Rapid Attenuation of AP-1 Transcriptional Factors Associated with Nitric Oxide (NO)-mediated Neuronal Cell Death*

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Stimulation of glutamate receptors causes several intracellular reactions including activation of activator protein-1 (AP-1) production and nitric oxide (NO) generation. Exposing mouse cerebellar granule cells to N-methyl-D-aspartate or kainate (KA) in culture induced an increase of AP-1 DNA binding activity that was blocked by further addition of sodium nitroprusside (SNP), a typical NO donor. Immunoblotting using anti-c-Fos antiserum revealed the specific attenuation of AP-1, although total protein synthesis was not affected. Since the level of c-fos mRNA expression stimulated by KA remained constant even after exposure to SNP, the AP-1 attenuation can be post-transcriptionally induced. SNP did not affect the Ca2+ influx into the cells stimulated by KA. The involvement of NO in the AP-1 attenuation was supported by the fact that potassium ferrocyanide (K2Fe(CN)6), an analogue of SNP but devoid of NO, failed to inhibit the AP-1 DNA binding activity stimulated by KA. SNP alone induced neuronal cell death, which was blocked by the simultaneous addition of antioxidants, superoxide dismutase and catalase, and an NO scavenger, suggesting a direct role of peroxynitrite in the cell death. In good agreement with these effects, the AP-1 attenuation by SNP was also blocked by antioxidants. These results indicated that post-transcriptional attenuation of AP-1 is involved in the early processes of NO-mediated neuronal cell death.

The stimulation of glutamate receptors elicits an extracellular Ca2+ influx into neurons through NMDA1 receptors or voltage-dependent calcium channels (VDCCs) (1). The increased cytosolic Ca2+ concentration resulting from the stimulated Ca2+ influx into neurons successively activates a variety of cellular reactions, including the activation of Ca2+-dependent protein kinases (2) and the induction of immediate early genes such as c-fos and zif268 (3, 4). The transduced calcium signals also enhance the activity of nitric oxide synthase through Ca2+/calmodulin (5), resulting in the increased production of NO. Synthesized NO then diffuses out to neighboring cells where it activates soluble guanylate cyclase that can produce cGMP (6). NO may be involved in long term potentiation (7) and long term depression (8) in the central nervous system. During ischemic brain injury, however, NO may be responsible for neuronal cell death (9), which can be achieved by the cytosolic Ca2+ overload evoked via overstimulating glutamate receptors. Thus, NO seems to have dual functions as an intercellular messenger for synaptic plasticity and, if in excess, as a neurodestructive effector of neuronal injury. The mechanisms proposed for NO-mediated neurotoxicity include inactivation of the mitochondrial respiratory chain (10), S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (11), inhibition of cis-aconitase (12), activation of poly(ADP-ribose) synthase, and DNA damage (13), most of which can be mediated by the formation of nitrosocompounds by cellular components. However, it remains uncertain which cellular mechanisms are exactly responsible for the NO-mediated neurotoxicity. To elucidate these mechanisms, intracellular reactions that can be induced with NO-mediated neuronal cell death must be identified.

Activator protein-1 (AP-1) mainly consists of c-Fos and c-Jun, which are the members of the basic leucine zipper (bZIP) family, and it mediates immediate-early cellular responses by controlling the transcription of a variety of genes carrying AP-1 recognition sequences termed 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) (14). The AP-1 DNA binding activity can be modulated by the reduction-oxidation (redox) of a single conserved cysteine residue in the DNA-binding domains of c-Fos and c-Jun (15, 16). NO from sodium nitroprusside (SNP), an NO donor, may directly modulate the AP-1 DNA binding activity in vitro (17). In addition, reactive oxygen species including superoxide anions also affect c-fos mRNA expression and AP-1 DNA binding activity in cultured cells (18, 19). Thus, AP-1 DNA binding activity seems to be sensitive to changes in the redox state of cells and, hence, provides a useful molecular tool for investigating the mechanisms responsible for NO-mediated neuronal cell death.

Although brain ischemia may cause neuronal injury via a production of reactive oxygen species or NO in vivo (20, 21), NO-mediated neuronal cell death can be reproduced in primary cultures of neurons, which can be driven by the formation of peroxynitrite from NO and superoxide anion (22). For further understanding of the molecular mechanisms of NO-mediated neuronal cell death, we investigated the effect of NO on the AP-1 DNA binding activity using primary cultures of cerebellar granule cells. Stimulation of cerebellar granule cells with NMDA and KA, which causes the Ca2+ influx into the cells through NMDA receptors and VDCCs, respectively, leads to the activation of AP-1 DNA binding activity resulting from an increase in c-fos mRNA expression (23, 24). In this study, we...

