- and time-dependent manners, starting as early as 3 h and persisting up to 48 h after the exposure of U87MG glioblastoma cells to SNAP. To elucidate whether the increase of AP-1 DNA binding activity by SNAP treatment was dependent on c-Jun phosphorylation, we examined the binding activity of AP-1 in DN-c-Jun transformants, which contains non-phosphorylating alanine residues in the place of serines 63 and 73, after treatment with SNAP. As shown in Figure 4C, the DNA binding activity of AP-1 in response to SNAP was significantly suppressed by the expression of DN-c-Jun. These results suggest that the increase in AP-1 DNA binding activity was mainly due to an increase in c-Jun phosphorylation.

**JNK mediates the SNAP-induced AP-1 activation**

To confirm whether JNK activation regulates AP-1 activation induced by SNAP, SP600125, and DN-JNK1 were applied to U87MG glioblastoma cells and found indeed that SP600125 and DN-JNK1 markedly blocked the induction of AP1 DNA binding activity and transcriptional activity. As shown in Figure 5A, pretreatment of the cells with SP600125 and DN-JNK1 markedly decreased the AP-1 DNA binding activity by SNAP. As shown in Figure 5B, up-regulation of AP-1 transcriptional activity by SNAP was also effectively suppressed in SP600125 and DN-JNK1 treated cells, indicating that JNK mediates AP-1 induction by SNAP.

**SNAP induces Bak and Bim up-regulation through JNK and c-Jun activation**

The Bcl-2 family of pro- and anti-apoptotic proteins constitutes a critical control point for apoptosis (Kroemer and Reed, 2000; Cory and Adams, 2002). To determine whether Bcl-2-related proteins changed during the NO-induced apoptosis of glioblastoma cells, the expression of anti-apoptotic Bcl-2 proteins (Bcl-xL and Bcl-2) and pro-apoptotic Bcl-2 family proteins (Bak, Bax, Bim) was examined. Interestingly, in the present...
study, untreated U87MG glioblastoma cells expressed relatively low levels of endogenous Bim and Bak proteins (Fig. 6), while the basal levels of protein expression of Bax, Bcl-2, and Bcl-xL were relatively high (data not shown). However, in the cells after 12 h of exposure to SNAP, the transcriptional and translational expression levels of Bak and Bim were dramatically increased (Fig. 6A), whereas the expression levels of Bax, Bcl-2, and Bcl-xL were not changed (data not shown), determined by RT-PCR and Western blot analysis. The expression of BimL protein was also marginally detected after 24 h of exposure to SNAP whereas BimS was not detected at all in this experimental system (data not shown). It has been known that Bak is an integral mitochondrial membrane protein and Bim can be translocated from cytosol to mitochondria when activated (Kirkin et al., 2004). To test whether up-regulated proteins of Bak and Bim were located in mitochondria, the expression of Bim and Bak proteins was examined in U87MG glioblastoma cells treated with 600 μM SNAP for over 48 h culture period. As shown in Figure 6B, increased levels of Bim were detected in both cytosolic and mitochondrial fractions, and those of Bak were only in mitochondrial fractions of SNAP treated U87MG glioblastoma cells.

To ascertain whether JNK activation regulates Bak and Bim expression, SP600125 and DN-JNK1 were applied to U87MG glioblastoma cells. As shown in Figure 6C, pretreatment of the cells with SP600125 and DN-JNK1 markedly decreased the up-regulation of Bak and Bim by SNAP. Next, in order to determine whether phosphorylation of the c-Jun transcription domain was necessary for the induction of Bak and Bim in U87MG glioblastoma cells, the cells were transfected with expression vector for DN-c-Jun. As shown in Figure 6D, Bak and Bim up-regulation by SNAP was effectively suppressed in DN-c-Jun transfected cells, indicating that AP-1 induction by JNK-c-Jun pathway plays a role in up-regulation of genes that induce apoptosis, such as Bak and Bim. Finally, using DN-c-Jun, we examined the role of c-Jun in the induction of apoptosis by SNAP. Figure 6E shows that DN-c-Jun transfection blocked the apoptotic cell death mediated by SNAP treatment. Therefore, these results indicate a role of JNK and c-Jun activation in NO-induced apoptosis of U87MG glioblastoma cells.

**De novo protein synthesis is required for SNAP-induced apoptosis**

To determine if macromolecular synthesis is required for induction of apoptosis by SNAP, we examined the effect of CHX, a protein synthesis inhibitor, on NO-induced apoptotic events in U87MG glioblastoma cells. As shown in Figure 7A, CHX markedly inhibited
Fig. 3. SNAP induces cell death via JNK activation. 

A: SNAP induces activation of JNK and c-Jun. U87MG human glioblastoma cells were treated with 600 μM SNAP for the indicated times and analyzed by Western blot.

