Nitric Oxide Promotes Survival of Cerebral Cortex Neurons with Simultaneous Addition of [Fe(II)(β-Citryl-L-glutamate)] Complex in Primary Culture

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It has been reported that the activity of mitochondrial aconitase (m-aconitase) is rapidly inhibited in a variety of cells when exposed to nitric oxide (NO). In present study, we found that NO significantly increased the number of surviving neurons via enhanced mitochondrial functions with simultaneous addition of the [Fe(II)(β-citryl-L-glutamate; β-CG)] complex. In vitro, a variety of aconitase-inhibitors, such as fluorocitrate, cyanide ion, ferricyanide ([Fe(NC)₆]₃⁻), and various oxidants including superoxide anion, inhibited the activity of m-aconitase even in the presence of Fe(II), whereas a NO-donor, nitroprusside (SNP) ([Fe(NC)₃NO]), was the only agent that significantly increased activity of that enzyme. Therefore, it is reasonable to assume that NO released from SNP promotes Fe-dependent activation of aconitase. All other tested NO-donors, including 3-morpholino-sydnonimine (SIN), Deta NONOate (NOC18), and NaN₃O₂, also promoted activation of m-aconitase in time- and dose-dependent manners in the presence of Fe(II). The promoting effects of the NO-donors on activation disappeared with the addition of NO-scavengers. In intact mitochondria, all tested NO-donors promoted reactivation of aconitase in a dose-dependent manner in the presence of Fe(II), whereas that was not seen in its absence. These findings suggest that NO released from NO-donors promotes Fe-dependent activation of aconitase. In mixed neuronal and glial cultures, NO-donors except for SNP enhanced mitochondrial activity at low concentrations. Furthermore, simultaneous addition of the [Fe(II)(β-CG)] complex significantly enhanced those activities and greatly increased the number of surviving neurons. Thus, NO can carry Fe ions into m-aconitase via the guide of the tag of β-CG addressed to the enzyme.

Key words nitric oxide; iron-carrier; beta-citrylglutamate; mitochondrial aconitase; neuron

Aconitase is a critical citric acid-cycle enzyme that catalyzes the stereospecific interconversion of citrate and isocitrate via the intermediate cis-aconitate, whose activities depend on an intact [4Fe–4S]²⁺ cluster. This enzyme contains a unique [4Fe–4S]²⁺ cubane cluster in its active catalytic sites, with one particularly labile Fe atom (so-called Feₕ). Loss of aconitase activity is commonly used as a biomarker of oxidative damage due to susceptibility of the [4Fe–4S]²⁺ cubane cluster to oxidative disassembly. Oxidatively inactivated aconitase is rapidly reactivated in vitro and in vivo, by cluster reduction and Fe(II) ion reinsertion. However, the physiological mechanisms for reduction of [3Fe–4S] and Fe(II) insertion into the [3Fe–4S]° center are currently unknown. In cells, aconitase is in a dynamic state of inactivation and reactivation, while the Fe–S center is under continuous threat from attack by physiological oxidants including superoxide anion, hydrogen peroxide (H₂O₂), molecular oxygen, and nitric oxide (NO), and perhaps even peroxynitrite (ONOO⁻). Significant inactivation of aconitase has been reported to occur after exposure to such oxidants.

NO is a free radical produced by a wide variety of cell types, that reacts predominantly with Fe(III) or Fe(II) ions of heme proteins and Fe–S centers, as well as molecular oxygen, and superoxide anion. Moreover, NO easily reaches mitochondria from cytosolic or extracellular sources, due to its low molecular radius and hydrophobic nature. It has also been reported that induction of NO synthesis or exposure of different cell types to NO-donors resulted in early loss of mitochondrial aconitase (m-aconitase) activity. NO-mediated inactivation of m-aconitase has been reported in a variety of cells including macrophages, fibroblasts, tumor cells, and Escherichia (E.) coli, though results from in vitro studies are somewhat contradictory. Indeed, in vitro studies that used porcine heart m-aconitase found that low concentrations of NO did not inactivate aconitase, whereas high concentrations led to moderate inhibition. Resistance of purified E. coli aconitase and human recombinant cytosolic aconitase (c-aconitase) to NO-dependent inactivation has also been reported. On the contrary, inactivation by either NO or a NO-donor was reported for both m-aconitase and c-aconitase in the presence and absence of a substrate.

In agreement with those findings, it was shown that the active form [4Fe–4S] of m-aconitase is rapidly and directly oxidized by ONOO⁻, to [3Fe–4S], causing a loss of catalytic activity. Another report also noted that E. coli aconitase is highly sensitive to NO-mediated inactivation apparently independent of ONOO⁻ formation. Recently, it was also reported that NO binds to Feₕ in the [4Fe–4S] cluster of recombinant porcine m-aconitase and slowly promotes complete cluster disassembly.