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1 The abbreviations used are: NMDA, N-methyl-D-aspartate; KA, kainate; VDCC, voltage-dependent calcium channels; AP-1, activator protein-1; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element; SNP, sodium nitroprusside; SOD, superoxide dismutase; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl; LDH, lactate dehydrogenase; HI, hypoxic ischemia; SE, status epilepticus; 8-Br-cGMP, 8-bromoguanosine-3',5'-cyclic monophosphate.

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found that NO, released from an NO donor, rapidly causes the post-transcriptional attenuation of AP-1, followed by NO-mediated neuronal cell death.

**EXPERIMENTAL PROCEDURES**

**Materials**—NMDA, KA, TPA, SNP, and 8-bromoguanosine-3’5’-cyclic monophosphate (8-Br-cGMP) were purchased from Sigma. Potassium ferrocyanide, bovine copper, zinc-superoxide dismutase (SOD), and catalase were purchased from Wako Pure Chemicals Co. 2(4-Carboxyphosphoryl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) was from Dojin Chemicals Co.

**Cell Culture and Drug Stimulation**—Primary cultures of cerebellar granule cell were prepared from 7-day-old mice (ICR) as described (23). In brief, cells were seeded on plastic culture dishes coated with 5 mg/ml poly-1-lysine (Pharmacia Biotech Inc.) and incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 25 mM KCl, 3.6 mM NaHCO3, 2.3 mM CaCl2 and 5.6 mM glucose, and incubated in the same solution for 1 h. The glutamate receptor agonist, NMDA or KA, was added at the indicated concentrations 10 min before 100 µM NMDA, 100 µM KA, or 100 ng/ml TPA. One hour later, nuclear mini-extracts were prepared and their gel mobility was assayed. B, the cells were incubated for 1 h with 100 µM SNP (lanes 3–5). After washing with Locke’s solution without SNP and incubating for another 1 h, 100 µM KA was added (lane 4) or not (lane 3) and the cells were incubated for 1 h before preparing nuclear mini-extracts. SNP was added again 10 min before KA (lane 5). When the pretreatment of cells with SNP was omitted, the cells were continuously incubated without SNP for 2 h before KA stimulation was added (lane 2) or not (lane 1). The relative levels of TRE binding activities are shown as -fold increase compared with that of the control. Cont. and pre mean the unstimulated control and the pretreatment of cells with SNP, respectively. T means the bands formed by the DNA-protein complexes on TRE. F means free TRE-probe. Bars represent the mean S.D. obtained from two to three experiments.

**Preparation of Nuclear Mini-extracts and the Gel Mobility Assay**—The procedures for extracting nuclear mini-extracts and the conditions for the gel mobility assay were as described (23). The synthetic 20-base pair oligonucleotides containing TRE (5’-GATTCGTGACTCAGCA-CAGG-3’) was end-labeled with [32P]dCTP (Amersham) and used as a DNA probe to detect AP-1 DNA binding activity. DNA-protein complexes were separated on a 4% polyacrylamide gel in 1 X TAE (6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 2.5% glycerol, and 0.1 mM EDTA) and transferred onto a nylon membrane (Hybond N, Amersham). The membrane was washed twice with 2 X SSPE–0.1% SDS, denatured salmon sperm DNA. Thereafter, heat-denatured [32P]labeled DNA probes were added, and the membrane was hybridized at 42°C overnight. The membrane was washed twice with 2 X SSPE and 0.1% SDS for 10 min at 60°C, once with 1 X SSPE and 0.1% SDS for 30 min at 60°C, and finally twice with 0.1% SSPE and 0.1% SDS for 15 min at room temperature. After autoradiography, the level of mRNA expression was quantified using an imaging scanner (BAS 2000, Fuji).

