Growth factors prevent changes in Bcl-2 and Bax expression and neuronal apoptosis induced by nitric oxide

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Abstract

Recent studies have shown that nitric oxide (NO) donors can trigger apoptosis of neurons, and growth factors such as insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) can protect against NO-induced neuronal cell death. The purpose of this study was to elucidate the possible mechanisms of NO-mediated neuronal apoptosis and the neuroprotective action of these growth factors. Both IGF-1 and bFGF prevented apoptosis induced by NO donors, sodium nitroprusside (SNP) or 3-morpholinosydnonimine (SIN-1) in hippocampal neuronal cultures. Incubation of neurons with SNP induced caspase-3-like activation following downregulation of Bcl-2 and upregulation of Bax protein levels in cultured neurons. Treatment of neurons with a bax antisense oligonucleotide inhibited the caspase-3-like activation and neuronal death induced by SNP. In addition, treatment of neurons with an inhibitor of caspase-3, Ac-DEVD-CHO, together with SNP did not affect the changes in the protein levels, although it inhibited NO-induced cell death. Pretreatment of cultures with either IGF-1 or bFGF prior to NO exposure inhibited caspase-3-like activation together with the changes in Bcl-2 and Bax protein levels. These results suggest that the changes in Bcl-2 and Bax protein levels followed by caspase-3-like activation are a component in the cascade of NO-induced neuronal apoptosis, and that the neuroprotective actions of IGF-1 and bFGF might be due to inhibition of the changes in the protein levels of the Bcl-2 family.

Keywords: Bcl-2; Bax; caspase-3; IGF-1; bFGF; nitric oxide

Abbreviations: Ac-DEVD-CHO, acetyl-DEVD-CHO; AMC, 7-amino-4-coumarin; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; ICE, interleukin 1 β -converting enzyme; IGF-1,

insulin-like growth factor-1; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside

Introduction

Nitric oxide (NO) is an unstable but multifunctional molecule which mediates a number of diverse physiological processes (Moncada *et al*, 1991). NO is synthesized from L-arginine by NO synthase (NOS), of which three distinct forms have been identified, that is, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Nathan, 1992; Moncada and Higgs, 1993). In the nervous system, NO produced by nNOS in response to receptor stimulation has several potential roles as a neuronal messenger molecule (Dawson and Dawson, 1995). However, excess production of NO has been implicated in the pathogenesis of neuronal death following overstimulation of N-methyl-D-aspartate (NMDA) receptors (Dawson *et al*, 1993) and of neuronal death after glial iNOS induction (Dawson *et al*, 1994; Chao *et al*, 1996). The mechanisms proposed for NO-mediated cytotoxicity include inactivation of the mitochondrial respiratory chain (Bolanos *et al*, 1997), S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (McDonald *et al*, 1993), and induction of DNA damage. However, the exact mechanism of NO-mediated neuronal death remains unclear.

Apoptosis can result either from developmentally controlled activation of endogenous execution programs or from transduction of death signals triggered by various adverse stimuli (Steller, 1995). One major path in the apoptotic program involves the activation of caspase-3, a member of a family of cysteine proteases which have recently been renamed caspases, and are homologous to the product of the *Caenorhabditis elegans* cell death gene *ced 3* (Kumar, 1995; Fernandes-Anemri *et al*, 1994). On the other hand, Bcl-2, which is homologous to the product of the negative regulator of *C. elegans*, *ced-9*, can prevent apoptosis during development and neuronal death after various adverse insults (Davies, 1995; Kroemer, 1997). In addition, Bcl-2 forms a heterodimer with a related protein, Bax, which has been suggested to oppose the anti-apoptotic function of Bcl-2 (Oltvai *et al*, 1993).