The present findings demonstrate that NO-donors promote Fe-dependent activation of m-aconitase ([3Fe–4S] form) in vitro. In intact mitochondria, the NO-donors also promoted Fe-dependent reactivation of the enzyme disassembled by ammonium peroxodisulfate (APS). In mixed neuronal and...
glial cultures, NO-donors except for sodium nitroprusside (SNP) enhanced mitochondrial activity at low concentrations. Furthermore, simultaneous addition of the [Fe(II)(β-citryl-l-glutamate; β-CG)] complex significantly enhanced mitochondrial activities and greatly increased the number of surviving neurons. Thus, NO plays a role as an Fe-carrier to mitochondrial aconitase, and then activates it, while β-CG is considered to function as a tag addressed to the enzyme.

MATERIALS AND METHODS

Materials  Aconitase (from porcine heart), isocitrate dehydrogenase recombinant (from yeast), xanthine oxidase (from buttermilk), and β-nicotinamide adenine dinucleotide phosphate (NADP+) were purchased from Oriental Yeast Co. (Tokyo Japan). Deferoxamine mesylate (Def: Fe(III) chelator), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dl-fluorocitric acid tribarium were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Amino-3-morpholynyl-1,2,3-oxazolium chloride (SIN chloride), came from Toecris Cookson Inc. (Missouri, U.S.A.). Fe(II)/O (Alfa Aesar), Fe(III)/O, ammonium Fe(III)citrate, Fe(II)gluconate (Alfa Aesar), and haemin chloride (Alfa Aesar), came from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), while 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazine (Deta NONOate; NOC18) came from Calbiochem (CA, U.S.A.). Hypoxanthine sodium, ammonium Fe(II) sulfate hexahydrate (Fe(II)AS), nitroprusside sodium (SNP), Fe(II) lactate, and amnonium peroxodisulfate (APS) were obtained from Nacalai特斯 (Tokyo Japan). d-eferoxamine mesylate (def: Fe(III) chelator) was synthesized as previously described.15) [Fe(II)(Citrate)] complex was prepared from Fe(II)/O and trisodium citrate as previously described.16) All other reagents were purchased from commercial sources at the highest grade available.

Determination of Aconitase Activity  Enzyme activities were assayed by determining the formation of cis-aconitate from isocitrate.11) UV assays were performed at room temperature. The final volume was 1 mL, including 25 mM Tris–HCl, pH 7.4, and 2 mM trisodium dl-isocitrate. After addition of the enzyme, changes in absorption at 240 nm were determined from 0.5 to 1.5 min. One unit was defined as the amount of enzyme necessary to produce 1 µmol of cis-aconitate/minute (ε240nm=3.6 mmol−1 cm−1).5) Unless otherwise noted, all specific activities refer to the UV assay. The presence of Fe(II) and a reducing agent, such as cysteine or dithiothreitol, is required for activation of aconitase.3) In some experiments, aconitase activity was determined using a coupled assay,1) with which activation of aconitase preparations were used as “activated aconitase” in the experiments.

Treatments of Intact Mitochondria with Oxidants: Effects of NO-Donor on Reactivation of Mitochondrial Aconitase  Mitochondrial fractions were prepared from Wistar rats hearts as previously described7) and stored at −80°C until use in the experiments.

Mitochondria were diluted to about 25 mg of protein/mL in 100 mM Tris–HCl buffer, pH 7.4, and then incubated at 10 min after addition of 100 µM ammonium peroxodisulfate (APS), a relatively mild oxidant, as previously described.3) Aconitase ([3Fe–4S] cluster form) damaged by APS loses its enzyme activities, while it can be reactivated with Fe(II) ion. All incubations were performed at 37°C. After incubation, 30-µL aliquots of the reaction mixtures were suspended in 30 µL of 1% Triton X-100, and solubilized, then 50-µL aliquots were used for determination of aconitase activity.16)

Primary Cell Cultures from Newborn Mouse Brain Tissues  Cerebroms of 1-d-old ddY mice were dissected and placed in Ca2+/Mg2+-free Dulbecco’s phosphate-buffered saline [PBS(−)]. Dissociation and cultures of neurons were performed as previously described,19) with slight modifications. In brief, cerebrum specimens were dispersed using a pipette. Aliquots (0.1 mL) of the cell suspension, usually 2×105 cells/mL, were placed in 96-well plates pre-coated with 0.1% poly-DL-ornithine. After incubation for 60 min at 37°C in a 5% CO2 atmosphere, the medium was changed to serum-free Dulbecco’s modified Eagle’s medium (DMEM) containing N1 constituents, consisting of 5 ng/mL of insulin, 5 ng/mL of transferrin, 7.3 ng/mL of progesterone, 16 µg/mL of putrescine, and 5 ng/mL of selenium, then the cells were successively incubated for 3 d under the same conditions described above for the neuron-enriched cultures. Contamination by non-neuronal cells in the cultured cell populations, estimated by determination with anti-β-III tubulin antibodies, was scarcely observed until day 7 after inoculation.