**Fig. 1. The inhibitory effect of SNP on the increase in TRE binding activity induced by NMDA, KA, or TPA and its reversibility.**

| Concentration of SNP (µM) | Cont. | NMDA | KA | TPA |
|--------------------------|-------|------|----|-----|
|                          |       | 50   | 100|     |
|                          |       | 50   | 100|     |
|                          |       | 100  |    |     |
|                          |       | 100  |    |     |
|                          |       | 50   |    |     |
|                          |       | 100  |    |     |

The culture medium was replaced with Locke’s solution and the cells were incubated for 1 h. A, SNP was added to the Locke’s solution at the indicated concentrations 10 min before 100 µM NMDA, 100 µM KA, or 100 ng/ml TPA. One hour later, nuclear mini-extracts were prepared and their gel mobility was assayed. B, the cells were incubated for 1 h with 100 µM SNP (lanes 3–5). After washing with Locke’s solution without SNP and incubating for another 1 h, 100 µM KA was added (lane 4) or not (lane 3) and the cells were incubated for 1 h before preparing nuclear mini-extracts. SNP was added again 10 min before KA (lane 5). When the pretreatment of cells with SNP was omitted, the cells were continuously incubated without SNP for 2 h before KA stimulation was added (lane 2) or not (lane 1). The relative levels of TRE binding activities are shown as -fold increase compared with that of the control. Cont. and pre mean the unstimulated control and the pretreatment of cells with SNP, respectively. T means the bands formed by the DNA-protein complexes on TRE. F means free TRE-probe. Bars represent the mean ± S.D. obtained from two to three experiments.
SNP was added at the indicated concentrations 10 min before 100 μM NMDA or KA. After a 1-h incubation, nuclear mini-extracts (A) or total cellular extracts (B) were prepared, then aliquots (30 μg of proteins) were immunoblotted (see “Experimental Procedures”). C, cells were incubated with Locke’s solution containing 200 μCi of [35S]methionine mixture for 1 h in the presence or absence of SNP (100 and 200 μM) and cycloheximide (CHX) (10 μg/ml). Molecular mass (kDa) is also indicated with the figures.

**FIG. 2. Inhibitory effect of SNP on the accumulation of c-Fos and its non-inhibitory effect on total protein synthesis.** SNP was added at the indicated concentrations 10 min before 100 μM NMDA or KA. After a 1-h incubation, nuclear mini-extracts (A) or total cellular extracts (B) were prepared, then aliquots (30 μg of proteins) were immunoblotted (see “Experimental Procedures”). C, cells were incubated with Locke’s solution containing 200 μCi of [35S]methionine mixture for 1 h in the presence or absence of SNP (100 and 200 μM) and cycloheximide (CHX) (10 μg/ml). Molecular mass (kDa) is also indicated with the figures.

**RESULTS**

**Effect of SNP on TRE Binding Activity**—To investigate whether or not NO modulates AP-1 DNA binding activity, we examined the effect of the NO donor, SNP, on the increase of TRE binding activity induced by stimulating cultured cerebellar granule cells with NMDA, KA, and TPA, a potent activator for protein kinase C (23, 24). As shown in Fig. 1A, NMDA, KA, and TPA in Locke’s solution increased the TRE binding activities (lanes 2, 5, and 8) but further addition of SNP inhibited these induced increases in a dose-dependent manner (lanes 3, 4, 6, 7, 9, and 10). When the KA was administered after exposure to SNP for 1 h (Fig. 1B, lane 4), the TRE binding activity increased in a manner similar to that obtained when KA was administered alone (lane 2). When KA was omitted after the exposure to SNP, the level of TRE binding activity remained constant (lane 3). In addition, SNP was effective in inhibiting the increase in TRE binding activity stimulated by KA even after the exposure to SNP (lane 5). These results indicated that the inhibitory effect of SNP on the increase in TRE binding activity was reversible and, moreover, that exposing cells to SNP for 1 h does not irreversibly damage the cells.

**Attenuation of AP-1 by SNP**—We investigated whether the amounts of AP-1 in nuclear extracts decreased in accordance with the inhibitory effects of SNP on the TRE binding activity, by means of immunoblotting using anti-c-Fos antisera (Fig. 2, A and B). The immunoblots showed that c-Fos accumulation was induced by NMDA and KA (Fig. 2A, lanes 2 and 5, respectively), but inhibited by SNP (lanes 3, 4, 6, and 7). In addition, the inhibition of c-Fos protein accumulation by SNP was also observed with the total cellular extracts prepared from the cells treated with NMDA or KA (Fig. 2B). This attenuation of c-Fos proteins induced by SNP does not seem to be caused by a nonspecific inhibition of total protein synthesis, because the intensity of other nonspecific protein bands detected by cross-