Neuronal death can be modified by several pharmacological agents including peptide growth factors such as IGF-1 and bFGF. Previous studies revealed that these growth factors prevent neuronal death *in vitro* due to glutamate toxicity (Mattson *et al*, 1989; Freese *et al*, 1992), hypoglycemia (Cheng and Mattson, 1992) or nitric oxide toxicity (Maiese *et al*, 1993). Moreover, administration of these growth factors protects against neuronal death after

Figure 1 NO donors, SNP and SIN-1, induced hippocampal neuronal apoptosis. **(A)** Hippocampal neurons were incubated with different concentrations of either SNP or SIN-1 for 24 h and neuronal viability was measured by LDH assay after 24 h treatment. Data are mean $\pm$ S.E.M. for three independent experiments performed in triplicate for both curves. * $P < 0.05$

cerebral hypoxia-ischemia *in vivo* (Jhonston *et al*, 1996; Yamada *et al*, 1991). Recent reports suggest that the mechanism whereby the growth factors protect neurons from ischemic damage involves stabilization of neuronal calcium homeostasis (Mattson and Cheng, 1993). However, the exact mechanism of the neuroprotective action against NO-mediated toxicity remains unknown.

In the current study, we examined the effects of NO exposure on expression of Bcl-2 and Bax and caspase-3-like activity in cultured hippocampal neurons, and how IGF-1 and bFGF exert neuroprotective effects in relation to these apoptosis-related proteins.

Results

NO induced apoptotic cell death in hippocampal neurons

We first examined the neurotoxic effects of NO donors, SNP and SIN-1, on cultured hippocampal neurons. Treatment of neurons with either SNP or SIN-1 dose-dependently induced neuronal degeneration (Figure 1A). The decomposition products, the NO donors (50 μM) dissolved in medium left at 37°C for 24 h prior to use, did not affect cell viability of neurons (data not shown), indicating that the toxicity seen with the NO donors is due to NO and not to the decayed compounds. Time course experiments revealed that incubation with either SNP or SIN-1 at 50 μM reduced neuronal viability in a time-dependent manner, and significant neuronal death was observed after 18 h of the treatment (Figure 1B).

Previous studies have shown that NO donors such as SIN-1, S-nitroso-N-acetyl-penicillamine, and S-nitrosoglutathione, induced apoptosis of cultured cortical neurons (Palluy and Rigaud, 1996). To confirm that NO-mediated neuronal death occurred by apoptosis in our hippocampal culture system, DNA fragmentation of neurons treated with NO donors were analyzed by conventional agarose gel electrophoresis. DNA ladder formation was induced by incubation of neurons for 24 h with either of the NO donors in a dose-dependent manner (Figure 1C).

Caspase-3-like activity in neurons treated with SNP

The caspase family has been suggested to play a role in neuronal death (Hara *et al*, 1997; Friedlander *et al*, 1997). To identify the apoptotic mechanism of NO-mediated neuronal death, we investigated the involvement of the caspase-3-like proteases in neurons treated with SNP for 24 h. Addition of the tetrapeptide inhibitor acetyl-DEVD-CHO (Ac-DEVD-CHO)

versus control cultures; two-way ANOVA followed by Scheffe's *post-hoc* test. **(B)** Hippocampal neurons were incubated with either SNP (50 μM) or SIN-1 (50 μM) for indicated times, and neuronal viability was measured by LDH assay. Data are mean $\pm$ S.E.M. for three independent experiments performed in triplicate for both curves. * $P < 0.05$ *versus* time 0 h; two-way ANOVA followed by Scheffe's *post-hoc* test. **(C)** Agarose gel electrophoresis of oligonucleosomal DNA fragments (DNA laddering). After 24 h exposure of neurons to SNP or SIN-1, DNA extracted from neuron cultures was subjected to conventional agarose gel electrophoresis. MK; 1 kb ladder molecular weight marker

inhibited NO-induced neuronal death in a dose-dependent manner with maximum effects at 1 μM (Figure 2A). Next, we measured caspase-3-like activity in neurons treated with SNP. Activity of caspase-3-like protease was measured spectrophotometrically by its ability to cleave Ac-DEVD-MCA. Incubation with SNP induced upregulation of caspase-3-like activity in neurons in a dose-dependent manner (Figure 2B). The level of caspase-3-like activity in neurons treated with different doses of SNP showed an inverse relationship with neuronal viability (Figure 1A). In addition, the ability of Ac-

DEVD-CHO to inhibit caspase-3-like activity was confirmed by the observation that the inhibitor inhibited NO-induced upregulation of caspase-3-like activity (Figure 2B), suggesting that the ability of the inhibitor to prevent NO-induced neuronal degeneration is due to inhibition of upregulation of caspase-3-like activity. Furthermore, time course experiments revealed a significant increase in caspase-3-like activity after 18 h of SNP treatment (Figure 2C).