B: SP600125 inhibits SNAP-induced activation of JNK and c-Jun. Cells were treated with the indicated concentrations of SNAP in the presence of 20 mM SP600125 or 20 mM GSH for 48 h and analyzed by Western blot.

C, D: SP600125 blocks SNAP-induced cell death (C) and loss of MMP and release of mitochondrial AIF and cytochrome c (D). Cells treated with 20 μM SP600125 for 30 min and then treated with 600 μM SNAP for 48 h.

E, F: Expression of dominant negative JNK1 (DN-JNK1) blocks SNAP-induced activation of JNK and c-Jun (E) and cell death (F). Cells were infected with Flag-tagged dominant negative JNK1 (DN-JNK1) retrovirus for 24 h and then treated with 600 μM SNAP for 48 h. Cell lysates were immunoprecipitated with anti-JNK1 antibody and used for JNK kinase assay using GST-c-Jun protein as described in Materials and Methods. The level of c-Jun phosphorylation was analyzed by Western blot using anti-p-c-Jun (Ser 63) antibody. Data are expressed as means ± SD from three independent experiments. Asterisk (*) indicates a significant difference compared to the SNAP treated groups.
Fig. 4. SNAP induces AP-1 binding activity via c-Jun phosphorylation. A, B: U87MG human glioblastoma cells were treated with the indicated concentrations of SNAP for 48 h (A) and further treated with 600 μM SNAP for the indicated times (B). AP-1 binding activity of nuclear protein extracts were prepared for EMSA assay using radioactive labeled AP-1, as described in Materials and Methods. Specificity of AP-1 binding activity was examined by adding 100-fold excess concentration of non-labeled AP-1 oligonucleotides competitor (C, 100-fold). C: Expression of dominant negative of c-Jun (DN-c-Jun) inhibits SNAP-mediated AP-1 binding activity. Cells were transiently transfected with DN-c-Jun for 24 h and then exposed to 600 μM SNAP for 48 h. C, 100× competitor.

Fig. 5. JNK mediates the SNAP-induced AP-1 activation. A: DN-JNK1 and SP600125 inhibit SNAP-induced AP-1 binding activity. U87MG human glioblastoma cells were infected with DN-JNK1 retrovirus for 24 h or treated with either 20 μM SP600125 or 20 mM GSH for 30 min. Subsequently, the cells were treated with 600 μM SNAP for 48 h and analyzed by EMSA assay. B: DN-JNK1 and SP600125 inhibit SNAP-induced AP-1 transcriptional activity. Cells were transiently transfected with empty vector control (pcDNA) or DN-JNK1 along with pAP-1-Luc (luciferase reporter construct) and pCMV-β-gal encoding β-galactosidase. After 24 h of transfection, cells were treated with either 20 μM SP600125 or 20 mM GSH for 30 min and subsequently treated with 600 μM SNAP for 48 h. Luciferase assay was measured and transfection efficiency was adjusted with β-galactosidase activity. Asterisk (**, P < 0.01) indicates a significant difference compared to the SNAP treated groups.
NO-induced apoptosis. Furthermore, the analysis of subcellular fractionation in combination with Western blot showed that the up-regulation of Bak and Bim protein levels and their increased localization in mitochondria upon SNAP treatment were also inhibited by CHX (Fig. 7B), thus implying that de novo protein synthesis is at least partially required for the apoptosis of U87MG glioblastoma cells induced by SNAP.

**DISCUSSION**

In the present study, we demonstrated the biological effects and intracellular signal cascades in response to NO in neuronal cells. NO donor SNAP induced apoptosis in U87MG human glioblastoma cells, which constitutively express relatively low levels of Bak and Bim, by changes in Bak and Bim expression. Up-regulation of Bak and Bim was regulated by the activation of AP-1 through the JNK-induced c-Jun activation. The inhibition of AP-1 with DN-JNK1 and DN-c-Jun reduced Bak and Bim protein expression. These results led us to propose that NO activates AP-1 via JNK, which in turn transactivates Bak and Bim, which then leads to mitochondrial membrane permeabilization and subsequent release of cytochrome c and AIF from mitochondria, resulting in glioblastoma cell apoptosis.

It is now generally believed that apoptosis occurring through the mitochondrial pathway is dependent on the balance between the pro-apoptotic BH3-only Bcl-2...
family members and the anti-apoptotic Bcl-2 family members, which are known to be regulators of caspase-dependent cell death (Kroemer and Reed, 2000; Bouillet and Strasser, 2002; Cory and Adams, 2002). However, it has also been shown that members of Bcl-2 family such as Bax are activated in several instances of caspase-independent regulation of mitochondrial integrity and cell death (Donovan and Cotter, 2004). We have recently showed that translocation of AIF and cell death induced by arsenic trioxide, an anticancer agent leading to an increase of reactive oxygen species, in human cervical cancer cells were not attenuated by caspase inhibitors (Kang et al., 2004). In the present study, our findings that treatment of U87MG glioblastoma cells with SNAP resulted in mitochondrial dysfunction, including the loss of MMP and release of cytochrome c and AIF from mitochondria, and that neither loss of MMP nor cell death were inhibited by a pan-caspase inhibitor (Fig. 2) also suggest the changes in MMP and apoptotic cell death in SNAP-treated cells are mainly resulted from the action of caspase-independent pro-death molecules, potentially including AIF and some of pro-apoptotic Bcl-2 family proteins, Bim and Bak.