**Measurement of [35S]Methionine Incorporation**—Total protein synthesis was measured by the incorporation of [35S]methionine into cellular protein fraction. Cells were labeled with 200 μCi of a [35S]methionine/cysteine mixture (Tran³⁵S-label, DuPont NEN) for 1 h in Locke’s solution in the presence or absence of NO donor and cycloheximide. After a 1-h incubation, the increase in TRE binding activity stimulated by KA even after the exposure to SNP (lane 5). These results indicated that the inhibitory effect of SNP on the increase in TRE binding activity was reversible and, moreover, that exposing cells to SNP for 1 h does not irreversibly damage the cells.

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Effect of SNP on the increase in \( c\)-\( fos \) mRNA expression induced by NMDA, KA, or TPA. SNP was added at the indicated concentrations 10 min before 100 \( \mu \)M NMDA (A), 100 \( \mu \)M KA (B), or 100 ng/ml TPA (C). After 1 h, cytoplasmic RNA was prepared and hybridized with a \( ^{32}P \)-labeled \( c\)-\( fos \) probe (see "Experimental Procedures"). Hybridization also proceeded with a \( \beta\)-actin probe, and the results are shown in the middle panels. The radioactivity of the bands after autoradiography was quantified using an imaging scanner and the relative levels of \( c\)-\( fos \) mRNA expression are shown as -fold increase in the bottom panels. Bars represent the mean \( \pm \) S.D., calculated from three separate experiments.

Effect of SNP on \( c\)-\( fos \) mRNA Expression—To further investigate whether or not the attenuation of \( c\)-Fos proteins corresponds to a decrease in the amount of \( c\)-\( fos \) mRNA in the cells, we examined the effect of SNP on the increase in \( c\)-\( fos \) mRNA expression induced by NMDA, KA, or TPA by means of Northern blotting. As shown in Fig. 3A, SNP inhibited the increase of \( c\)-\( fos \) mRNA expression induced by NMDA in a dose-dependent manner. In contrast, SNP did not change the level of \( c\)-\( fos \) mRNA expression stimulated by KA (Fig. 3B) or TPA (Fig. 3C).

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Even a higher concentration (1 mM) of SNP did not change the level of \( c\)-\( fos \) mRNA expression induced by KA (data not shown). SNP alone did not affect the \( c\)-\( fos \) mRNA expression, and \( \beta\)-actin mRNA expression in the cells, although cycloheximide (10 \( \mu \)g/ml) completely abolished the \( ^{35}S \)methionine incorporation (lane 4).

Effect of SNP on 45Ca\(^{2+}\) Uptake—Since the activation of TRE binding activities and \( c\)-\( fos \) mRNA expression induced by NMDA or KA depends upon the extracellular Ca\(^{2+}\) influx through NMDA receptors or VDCCs (24), we examined the effect of SNP on the Ca\(^{2+}\) influx into the cells. As shown in Fig. 4A, stimulating cells with NMDA or KA induced the increase of 45Ca\(^{2+}\) uptake by the cells, and the addition of 100 \( \mu \)M SNP decreased the 45Ca\(^{2+}\) uptake stimulated by NMDA to the level of the control, but did not affect that stimulated by KA. In addition, SNP inhibited the NMDA- but not the KA-evoked 45Ca\(^{2+}\) uptake in a dose-dependent manner (Fig. 4B). Potassium ferrocyanide (K\(_{4}\)Fe(CN)\(_{6}\)), a chemical analogue of SNP (Na\(_{2}\)Fe(CN)\(_{5}\)NO), mimicked the effects of SNP on the 45Ca\(^{2+}\) uptake by the cells, in which K\(_{4}\)Fe(CN)\(_{6}\) inhibited the NMDA-evoked 45Ca\(^{2+}\) uptake but not that by KA (Fig. 5A).