Bcl-2 and Bax protein levels in neurons treated with SNP

Since Bcl-2 has been shown to prevent apoptosis by functioning upstream of caspase-3 (Chiannaiyan *et al*, 1996), we investigated the involvement of Bcl-2 and a related protein, Bax, in NO-induced cell death in neurons treated with 50 μM SNP. Western blot analysis revealed that incubation of neurons with SNP induced downregulation of Bcl-2 and upregulation of Bax in a time-dependent manner, and the changes in the protein levels started at latest 12 h after SNP treatment (Figure 3). Addition of Ac-DEVD-CHO (1 μM) to the neuronal culture, which protected neurons (Figure 2A), did not affect the changes in protein levels in neurons treated with 50 μM SNP (Figure 3), suggesting that the changes in Bcl-2 and Bax levels occur upstream to caspase-3-like activity. Furthermore, these results suggest that the observed changes in Bcl-2 and Bax expression are not secondary to cell death as they are not blocked by Ac-DEVD-CHO, a compound that inhibited NO-induced neuronal apoptosis. β -actin protein level as the internal control at each time point

Figure 2 Involvement of caspase-3-like activation in NO-induced neuronal death. (A) Effect of Ac-DEVD-CHO on cell viability of neurons treated with NO donors. Neuronal cultures were treated for 24 h with 50 μM SNP with or without different concentrations of an inhibitor of the caspase-3-like protease, Ac-DEVD-CHO, and neuronal viability was determined by LDH assay. Data are mean $\pm$ S.E.M. of four independent experiments performed in triplicate. * $P < 0.05$ versus control, ** $P < 0.05$ versus 50 μM SNP; ANOVA with Scheffe's *post-hoc* test. (B) Caspase-3-like activity in cultured neurons treated with SNP. Hippocampal neurons were treated with different concentrations of SNP in combination with or without Ac-DEVD-CHO for 24 h and then caspase-3-like activity was measured spectrophotometrically by its ability to cleave Ac-DEVD-MCA. Data are mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ versus control, ** $P < 0.05$ versus 50 μM SNP alone; ANOVA with Scheffe's *post-hoc* test. (C) Time-course of caspase-3-like activity in neurons treated with 50 μM SNP. Neurons were incubated with 50 μM SNP for 6, 12, 18, and 24 h and then caspase-3-like activity was measured. * $P < 0.01$ versus control (0 h); ANOVA with Scheffe's *post-hoc* test

Figure 3 Bcl-2 and Bax protein levels in neurons treated with SNP. After neurons were treated with SNP (50 μM) for the indicated times with or without Ac-DEVD-CHO (1 μM), extracts of neurons were subjected to SDS-PAGE and the blots were probed with antibodies to Bcl-2, Bax or β -actin. Visualization of the proteins was performed with ECL

Figure 4 Effect of Bax antisense oligodeoxynucleotide on Bcl-2 and Bax protein levels, caspase-3-like activity and cell viability in neurons treated with SNP. Hippocampal neurons were exposed for 24 h to 50 μ M SNP in the absence or presence of Bax antisense ODN (AS) or mismatch control ODN (MC). (A) Extracts of neurons were subjected to SDS-PAGE, and the blots were probed with antibodies to Bcl-2, Bax, or β -actin. (B) Caspase-3-like activities in neurons were measured spectrophotometrically by its ability to cleave Ac-DEVD-MCA. Values are mean $\pm$ S.E.M. of the four independent experiments. * P < 0.01 versus control, ‡ P < 0.01 versus SNP; ANOVA with

confirmed that equal amounts of the protein were loaded in each well of the gel (Figure 3).