In separate experiments, aliquots (0.1 mL) of the cell suspension detailed above were placed in 96-well plates pre-coated with 0.1% poly-DL-ornithine. After incubation for 60 min at 37°C in a 5% CO2 atmosphere, the medium was changed to DMEM containing 5% fetal calf serum (FCS). The next day, the medium was changed to that containing 20 µM cytosine arabinoside, and the cells were successively incubated for 3 d (mixed neuronal and glial cultures). The time of conversion to FCS was designated as day 0. The populations of neuronal cells in the culture dishes ranged from about 50–60% on day 5 after inoculation, as estimated by the ratio of cells reacting with anti-β-III tubulin antibodies to the total number of cells, with about 10% of the total cells stained with anti-glia fibrillary acidic protein (GFAP) (astrocyte-specific marker) antibodies.

Cultured cells were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS(−) for 10 min, and treated with Blocking One solution (Nakalai, Japan). Next, they were incubated for 2 h with anti-β-III tubulin (Sigma, U.S.A.; 1:500) or GFAP (Becton Dickinson U.S.A.; 1:1000) primary antibodies at 4°C, then washed in TTBS (50 mM Tris–HCl, pH 7.4, 150 mM
NaCl, 0.05% Tween20) and incubated for 2h with fluorescent-conjugated secondary antibodies (Alexa fluoros546, Molecular Probes, U.S.A.; 1: 1000) at room temperature. The cells were observed using an IX70 fluorescence microscope (Olympus, Japan).

**MTT Reduction Assay of Primary Cultured Cells** An MTT reduction assay assesses the integrity of mitochondrial functions, as described by Mosmann. In this assay, a principle that the tetrazolium ring on MTT is reduced by the electron transfer system in active mitochondria. The primary enzymes to reduce MTT have been shown to be succinate dehydrogenase in complex II (sucinate: ubiquinone oxidoreductase) and antimycin A-sensitive cytochrome c oxidase in complex III. In the present study, the assay was performed according to the specifications of the manufacturer (MTT kit I; Roche, Mannheim, Germany).

The contents of DNA in the cells were determined according to a method previously described. In brief, 2×10^6 cells were cultured in 24-well plates for 3d, then solubilized in 1mL of lysis buffer, and treated with a proteinase K and RNase A solution. DNA was extracted once with phenol-chloroform and precipitated twice, first with isopropyl alcohol and then with 70% ethanol, after which air-dried pellets were solubilized. DNA concentrations were calculated by determining the OD_260 value.

**RESULTS**

**Comparison of Effects of Nitroprusside with Those of Other Inhibitors on Aconitase Activities** Aconitase was obtained from a commercial source in an inactive state and required activation, as a previous study indicated that Fe(II) is needed in the presence of a reducing agent such as cysteine and dithiothreitol for that activation. In the present study, commercial porcine heart aconitase ([3Fe–4S] form) was activated for 20min at 37°C in the presence of Fe(II)AS (0.05mM), then inhibitory activity was determined at room temperature using a UV assay. FC, EDTA, Def and hydrogen peroxide were used at a concentration of 0.1mM, while the concentrations of SNP [Fe(CN)NO], APS, ferricyanide [Fe(CN)_6^3-], and KCN were 0.05, 0.25, 2, and 5mM, respectively. X: XO: superoxide anion generated by pre-incubation at 37°C for 20min with hypoxanthine (X) (0.25mM) and xanthine oxidase (XO) (125 unit/mL); FC: 0.1-fluorocitrate, Def: deferoxamine, APC: ammonium peroxodisulfate. Blank: activity only in the presence of DTT. C: control, activity in the presence of DTT and Fe(II)AS. Data are expressed as the mean±S.E.M. (bar). n=3–5; *p<0.01 to control (one-way ANOVA followed by a Tukey–Kramer multiple comparisons test).