K\(_{4}\)Fe(CN)\(_{6}\) Does Not Decrease the TRE Binding Activity Stimulated by KA—As shown in Fig. 5B (lanes 2–4), the increase in TRE binding activity induced by NMDA was also inhibited by K\(_{4}\)Fe(CN)\(_{6}\) as was observed with SNP (Fig. 1A). In contrast, however, K\(_{4}\)Fe(CN)\(_{6}\) did not inhibit the increase in TRE binding activity induced by KA (Fig. 5B, lanes 5–7), whereas SNP did (Fig. 1A). Since the main difference in the chemical structures of SNP and K\(_{4}\)Fe(CN)\(_{6}\) is NO, we examined whether or not NO could be released from SNP in culture. We monitored the levels of intracellular cGMP after incubating cells with SNP or K\(_{4}\)Fe(CN)\(_{6}\) and found that cGMP accumulated with increasing concentrations of SNP in a time- and dose-dependent manner but not with those of K\(_{4}\)Fe(CN)\(_{6}\) (data not shown), suggesting that NO is released from SNP in culture and that it affects the cGMP production in the cells.

Cytotoxic Effect of SNP—Since NO can be largely involved in neuronal cell death (9, 13, 20–22, 28), we examined whether or not SNP causes the death of cerebellar granule cells. As shown in Fig. 6A, exposing cells to SNP for 1 h elicited only a small increase in LDH release, whereas for 4 h significantly increased it. SNP induced a high level of LDH release regardless of KA. The LDH release detected at 4 h increased in a dose-dependent manner, starting at 50 \( \mu \)M and reaching a plateau at 500 \( \mu \)M SNP (data not shown). K\(_{4}\)Fe(CN)\(_{6}\) did not cause LDH release (Fig. 6A). The addition of SOD, a superoxide anion scavenger, significantly reduced the LDH release in-
duced by SNP, whereas catalase did not (Fig. 6B). The simultaneous addition of SOD and catalase reduced the level of LDH release but not completely (Fig. 6B). The addition of SOD and catalase alone had no effect on the LDH release (Fig. 6B).

Furthermore, the addition of carboxy-PTIO, an NO scavenger (29), also reduced the LDH release induced by SNP in a dose-dependent manner (Fig. 6C). The simultaneous addition of carboxy-PTIO with SOD and catalase reduced the LDH release almost to that of the control. Carboxy-PTIO alone had no effect on the LDH release from the cells.

**DISCUSSION**

In this study, we found that SNP, a potent NO donor, effectively inhibited the increases in TRE binding activity induced by NMDA or KA in primary cultures of mouse cerebellar granule cells (Fig. 1A). Corresponding to this inhibitory effect of SNP, the amounts of c-Fos proteins in the nuclear extracts decreased when the cells were incubated with SNP (Fig. 2A). These results indicated that the attenuation of AP-1 was induced in the cells incubated with SNP. However, the mode of this AP-1 attenuation by SNP differed in cells stimulated with NMDA or KA. Since the increase in TRE binding activity stimulated by NMDA and KA is dependent upon the Ca\(^{2+}\) influx into neurons through NMDA receptors and VDCCs, respec-

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**Fig. 4.** Effect of SNP on the \(^{45}\)Ca\(^{2+}\) uptake induced by NMDA or KA. A, time course of \(^{45}\)Ca\(^{2+}\) uptake. SNP (100 \(\mu\)M) was added 10 min before adding 100 \(\mu\)M NMDA or KA with 1 \(\mu\)Ci \(^{45}\)CaCl\(_2\), and the cells were incubated for the indicated times. Cont. means the unstimulated control. B, dose dependence of the SNP effect on \(^{45}\)Ca\(^{2+}\) uptake. SNP was added at the indicated concentrations 10 min before 100 \(\mu\)M NMDA or KA, and the cells were incubated with \(^{45}\)CaCl\(_2\) for 10 min before measuring the \(^{45}\)Ca\(^{2+}\) uptake by the cells. Results are shown as a percent of control (100%) in which the cells stimulated with NMDA or KA were incubated without SNP.

**Fig. 5.** Effects of potassium ferrocyanide on the increase in TRE binding activity and \(^{45}\)Ca\(^{2+}\) uptake induced by NMDA or KA. A, potassium ferrocyanide (100 \(\mu\)M) was added 10 min before 100 \(\mu\)M NMDA or KA, and the cells were incubated with 1 \(\mu\)Ci \(^{45}\)CaCl\(_2\) for 10 min. Relative levels of \(^{45}\)Ca\(^{2+}\) uptake are shown as a fold increase compared with that of the unstimulated control. Bars represent the mean \(\pm\) S.D., calculated from two separate experiments. B, potassium ferrocyanide (100 \(\mu\)M) was added at the indicated concentrations 10 min before stimulating the cells with 100 \(\mu\)M NMDA or KA. After 1 h, nuclear mini-extracts were prepared for the gel mobility assay.