Effect of the changes in the ratio of Bax to Bcl-2 to the caspase-3-like activation and neuronal survival

To explore a role for the ratio of Bax to Bcl-2 protein levels in NO-induced caspase-3-like activation and neuronal death, antisense ODN for Bax were employed. In preliminary studies the specificity of the antisense for suppressing upregulation of Bax expression was confirmed by immunoblotting of neuronal extracts after exposure to 50 μ M SNP for 24 h in the absence or presence of antisense or control ODN. Upregulation of Bax expression in hippocampal neurons induced by 24 h exposure to SNP was completely blocked by addition of Bax antisense (50 μ M), but not by addition of a mismatch control ODN (50 μ M) (Figure 4A). We next measured cell viability and caspase-3-like activity in neurons exposed for 24 h to 50 μ M SNP in the absence or presence of Bax antisense or mismatch control ODN. Addition of Bax antisense to neuronal cultures significantly inhibited the caspase-3-like activation and cell death of neurons induced by NO exposure (Figure 4B and C). In contrast, administration of a control mismatch ODN for Bax had no effect on the caspase-3-like activity and cell viability in neurons treated with SNP (Figure 4B and C). Sense and random sequence ODNs also had no effect on Bcl-2 and Bax expression, cell viability and caspase-3-like activation in neurons treated with SNP (data not shown).

Effects of IGF-1 and bFGF on neuronal viabilities, Bcl-2 and Bax protein levels and caspase-3-like activity

Peptide growth factors such as IGF-1 and bFGF have been proposed to play a neuroprotective role against ischemic insult (Jhonston *et al*, 1996; Yamada *et al*, 1991), but the mechanism by which they do so remains largely unknown. To examine the neuroprotective role of these growth factors in NO-mediated neuronal death, neurons were pretreated or not treated with increasing concentrations (1, 10, 100 ng/ml) or either IGF-1 or bFGF for 6 h, and then exposed to 50 μ M SNP for 24 h. Administration of IGF-1 dose-dependently increased survival of neurons exposed to SNP, where 100 ng/ml of IGF-1 rescued 65% of cells from death induced by SNP (Figure 5A). In a similar fashion, bFGF also dose-dependently inhibited NO-induced neuronal death, wherein 100 ng/ml bFGF rescued 60% of neurons from death induced by SNP (Figure 5A). Consistent with these results, pretreatment with either IGF-1 (100 ng/ml) or bFGF (100 ng/ml) attenuated DNA laddering induced by 50 μ M SNP (Figure 5B).

Since caspase-3-like protease was involved in NO-induced neuronal death, we examined whether the growth factors alter NO-induced upregulation of caspase-3-like activity. Hippocampal neurons that were pretreated with

Scheffe's *post-hoc* test. (C) Neuronal viabilities after 24 h exposure to SNP were assessed by LDH assay. Values are mean $\pm$ S.E.M. of the four independent experiments. * P < 0.01 versus control, ‡ P < 0.01 versus SNP; ANOVA with Scheffe's *post-hoc* test

Figure 5 Effects of IGF-1 and bFGF on NO-induced neuronal apoptosis. **(A)** Hippocampal neurons pretreated with different concentrations of either IGF or bFGF were incubated with 50 μ M SNP for 24 h, and neuronal viability was determined by LDH assay. Data are mean $\pm$ S.E.M. of three independent experiments performed in triplicate. * $P < 0.05$ versus SNP alone; two-way ANOVA followed by Scheffe's *post-hoc* test. **(B)** Agarose gel electrophoresis of oligonucleosomal DNA fragments (DNA laddering). Neurons pretreated with either IGF (100 ng/ml) or bFGF (100 ng/ml) were incubated with 50 μ M SNP for 24 h, and then DNA extracted from neuron cultures was subjected to conventional agarose gel electrophoresis. MK, 1 kb ladder

either IGF-1 or bFGF were incubated with 50 μ M SNP for 24 h, and caspase-3-like activity was measured. As shown in Figure 6A, IGF-1 inhibited NO-induced upregulation of caspase-3-like activity in a dose-dependent manner, where 100 ng/ml IGF-1 inhibited 71% of the increase in caspase-3-like activity. In a similar fashion, bFGF also dose-dependently inhibited NO-induced caspase-3-like activation, where 100 ng/ml bFGF inhibited 68% of the increase in the activity (Figure 6A).