Figure 2A shows that all NO-donors including SNP promoted aconitase-activation in the presence of Fe(II)AS, whereas only SNP increased it slightly even in the absence of Fe(II)AS. Fe released from SNP may be responsible for the observed effects on activation of aconitase. Figure 2B indicates that incubation of aconitase with all NO-donors resulted in time-dependent promotion of aconitase-activation in the presence of Fe(II)AS. This activation was significantly promoted by the NO-donors as compared with Fe(II)AS alone. As shown in Fig. 2C, SNP strongly promoted aconitase-activation in a dose-dependent manner in the presence of Fe(II)AS, while that promotion was dose-dependent and moderate in its absence. Activation by SNP alone may be mediated by Fe...
ion released from the SNP molecule. Indeed, pre-incubation of SNP (0.025 mM) with dithiothreitol (dTT) (2.5 mM) at 37°C led to time-dependent activation as compared to without pre-incubation (data not shown). Figure 2D demonstrates that both SIN and NOC18 strongly promoted that activation in a dose-dependent manner in the presence of Fe(II)AS. The effects of NO-donors on aconitase-activation were examined in the presence of X/XO, with the results shown in the Fig. 3A. X/XO strongly counteracted the promoted activation by SIN and NOC18, and moderately decreased that by SNP. Fe ions released from the SNP molecule were estimated to become reinserted into aconitase damaged by ONOO−, as Fe ion can reinsert into aconitase damaged by various oxidants.3 However, that mechanism is unclear.

NO is also known to be counteracted by various NO-scavengers including heme33) and imidazolineoxyln N-oxide derivative (PTIO).34) Therefore, the effects of heme and PTIO on NO-mediated promotion of aconitase-activation were examined. As shown in Fig. 3B, the promoting effect of all tested NO-donors on activation was completely prevented by PTIO, while that was moderately prevented by heme. Taken together, these findings suggest that NO released from NO-donors promotes aconitase-activation.

Effects of NO-Scavengers on Aconitase-Activation Promoted by NO Since NO reacts with superoxide anion, yielding peroxynitrite, it was also of interest to explore the effects of NO under conditions generating superoxide anion. Superoxide was generated by pre-incubation at 37°C for 20 min with hypoxanthine (X) and xanthine oxidase (XO).26,27) The effects of NO-donors on aconitase-activation were examined in the presence of X/XO, with the results shown in the Fig. 3A. X/XO strongly counteracted the promoted activation by SIN and NOC18, and moderately decreased that by SNP. Fe ions released from the SNP molecule were estimated to become reinserted into aconitase damaged by ONOO−, as Fe ion can reinsert into aconitase damaged by various oxidants.3 However, that mechanism is unclear.

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Fe-Dependence for Promotion of Aconitase-Activation by NO-Donors Our findings clearly indicate that NO strongly promoted Fe-dependent activation of aconitase in time- and
On the other hand, it has also been reported that NO predominantly reacts with Fe(III) or Fe(II) ions in heme, Fe-S centers in various enzyme proteins, and reported that NO is known to be counteracted by heme and PTIO. The effects of heme (0.05 mM) and PTIO (1 mM) on NO-donor-mediated promotion of aconitase-activation were examined in the presence of Fe(II)AS (0.02 mM) and SNP (0.025 mM), SIN (1 mM), NaNO 2 (10 mM), and NOC18 (0.2 mM). Data were obtained from 3–4 experiments and are expressed as the mean±S.E.M. (bar). Fe: activity in the presence of DTT and Fe(II)AS. X/XO: activity in the presence of hypoxanthine and xanthine oxidase. *p<0.01, **p<0.05 vs. (Fe alone) (two-way ANOVA followed by a Tukey–Kramer multiple comparisons test). (B) Effects of heme and PTIO on aconitase-activation promoted by NO-donors. NO is known to be counteracted by heme and PTIO. The effects of heme (0.05 mM) and PTIO (1 mM) on NO-donor-mediated promotion of aconitase-activation were examined in the presence of Fe(II)AS (0.02 mM) and SNP (0.025 mM), SIN (1 mM), NaNO 2 (10 mM), and NOC18 (0.2 mM). Data were obtained from 3–4 experiments and are expressed as the mean±S.E.M. (bar). B: Blank, activity only in the presence of DTT. C: control, activity in the presence of DTT and Fe(II)AS. N: none, without TPIO and Heme. *p<0.01, **p<0.05 vs. control or N (two-way ANOVA followed by a Tukey–Kramer multiple comparisons test).

Concentration-dependent manners. However, the relationship of NO and Fe ion during promotion of aconitase-activation is yet to be clarified. Therefore, we used various concentrations of Fe(II)AS during activation of aconitase in the presence and absence of the NO-donors, SNP, SIN, NaNO 2 and NOC18. As shown in Fig. 4A, Fe(II)AS caused a dose-dependent promotion of aconitase-activation at lower concentrations in the presence of all tested NO-donors as compared to Fe(II)AS alone. However, the maximum levels of aconitase-activation in the presence of each donor was nearly the same as with Fe(II)AS alone.