Recovery of TRE Binding Activity by SOD and Catalase—We investigated the relationship between the SNP-mediated inhibition of the TRE binding activity stimulated by KA (Fig. 1A) and the SNP-mediated cytotoxicity (Fig. 6). Although a slight recovery of TRE binding activity from the SNP-mediated inhibition was detected after adding SOD (Fig. 7, compare lane 4 with lane 3), the simultaneous addition of SOD and catalase markedly recovered the TRE binding activity to the same level as that obtained by KA stimulation (Fig. 7, compare lane 8 with lane 2). SOD and catalase alone had no effect on the TRE binding activity (lanes 7 and 8). When KA stimulation was omitted, SNP did not significantly decrease TRE binding activity and subsequent addition of SOD and catalase did not affect the level of TRE binding activity (lanes 9–11).
Fig. 6. SNP-induced cytotoxicity and its inhibition by antioxidants and NO scavenger. The cytotoxic activity of SNP is shown as a percentage of LDH released from the cells into the Locke's solution (see “Experimental Procedures”). Data are shown as mean ± S.D. obtained from three to six separate experiments and were assessed by Student's t test. A, KA (100 μM), SNP (100 μM), or potassium ferrocyanide (100 μM) were added and the cells were incubated for 1 or 4 h. Asterisks represent a difference from the control (cont.) of 4-h incubation (***, p < 0.001). Ferro. means potassium ferrocyanide. B, SOD (100 units/ml) and catalase (cat.; 100 units/ml) were added 10 min before SNP, and LDH release was measured after incubating the cells for 4 h. Asterisks represent differences from the results obtained in the presence of SNP alone (*, p < 0.05; **, p < 0.01). C, carboxy-PTIO (cPTIO; 10 or 50 μM), SOD (100 units/ml), and catalase (100 units/ml) were added 10 min before 100 μM SNP. LDH release was measured after incubating the cells for 4 h. Asterisks represent a difference from the results obtained in the presence of SNP alone (**, p < 0.01, *** ,p < 0.001).

Fig. 7. Recovery of the inhibitory effect of SNP on the KA-induced increase in TRE binding activity by antioxidants. SOD (100 units/ml) and catalase (cat.; 100 units/ml) were added 20 min, and 100 μM SNP was added 10 min before 100 μM KA. When KA was omitted, SOD and catalase were added 10 min before SNP. After 1 h, the cells were collected to prepare nuclear mini-extracts.

Potassium ferrocyanide (K₃Fe(CN)₆) mimicked the effects of SNP on the Ca²⁺ influx when the cells were incubated with NMDA or KA, where it blocked the NMDA- but not the KA-induced Ca²⁺ influx (Figs. 4 and 5A). The blockade of the NMDA-induced Ca²⁺ influx by SNP and K₃Fe(CN)₆ is assumed to be due to the ferrocyanide portion of these molecules, which has been suggested by Costa et al. (30). Since SNP did not block the Ca²⁺ influx evoked via Bay K8644 (data not shown), a potent agonist for VDCCs, the ferrocyanide portion of these molecules appears to be unable to block the Ca²⁺ influx through VDCCs, accounting for the failure of SNP and K₃Fe(CN)₆ to block the KA-induced Ca²⁺ influx that is caused through VDCCs (24). Potassium ferricyanide (K₃Fe(CN)₆) did not block the increase in ⁴⁵Ca²⁺ uptake stimulated by NMDA or KA (data not shown).

The possible involvement of NO in the AP-1 attenuation by SNP was supported by the failure of K₃Fe(CN)₆ to inhibit the increase in TRE binding activity induced by KA (Fig. 5B). The release of NO from SNP and the action of NO on the cells were confirmed by the accumulation of intracellular cGMP in the cells incubated with SNP (data not shown), which can be mediated by guanylate cyclase stimulated by NO (6). However, the AP-1 attenuation observed with the KA stimulation is independent of cGMP signals because 8-Br-cGMP had no effect on the NMDA- and KA-induced increases in TRE binding activity (data not shown). Since ferrocyanide ions do not pass through the cytoplasmic membrane (30), it seems reasonable that NO released from SNP can act on the cells, leading to AP-1 attenuation, directly or indirectly.