We next determined whether the growth factors influence NO-induced changes in Bcl-2 and Bax expression. Hippocampal neurons pretreated with either IGF-1 (100 ng/ml) or bFGF (100 ng/ml), the concentration that most effectively inhibited NO-induced neuronal death (Figure 6A) and caspase-3-like activation (Figure 6A), were incubated with 50 μ M SNP for 24 h, and the protein levels were examined by Western blot analysis. Administration of either IGF-1 or bFGF significantly prevented downregulation of Bcl-2 in parallel with upregulation of Bax protein levels induced by NO (Figure 6B).

Discussion

A recent study has demonstrated that cultured neurons undergo apoptosis with a delayed time-course after mild insult delivered by NO donors, whereas intense exposure to high concentrations of NO donors induces necrosis with a faster time-course (Bonfoco *et al*, 1995). In the present study, 50 μ M NO donors was used to examine possible mechanisms by which NO induces neuronal apoptosis, since this concentration of the NO donors most evidently induced DNA laddering formation among the concentrations examined (5–100 μ M, data not shown). Furthermore, the close correlation of the LDH-based viability assay which presumably measures necrosis and apoptosis and the caspase assays which presumably measures only apoptosis in the experiments using 50 μ M SNP suggests that the cell death seen is primarily apoptotic.

We found that NO-induced apoptosis is accompanied by downregulated Bcl-2 and upregulated Bax expression in addition to activation of caspase-3-like protease in primary cultured hippocampal neurons. In splenic B cells, NO induces Bcl-2 expression and prevent apoptosis (Genaro *et al*, 1995). Thus, different types of cells appear to differ in the regulation of Bcl-2 expression. NO-induced upregulation of Bax expression observed here agrees with a recent observation that Bax expression is increased during neuronal apoptosis following cerebral ischemia (Krajewski *et al*, 1995), suggesting that NO produced in ischemic brain may play a role in inducing the expression. The temporal profile during NO-induced neuronal death in the present study consisted of initial changes in Bcl-2 and Bax protein levels followed by caspase-3-like activation. Recent studies have demonstrated that overexpression of Bcl-2 protects against NO-induced cell death in the hypothalamic neural cell line GT1-7 cells and in non-neuronal cells (Bonfoco *et al*, 1996; Melkova *et al*, 1997) and that overexpression of Bcl-2 abrogated staurosporine-induced activation of caspase-3 and apoptosis in Jurkat cells, suggesting that Bcl-2 functions upstream of caspase-3 (Chinnaiyan *et al*, 1996).

In the present study, the ratio of Bax to Bcl-2 was increased during NO-induced neuronal death, which supports the hypothesis that the balance between expression of these proteins determines cell survival or death following an apoptotic stimulus (Oltavi *et al*, 1993). Furthermore, Bax antisense ODN inhibited NO-induced activation of the caspase-3-like protease and neuronal death, and application of a caspase-3-like protease inhibitor

did not inhibit NO-induced alterations in Bcl-2 and Bax expression in spite of its capability to prevent NO-induced neuronal death. Taken together, the results of the present study suggest that NO-mediated changes in the ratio of Bax to Bcl-2 expression trigger activation of caspase-3-like protease in hippocampal neurons, resulting in cell death.