Next, we examined whether NO utilizes bound Fe in various Fe-complexes during aconitase-activation. It has been reported that NO predominantly reacts with Fe(III) or Fe(II) ions in heme, Fe-S centers in various enzyme proteins, and ferritin.35 On the other hand, it has also been reported that there is virtually no free Fe in cells.35 Therefore, SNP was initially chosen for the Fe-complex. The SNP concentration varied, whereas when SIN was used as a NO-donor, the concentrations were kept constant (1 mM). As shown in Fig. 4B, SNP showed only a slight increase in aconitase-activation in both the presence and the absence of SIN, while Fe(II)AS caused a dose-dependent promotion of that activation in the presence of SIN. These findings suggest that NO can neither utilize Fe in the SNP molecule for aconitase-activation.

We also examined the effects of [Fe(II)(Lactate)] and [Fe(III)(Citrate)] as Fe-complexes on aconitase-activation in the presence and the absence of SIN. As shown in Fig. 4C, incubation of aconitase with [Fe(II)(Lactate)] resulted in a dose-dependent promotion of aconitase-activation in the presence of SIN, whereas that with [Fe(III)(Citrate)] had no effects. The complex could not promote activation even when a high concentration (20 mM) of the reducing compound, DTT, was used to reduce Fe(III) in the complex (data not shown). Moreover, [Fe(II)(Citrate)] and [Fe(II)(Glutamate)] complexes prepared from long-term incubations of citrate and glutamate with Fe(II)O powder did not activate the [3Fe–4S] form of aconitase, as shown in our previous study.36 In addition, [Fe(II)(Glucurate)] showed nearly the same activity as [Fe(II)(Lactate)] in the presence of SIN (data not shown). These findings suggest that NO dose not utilize strongly bound Fe such as that in the [Fe(III)(Citrate)] complex.

Recently, we found that β-citrylglutamate (β-CG), isolated from newborn rat brains, was an endogenous low molecular weight Fe chelator,36 while the [Fe(II)(β-CG)] complex plays a role as an Fe-carrier for mitochondrial aconitase, and then activates it.36 Therefore, we used the[Fe(II)(β-CG)] complex as an Fe-complex containing moderately bound Fe in our experiments. As shown in Fig. 4D, [Fe(II)(β-CG)] promoted dose-dependently aconitase-activation in the presence of SIN (1 mM), while it activated that only moderately without SIN as previously described.36

These findings suggest that NO essentially requires Fe(II) ion for binding to itself for aconitase-activation.

Questions regarding why contradictory results have been reported showing that NO either does or does not inactivates aconitase have not been answered. To address this issue, we prepared Fe-free activated aconitase. Aconitase was activated by Fe(II)AS (100 μM) at 37°C for 10 min. To remove excess low molecular activating agents such as Fe ion, aconitase protein preparations were rapidly desalted using a Bio-Gel p-6 cartridge (1.6×4 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol, then the effects of the NO-donors were immediately examined using the Fe-free aconitase preparations. As shown in Table 1, SIN and NOC18 inactivated aconitase at 4 mM, whereas SNP activated that at a low concentration (0.1 mM), and inactivated it at a high concentration (0.4 mM). NaNO 2 had no effect at either low or high concentrations, whereas it inactivated the enzyme at very high concentrations (>10 mM) (data not shown). Activation of aconitase by SNP at low concentrations may be due to Fe ion released from SNP molecules during incubation in the
The inactivation of aconitase by NO-donors seen in the present study agrees with previously reported \textit{in vitro} studies. Interestingly, the activities of these Fe-free aconitase were strongly promoted by adding Fe(II)AS in the presence of all NO-donors, though the degrees of promotion varied by type of donors. Our findings suggest that NO inhibits aconitase activity at high concentrations in the absence of Fe(II), whereas it has either no effect apparently or promotes Fe-dependent activation of the enzyme in the presence of Fe(II).

\textbf{Effects of NO-Donors on Aconitase-Reactivation in Mitochondria Treated with APS} Aconitase activity in mitochondria has been reported to consist of a sensitive redox sensor of reactive oxygen and nitrogen species in cells. It has been shown that aconitase is inhibited by various oxidants such as H$_2$O$_2$ by damaging Fe in [4Fe–4S] aconitase clusters. Also, APS is known to inactivate aconitase as a relatively mild oxidant as compared with H$_2$O$_2$. We examined the effects of APS using intact mitochondria prepared from rat hearts. As shown in Fig. 5A (blank column) the aconitase activity decreased when APS was added into mitochondria. Therefore, we determined whether the present NO-donors had effects to reactivate aconitase damaged by APS. Intact mitochondria suspensions were pre-incubated with APS for 10 min at 37°C, followed by incubation with the NO-donors in the presence or absence of Fe(II)AS for 10 min at 37°C. The mitochondria were then solubilized in 0.5% Triton X-100 and aconitase activities in the solution were determined. As shown in Fig. 5A, all of the tested NO-donors promoted reactivation of aconitase in a dose-dependent manner in the presence of Fe(II)AS (0.04 mM), but not in its absence (data not shown). However, the degrees of reactivation of aconitase by SIN and NaNO$_2$ were relatively lower than that by SNP and NOC18.