Treatment of SNAP induced increment in Bim concentration, and induced relocation of Bim from cytosolic fraction to mitochondria-containing residual fraction (Fig. 6). In our experimental systems using a commercially available anti-Bim antibody which is capable of detecting all three isoforms, however, BimS protein was not detected at all in U87MG glioblastoma cells while the expression of BimL protein was marginally detected after 24 h of exposure to SNAP (data not shown). Although the precise role of BimL in SNAP-treated U87MG glioblastoma cell death was not examined in the present study, our data that the expression levels of BimEL were dominantly higher than those of BimL in response to NO, and a recent publication by Jiang et al. (2004) that lovastatin induces up-regulation of BimEL and apoptosis and overexpression of BimEL alone is sufficient to induce cell death in U87 glioblastoma cells, indicate that BimEL might be a major isoform of Bim proteins in our experimental setups.

In addition to Bim, the multi-domain Bak proteins were also up-regulated (Fig. 6). These changes and relatively slow onset of apoptosis by SNAP led us to hypothesize that NO may induce apoptosis in U87MG human glioblastoma cells by transcriptional regulation of genes rather than more rapid direct effects on existing caspases or post-translational effects on existing pro-apoptotic proteins. It has been shown that the forkhead transcription factor induces Bim expression in T lymphocytes and is to be suppressed by survival-promoting cytokines through the PI3K/Akt anti-apoptotic pathway (Dijkers et al., 2000). Furthermore, Whitfield et al. (2001) have reported that DN-c-Jun prevents Bim up-regulation and inhibits mitochondrial cytochrome c release in cultured neurons after NGF withdrawal, suggesting that activation of the JNK/c-Jun pathway may also participate in the transcriptional regulation of Bim. Little is known about the regulation of transcription and translation of Bak. A recent report found that Bak was found to be one of the genes up-regulated in neuroblastoma cells, when treated with oxidative stress (Lovat et al., 2003). Based on data presented here it might be hard to conclude whether AP-1 complex binds to promoters of Bim and Bak directly. We failed to find potential binding sites for AP-1 in upstream regions of these genes, which remains to be determined.

We have observed there are certain discrepancies in response levels of Bim and Bak expression between DN-JNK1- and DN-c-Jun-overexpressed U87MG glioblastoma cells treated with SNAP (Fig. 6C,D). These might be because of increased total amount of endogenous c-Jun proteins in the samples. We have observed that the levels of endogenous c-Jun protein are highly up-regulated in response to SNAP in both dose and time dependent manners (Fig. 3), probably caused by direct stimulation of c-Jun transcription and translation by its own gene product in response to activation of JNK (Angel et al., 1988). In comparison with the experiment with DN-JNK1 (Figs. 5 and 6C), therefore, when DN-c-Jun-overexpressed cells were treated with 600 μM of SNAP for 48 h, the relatively high amount of exogenously induced DN-c-Jun (Fig. 6E) might still be insufficient for overruling the total amount of hugely up-regulated endogenous c-Jun, which might lead to weaker effects on inhibition of SNAP-induced AP-1 binding activity (Fig. 4C) as well as Bim and Bak expression (Fig. 6D).
It has critically been evaluated that AIF which can be released from mitochondria in a caspase-dependent as well as a caspase-independent manner mediates caspase-independent death (Cande et al., 2004). Our data that inhibition of caspase activation has no effects on blocking NO-induced cell death and loss of MMP (Fig. 2) while inhibition of JNK markedly abrogates NO-induced cell death, loss of MMP, and translocation of AIF to the cytosol (Fig. 3) imply that up-regulated Bak and Bim proteins via JNK downstream pathways induce loss of MMP and translocation of AIF in a caspase-independent manner and that released AIF to the cytosol might play a role as one of key pro-death molecules in caspase-independent cell death in U87MG glioblastoma cells treated with SNAP.

Finally, the data presented herein may serve useful not only in the study of signal pathways involved in NO-induced apoptosis, but also help understand possible significance of NO synthase induction and clinical application of NO donors in the treatment of malignant brain tumor.

ACKNOWLEDGMENTS

We would like to thank Dr. Dirk Bohmann (Department of Biochemical Genetics, University of Rochester Medical Center, Rochester, NY) for the generous gift of DN-c-Jun plasmid and Dr. Yun-Sil Lee (Laboratory of Radiation Effect, Korea Institute of Radiological and Medical Sciences, Seoul, Korea) for DN-JNK1 retrovirus.

LITERATURE CITED