Neuronal death from various insults can be modified by several pharmacological agents including peptide growth factors such as IGF-1 and bFGF. Previous studies revealed that the growth factors prevent neuronal death *in vitro* due to glutamate toxicity (Mattson *et al*, 1989; Freese *et al*, 1992), hypoglycemia (Cheng and Mattson, 1992) or nitric oxide toxicity (Maiese *et al*, 1993). Moreover administration of these growth factors protects against neuronal death after cerebral hypoxia-ischemia *in vivo* (Jhonston *et al*, 1996; Yamada *et al*, 1991). Our results demonstrated that these growth factors can partly prevent NO-induced neuronal death, consistent with previous reports (Maiese *et al*, 1993). This incomplete rescue observed here could be due to a necrotic component of the cell death which is not inhibited by the growth factors or, alternatively, an inability of the growth factors to completely counteract apoptotic stimuli. Moreover, our finding that either of the growth factors prevented NO-induced changes in Bcl-2 and Bax protein levels and activation of caspase-3-like protease provides a possible explanation for the neuroprotective effect of these growth factors against NO-mediated neurotoxicity. Taking into account the previous observation that Bcl-2 acts upstream of caspase-3 (Chinnaiyan *et al*, 1996) and the present observation that a caspase-3 inhibitor does not inhibit NO-induced changes in Bcl-2 and Bax protein levels, it is likely that the growth factors inhibit changes in Bcl-2 and Bax protein levels and thereby prevent activation of caspase-3-like protease, resulting in prevention of NO-induced neuronal death. During periods of cerebral ischemia, expression of both IGF-1 and bFGF is increased (Gluckman *et al*, 1992; Finkelstein *et al*, 1990; Kiyota *et al*, 1991). Therefore, it may be that the upregulation of the growth factors following hypoxic-ischemic insult functions in protecting against NO-induced neuronal death by preventing changes in Bcl-2/Bax expression *in vivo*.

Recent data suggest that several growth factors can prevent the loss of mitochondrial membrane potential in neurons caused by glucose deprivation (Mattson *et al*, 1993). In addition, various apoptotic stimuli release cytochrome C from mitochondria into the cytoplasm followed by a decline of mitochondrial membrane potential, which is prevented by Bcl-2 overexpression (Yang *et al*, 1997; Kluck *et al*, 1997). Since NO disturbs the mitochondrial membrane potential (Bolanos *et al*, 1997), it is likely that the growth factors maintain mitochondrial membrane potential by inhibiting NO-induced changes in Bcl-2/Bax expression, thereby preventing neuronal damage. The mechanism by which the growth factors prevent NO-induced changes in Bcl-2/Bax expression is not known. Recently phosphoinositide 3-kinase (PI3-kinase) and its down stream protein kinase effector Akt have been implicated in the transduction of survival signals by growth factors in neurons (Dudek *et al*, 1997) and in

Figure 6 Effects of IGF-1 and bFGF on NO-induced caspase-3-like activation and changes in Bcl-2/Bax protein levels. **(A)** Both IGF-1 and bFGF inhibited NO-induced upregulation of caspase-3-like activity. Hippocampal neurons pretreated with different concentrations of either IGF or bFGF were incubated with 50 μ M SNP for 24 h and caspase-3-like activities were measured. Data are mean $\pm$ S.E.M. of three independent experiments. * P < 0.05 versus SNP alone; two-way ANOVA followed by Scheffe's *post-hoc* test. **(B)** Both IGF-1 and bFGF inhibited NO-induced changes in Bcl-2 and Bax protein levels. Hippocampal neurons pretreated with either IGF (100 ng/ml) or bFGF (100 ng/ml) were incubated with or without 50 μ M SNP for 24 h, the cells were subjected to gel electrophoresis and the blots were probed with antibodies to Bcl-2, Bax of β -actin