The actions of NO are known to be counteracted by NO-scavengers such as PTIO. Therefore, the effects of PTIO on NO-donor-mediated promotion of reactivation of aconitase in mitochondria were examined. As shown in Fig. 5B, the promoting effects of all the NO-donors on reactivation were prevented by PTIO. These findings suggest that NO released...
Commercial aconitase (purified from pig hearts) was activated by Fe(II)AS and dithiothreitol, then the enzyme preparation was rapidly desalted using a Bio-Gel P-6 cartridge. Fe-free activated aconitase was pre-incubated for 20 min at 37 °C in the presence of both the NO-donors and Fe(II)AS (0.05 mM). The concentrations of the NO-donors used were at 0.1 mM of SNP, 2 mM of SIN, 2 mM of NaNO₂, and 1 mM of NOC18. Data are expressed as the mean ± S.E.M. (bar). Asterisk indicates significant difference with value for control (100%) (*p < 0.01, n = 3, two-way ANOVA followed by a Tukey–Kramer multiple comparisons test).

Effects of NO-Donors on Primary Cultures of Neurons from 1-d-Old Mouse Brain

The number of mitochondria per neuron in the solution were determined within 30 s. SNP, SIN, NaNO₂, and NOC18 were used at 0.02–0.1 mM, 0.4–2 mM, 4–20 mM, and 0.08–0.4 mM, respectively. Blank: absence of Fe(II)AS, control: presence of Fe(II)AS. Data were obtained from 3–6 experiments and are expressed as a percentage of the control, [mean ± S.E.M. (bar)]. *p < 0.01 vs. control (one-way ANOVA followed by a Tukey–Kramer multiple comparisons test). (B) Effects of NO-scavengers on reactivation of aconitase promoted by NO-donors. The effects of NO are counteracted by NO-scavengers such as PTIO. The effects of PTIO (1 mM) on NO-donor-mediated promotion of aconitase-reenzyme inactivation were examined in the presence of Fe(II)AS (0.02 mM) and SNP (0.025 mM), SIN (2 mM), NaNO₂ (10 mM), and NOC18 (0.4 mM). Blank: absence of Fe(II)AS, control: presence of Fe(II)AS, N: none, without PTIO in the presence of Fe(II)AS. Data were obtained from 3–6 experiments and are expressed as a percentage of the control, [mean ± S.E.M. (bar)]. *p < 0.01 vs. N (two-way ANOVA followed by a Tukey–Kramer multiple comparisons test).

Table 1. Effects of NO-Donors on Activated Aconitase in Presence and Absence of Fe(II)AS

<table>
<thead>
<tr>
<th>NO-donors</th>
<th>Aconitase activity (% of control)</th>
<th>Added Fe</th>
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<tbody>
<tr>
<td></td>
<td>0.1 mM</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>SNP</td>
<td>174.4 ± 39.5*</td>
<td>22.5 ± 1.0*</td>
</tr>
<tr>
<td>SIN</td>
<td>97.8 ± 5.4</td>
<td>33.8 ± 2.8*</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>97.4 ± 2.3</td>
<td>107.5 ± 7.5</td>
</tr>
<tr>
<td>NOC18</td>
<td>60.6 ± 4.0*</td>
<td>30.3 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>4 mM</td>
</tr>
<tr>
<td>SNP</td>
<td>194.0 ± 3.5*</td>
<td></td>
</tr>
<tr>
<td>SIN</td>
<td>1553.0 ± 12.8*</td>
<td></td>
</tr>
<tr>
<td>NaNO₂</td>
<td>662.8 ± 8.0*</td>
<td></td>
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<tr>
<td>NOC18</td>
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</table>

Fig. 5. Treatments of Mitochondria with Oxidants, Effects of NO-Donors on Aconitase-Reactivation

(A) Effects of NO-donors on reactivation of aconitase damage by APS. Intact mitochondria suspensions were pre-incubated with APS (0.1 mM) for 10 min at 37 °C, followed by incubation with the NO-donors in the presence of Fe(II)AS (0.04 mM) or its absence for 10 min at 37 °C. Mitochondria were then solubilized in 0.5% Triton X-100, and aconitase activities in the solution were determined within 30 s. SNP, SIN, NaNO₂, and NOC18 were used at 0.02–0.1 mM, 0.4–2 mM, 4–20 mM, and 0.08–0.4 mM, respectively. Blank: absence of Fe(II)AS, control: presence of Fe(II)AS. Data were obtained from 3–6 experiments and are expressed as a percentage of the control, [mean ± S.E.M. (bar)]. *p < 0.01 vs. control (one-way ANOVA followed by a Tukey–Kramer multiple comparisons test). (B) Effects of NO-scavengers on reactivation of aconitase promoted by NO-donors. The effects of NO are counteracted by NO-scavengers such as PTIO. The effects of PTIO (1 mM) on NO-donor-mediated promotion of aconitase-refunctional properties of the NO-donors, SNP, SIN, NaNO₂, and NOC18 were examined using mixed neural and glial cultures, whereas mitochondrial activity decreased in a dose-dependent manner with high concentrations of all of the tested NO-donors.