SNP requires reduction before NO is released (31). Actually, only a small signal of NO release from SNP was detected in the culturing medium, which was indirectly examined by the formation of nitrite, while the addition of diethiothreitol, a reducing agent, with SNP dramatically enhanced its formation (data not shown). Therefore, it can be speculated that in culturing medium NO could be released from SNP through the interaction with some reducing equivalents of the cells and subsequently incorporated into the cells. Thus, the mode of action of SNP on the cells remains to be further elucidated.

As shown in Fig. 6, SNP potently induced cell death. The cytotoxic effect of SNP can be largely avoided by adding SOD, suggesting that superoxide anion is key to the cell death caused by SNP. Furthermore, the finding that carboxy-PTIO prevented the LDH release (Fig. 6C) revealed the involvement of...
NO in the SNP cytotoxic effect. The involvement of NO in the SNP-cytotoxic effect was further supported by the failure of K$_2$Fe(CN)$_6$ to induce cell death (Fig. 6A). The simultaneous addition of SOD, catalase, and carboxy-PTIO almost totally prevented SNP-mediated cell death (Fig. 6C). These results indicated that peroxynitrite, a potent oxidant, which can be produced by NO and superoxide anion, is a direct cause of SNP-mediated neuronal cell death rather than NO or superoxide anion alone. However, we do not know exactly how superoxide anion is supplied from the cells.

The inhibitory effect of SNP on the increase in TRE binding activity induced by KA was effectively overcome by SOD and catalase (Fig. 7), suggesting that the AP-1 attenuation is associated with neuronal cell death induced by SNP. If so, AP-1 attenuation should be immediately caused before cell death because SNP inhibited the KA-stimulated TRE binding activity within 1 h (Fig. 6A), whereas significant cytotoxic effects were detected 4 h after incubating cells with SNP (Fig. 6A). Thus, the AP-1 attenuation might be an acute response of cells that is required to promote NO-mediated cell death.

Reduction-oxidation (redox) regulation is one factor that controls AP-1 DNA binding activity (15, 16). The conserved cysteine residues in the DNA-binding domain of AP-1 can be modified by the redox regulation, leading to the control of AP-1 DNA binding activities (15, 16). Cysteine residues interact with NO to produce the S-nitrosyl compounds through redox-regulatory mechanisms (32, 33). On the other hand, proteolytic events involving the cysteine proteases such as interleukin 1β-converting enzyme (34) are necessary for apoptotic cell death. Several kinds of cellular proteins including poly(ADP-ribose) polymerase (35, 36) are proteolytically degraded in cells undergoing apoptosis. These results support the notion that AP-1 can be attenuated by its proteolytic decomposition, which might be mediated by S-nitrosylation of AP-1.

Although SNP inhibits the total protein synthesis in hepatocytes at concentrations over 500 μM (37), we did not detect a decrease in the rate of protein synthesis when SNP was added at a concentration of 100 or 200 μM (Fig. 2C). Nevertheless, it seems likely that a selective blockade of c-Fos protein synthesis by SNP is involved in the AP-1 attenuation. Since c-Fos proteins can be rapidly degraded by the ubiquitin-related proteolytic pathway after their syntheses (38), c-Fos proteins should be secreted from the cells immediately after inhibiting protein synthesis. Other scenarios in which AP-1 attenuation involving its translocation to the nucleus being blocked by SNP can be excluded, because the amounts of c-Fos proteins decreased in both the nuclear and total cellular extracts (Fig. 2, A and B).

Various insults to the brain such as hypoxic ischemia (HI) and status epilepticus (SE) can cause two types of neuronal cell death; moderate HI and SE lead to apoptosis, whereas severe HI leads to necrosis (39). While moderate HI and SE induce a prolonged c-fos mRNA expression in brain neurons (39), severe HI rapidly induces c-fos mRNA expression but does not cause the accumulation of c-Fos proteins in the brain (40), suggesting that the attenuation of c-Fos proteins could be posttranscriptionally induced in brain neurons by severe HI. Even in primary cultures of neurons, production of peroxynitrite can elicit not only apoptotic neuronal cell death with moderate exposure, but also necrosis with severe exposure (41). In addition, apoptotic and necrotic cell death can also be induced in cerebellar granule cells incubated with glutamate (42). Thus, it seems likely that the rapid attenuation of AP-1 is one indicator of acute neuronal cell death mediated by NO, which could be caused by ischemic brain injury.

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