non-neuronal cells (Kauffmann-Zeh *et al*, 1977; Kulik *et al*, 1997). Therefore, the PI3-kinase-Akt signaling pathway might be involved in inhibitory effect of the growth factors on NO-induced changes in Bcl-2/Bax expression or might function to inhibit neuronal death without any effect on Bcl-2/Bax expression. Another possible component of the neuroprotective mechanism of the growth factors is the modulation of intracellular calcium levels. It has been reported that growth factors can stabilize neuronal calcium homeostasis and thereby protect against ischemic insults (Mattson *et al*, 1993). NO causes disruption of intracellular calcium homeostatic mechanisms in cultured hippocampal neurons (Brorson and Zhang, 1997). In addition, overexpression of Bcl-2 in lymphoma cells reduces the efflux of Ca from endoplasmic reticulum that is caused by thapsigargin, an inhibitor of the endoplasmic reticulum-associated Ca pump, and prevents thapsigargin-induced apoptosis (Lam *et al*, 1994). Therefore, the Bcl-2 expression maintained by the growth factors might play a role in inhibiting NO-induced disruption of calcium homeostasis.

Based on these findings, we propose a hypothesis for the cascade of events during NO-induced neuronal death and the protective mechanism of the growth factors; NO induces downregulation of Bcl-2 and upregulation of Bax expression in hippocampal neurons. The changes in Bcl-2 and Bax expression cause caspase-3-like activation, resulting in neuronal death. IGF-1 and bFGF protect cells from NO-induced neuronal death by inhibiting changes in Bcl-2 and Bax expressions and thereby preventing activation of caspase-3-like protease. Further investigations will establish the upstream mechanism of NO-mediated changes in the mitochondrially expressed proteins in relation to the neuroprotective action of these growth factors.

Materials and Methods

Cell culture

Cultures of rat primary hippocampal neurons were prepared from embryonic day 18 Sprague-Dawley rat embryos as described previously (Murphy *et al*, 1990) with slight modifications. Fetal hippocampi were dissected and digested with calcium/magnesium-free Hank's balanced salt solution (GIBCO, Scotland) containing 0.25% trypsin for 20 min at room temperature. Tissues were dissociated by repeated trituration. The cells were seeded at a density of 1×10^6 cells/cm² on poly-L-lysine (10 µg/ml)-coated plates (Falcon Lab and Maintainware, Lincoln Park, NJ, USA) and maintained in growth medium at 37°C in a humidified atmosphere of 5% CO₂ and 95% room air. The growth medium consisted of DMEM supplemented with 10% inactivated fetal calf serum, 30 mM glucose and 0.5% (v/v) penicillin-streptomycin. To prevent growth of glial cells, cytosine arabinoside (10 µM) was added to the cultures 48 h after seeding. All experiments were performed in 8- to 10-day old cultures.

Experimental treatments

The growth medium was replaced with serum-deprived medium which consisted of DMEM supplemented with 30 mM glucose and 0.5% (v/v)

penicillin-streptomycin and then the cells were treated with NO donors. In experiments involving growth factors, cells were pretreated with either insulin-like growth factor-1 (IGF-1) (recombinant human IGF-1; Sigma) or basic fibroblast growth factor (bFGF) (recombinant human bFGF; Sigma) for 6 h before treatment with NO donors, and the growth factors were added directly to the cultures when the growth medium was replaced with serum-deprived medium. In experiments involving an inhibitor of caspase-3, acetyl-DEVD-CHO (Ac-DEVD-CHO) (Peptide Institute Inc., Osaka, Japan) was directly added to the cultures when the growth medium was replaced with serum-deprived medium prior to treatment with NO donors. Oligodeoxynucleotides (ODNs) were purchased from Yuki Gosei Kogyo Co (Tokyo, Japan). The sequence of the Bax antisense ODN was 5'-TGCTCCCCGGACCCGTCCAT-3', complementary to the translation initiation site of mouse bax mRNA. Three different control ODNs were used: sense ODN; mismatch control ODN, 5'-TCGTCCGGC-CACCGCTCACT-3', random sequence ODN, 5'-CGGCCATAA-TGTTCTCG-3'.