Previously, the effects of NO and related nitroso-compounds were examined using mixed neural and glial cultures, and redox-based mechanisms for neuroprotective and destructive effects of NO were reported. In the present study, the effects of SNP, SIN, and NOC18 in mixed neuronal and glial cultures were examined at concentrations ranging from 0.1–1000 µM. The results obtained were nearly the same as those with neuron-enriched cultures (data not shown). Indeed, SIN and NOC18 enhanced MTT reduction activity at low concentrations in primary cultures of neurons, whereas MTT reduction activity was decreased in a dose-dependent manner with high concentrations of all of the tested NO-donors.

from NO-donors promotes reactivation of aconitase in a mitochondrial matrix.

Effects of NO-Donors on Primary Cultures of Neurons from 1-d-Old Mouse Brain

The number of mitochondria present in a cell depends on the metabolic requirements of that cell. An adequate energy supply by mitochondria is essential for neuronal survival and based on their role as a cellular powerhouse, mitochondria are emerging as key participants in cell survival. Furthermore, mitochondrial oxidase phosphorylation provides the major source of ATP in cortical neurons. It is known that an assay of MTT reduction activity can assess the integrity of mitochondrial function. The resultant reaction is mainly attributed to mitochondrial enzymes and electron carriers, and can also be used to detect cell viability. In addition, NO has been shown to have markedly different biological effects in neural cells depending on its redox state. NO has a neurotoxic effect by reacting with superoxide anion to produce peroxynitrite (ONOO⁻). In contrast, the nitrosonium ion (NO⁺) has a neuroprotective effect via S-nitrosylation of thiol groups on the N-methyl-d-aspartate receptor. Therefore, mitochondrial functions were examined using an MTT reduction assay with neuron-enriched cultures after exposure to 0.1–1000 µM of the NO-donors, SNP, SIN, and NOC18, for the first 3 d after seeding. As shown in Fig. 6A, all NO-donors except for SNP enhanced MTT reduction activity at low concentrations in primary cultures of neurons, whereas MTT reduction activity was decreased in a dose-dependent manner with high concentrations of all of the tested NO-donors.
Fig. 6. Effects of NO-Donors on Neurons Cultured from 1-d-Old Mouse Cerebrum Tissues

Mitochondrial functions were determined using an MTT reduction assay in primary cultures of neurons exposed to 0.1–1000 µM of the NO-donors, SNP, SIN, and NOC18, for the first 3 d after seeding. Data were obtained from 3–6 experiments and analyzed for statistical significance by a two-way ANOVA. Significant differences between groups were determined using a Tukey–Kramer multiple comparisons test, with the level of significance set at *p < 0.05 vs. control (100%).

(A) Concentration-dependent effects of NO-donors on MTT reduction activity in neuron-enriched cultures. SIN and NOC18 significantly enhanced MTT reduction activity at low concentrations in cultures of neurons, whereas they decreased that activity at high concentrations. Contrary to the other NO-donors, SNP decreased MTT reduction activity at both low and high concentrations.

(B) Concentration-dependent effects of NO-donors on MTT reduction activity in mixed neuronal and glial cultures. All of the NO-donors except for SNP significantly enhanced MTT reduction activity at low concentrations.

(C) Effects of NO-donors on DNA content in mixed neuronal and glial cultures. DNA contents from cells in wells treated with the NO-donors were determined. Those contents in wells treated with SIN and NOC18 at both 10 and 30 µM were slightly increased, whereas those in wells treated with SNP were significantly decreased.

(D) Dose-dependent effects of β-CG and [Fe(II)(β-CG)] on MTT reduction activity in presence of NO-donors in mixed neuronal and glial cultures. The [Fe(II)(β-CG)] showed a higher MTT reduction activity even at 50 µM in the presence of SIN, whereas β-CG had only a lower activity at 50 µM, then increased gradually the activity until 200 µM. On the contrary, Fe(II)AS decreased the activity in the presence of SIN.