Assessment of cell viability

Neuronal cell viability was assessed by the release of lactate dehydrogenase (LHD) into the culture medium, which indicates loss of membrane integrity and cell death. LDH activity was measured using a commercial kit (Kyokuto Chemical Co., Tokyo, Japan), in which a colorimetric assay measures the pyruvate-mediated conversion of 2,4-dinitrophenylhydrazine into a visible hydrazone precipitate. Percent neuronal viability was expressed as $(1 - \text{experimental value}/\text{maximum release}) \times 100$, where the maximum release was obtained after exposure of untreated control cultures to 0.2% Triton X-100 for 15 min at 37°C.

Analysis of DNA fragmentation in agarose gel

Cortical cells (3×10^7) were lysed in 1 mL DNA extraction solution containing 20 mM Tris-HCl (pH 7.4) 0.1 M NaCl, 5 mM EDTA and 0.5% sodium dodecyl sulfate. The lysates were incubated with 100 µg/mL proteinase K at 37°C for 16 h. After incubation, 1 mL phenol/chloroform (1:1, v/v) was mixed well with the cell lysates, which were then centrifuged at 20 000 g for 10 min. DNA in the aqueous phase was incubated with 5 µg/mL DNase-free RNase A at 37°C for 1 h and extracted with phenol/chloroform again, and then with chloroform. DNA was collected by precipitation with two volumes of absolute ethanol in the presence of 5 M NaCl. After centrifugation, the DNA pellets were washed with 70% ethanol and air dried. The DNA was dissolved in 10 mM Tris-HCl and 1 mM EDTA, and its concentration was determined at 260 nm by spectrophotometry. DNA was separated on 1.8% agarose gel containing 1 µg/mL ethidium bromide and DNA fragments were visualized by exposing the gel to UV light.

Caspase-3-like enzyme activity

Caspase-3-like activity was measured by spectrophotometric assay as described previously (Shimizu *et al*, 1996). Briefly, neurons were suspended in buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 10 mM EGTA) and then incubated with 10 µM digitonin (Sigma, St. Louis, MO, USA) at 37°C for 10 min, followed by centrifugation at 15 000 r.p.m. for 3 min. Protein concentration in the resulting supernatant was measured using a DC protein assay kit (Bio-Rad, Richmond, CA, USA). Then the supernatant containing 30 µg protein was incubated with 50 µM of the enzyme substrate, 7-amino-4-coumarin (AMC)-DEVD at 37°C for 1 h. Levels of released AMC were measured using an excitation wavelength of 380 nm and an emission

wavelength of 460 nm with a spectrofluorometer (Hitachi F-3000, Hitachi, Tokyo, Japan). One unit was defined as the amount of enzyme required to release 0.22 nmol AMC per min at 37°C. Caspase-3-like activity of control neurons cultured alone and of neurons cocultured with astrocytes was 51 ± 5.3 and 48.5 ± 4.1 units/mg protein, respectively.

Western blot analysis

Cell extracts for Western blot analysis were prepared by washing the cells three times with phosphate-buffered saline (PBS) and lysing them in sample buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS) and 100 mM NaCl). The samples were boiled for 5 min before subjecting 20 µg of aliquots to electrophoresis on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After the proteins were transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA), the membrane was incubated in blocking buffer (1 × PBS, 5% non-fat dried milk) for 1 h at room temperature and then probed with a primary antibody in blocking buffer overnight at 4°C. The membrane was washed four times in PBS containing 0.3% Tween-20, probed with the secondary antibody in blocking buffer for 1 h at room temperature, and washed again in PBS containing 0.05% Tween-20. Detection of signals was performed with an enhanced chemiluminescence detection kit (Amersham International, Little Chalfont, UK). The primary antibodies used were anti-mouse iNOS polyclonal antibody (NO. M-19: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-rat Bcl-2 monoclonal antibody (MBL, Nagoya, Japan), anti-mouse Bax polyclonal antibody (No. P-19: Santa Cruz Biotechnology, Inc.), and anti-mouse β-actin monoclonal antibody (Amersham International).

Statistical analysis

Data are expressed as mean ± S.E.M. Significance was assessed by one-way or two-way ANOVA, followed by Scheffe's *post-hoc* test. *P* values less than 0.05 was considered as significant.

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