(E) Effects of NO-donors on MTT reduction activity in mixed neuronal and glial cultures in presence of [Fe(II)(β-CG)] complex. SIN and NOC18 significantly enhanced MTT reduction activities in the presence of the [Fe(II)(β-CG)] complex, whereas neither NO-donor had an effect in the presence of Fe(II)AS and [(Fe(II)(Citrate)]. All NO-donors were used at 30 µM, while Fe(II)AS, [Fe(II)(Citrate)], β-CG and [Fe(II)(β-CG)] at 200 µM. —: none, Fe(II)AS, Fe(II)AS: [Fe(II)(Citrate)], Fe-β-CG: [Fe(II)(β-CG)]

*p < 0.05 vs. Fe-β-CG alone, **p < 0.05 vs. SIN or NOC18 alone.
and its gene expression in prostate carcinoma cells at low concentrations. Therefore, the effects of the present NO-donors on MTT reduction activities in the mixed cultures were examined at low concentrations ranging from 0.1–10 µM, with the results shown in Fig. 6B. Both SIN and NOC18 significantly enhanced MTT reduction activity in a dose-dependent manner, whereas SNP decreased it slightly.

In separate experiments, DNA contents in cultured cells treated with NO-donors were determined, with the results presented in Fig. 6C. Those DNA contents in cells treated with SIN and NOC18 at both 10 and 30 µM were slightly increased, whereas those in cells treated with SNP were significantly decreased. However, the changes in DNA contents in SIN and NOC18-treated cells were not statistically significant. In this culture system, the number of neuronal cells declined to about 70% after 3 d of culture, which was determined based on the DNA contents in the dishes. Therefore, the increase in cell number by NO-donor treatment was considered to reflect an increased number of surviving cells.

In our earlier in vitro experiment (Fig. 4D), SIN increased the activation of aconitase by about 2–3-fold with the addition of the [Fe(II)β-CG] complex (about 200 µM) as compared to SIN alone. Therefore, concentration-dependent effects of β-CG and [Fe(II)β-CG] on MTT reduction activity were examined in presence of SIN using mixed neuronal and glial cultures. As shown in Fig. 6D, the [Fe(II)β-CG] showed a higher MTT reduction activity even at 50 µM in the presence of SIN, whereas β-CG had only a lower activity at 50 µM, but then gradually increased the activity until 200 µM. On the contrary, Fe(II)AS decreased MTT reduction activity in the presence of SIN. NOC18 also showed nearly the same results (data not shown). In addition, SIN and NOC18 showed fairly effective promotion of MTT reduction activities in the presence of β-CG (200 µM). β-CG is considered to form an [Fe(II)β-CG] complex with Fe ion (total about 2 µM) in culture medium containing bovine calf serum, as we previously demonstrated that β-CG can form a relatively strong complex with Fe ion in neutral pH. Therefore, β-CG would show effective compatibility to that of the [Fe(II)β-CG] complex.

Next, the effects of SIN, and NOC18 were examined in the presence of Fe(II)AS, citrate, [Fe(II)Citrate] and [Fe(II)β-CG] complex using mixed neural and glial cultures. As shown in Fig. 6E, SIN and NOC18 significantly enhanced MTT reduction activities in the presence of [Fe(II)β-CG] complex (200 µM), whereas neither of those NO-donors had effects in the presence of Fe(II)AS, citrate and [Fe(II)Citrate].

In separate experiments, cultured cells were exposed to 30 µM of NOC18 for the first 3 d after seeding in the presence of [Fe(II)β-CG]), then surviving cells were stained on day 5 with β-tubulin-III antibody (neuro-marker). Stained neurons in NOC18-treated wells were more abundant than those in wells treated with [Fe(II)β-CG]) alone. In wells treated with Fe(II)AS alone or NOC18 with Fe(II)AS, stained neurons were nearly non-existent. Bar=50 µM. (A) Control culture. (B) Culture with NOC18 (30 µM). (C) Culture with Fe(II)AS (200 µM). (D) Culture with NOC18 (30 µM) and Fe(II)AS (200 µM). (E) Culture with [Fe(II)β-CG] (200 µM). (F) Culture with NOC18 (30 µM) and [Fe(II)β-CG] (200 µM).

DISCUSSION

NO-mediated inactivation of m-aconitase has been reported to occur in a variety of mammalian cells, though results from in vitro studies are somewhat contradictory. Indeed, results of experiments with porcine heart m-aconitase revealed that low concentrations of NO did not inactivate aconitase, whereas high concentrations led to moderate inhibition. In the present study, we anticipated that SNP ([Fe(III)(CN),NO]) would inhibit the enzyme due to its ferricyanide moiety, because it is known that cyanide ion and ferricyanide ([Fe(III)(CN),]) have such inhibition activity. However, SNP significantly increased aconitase activity. Therefore, it is reasonable to assume that NO released from the SNP molecule promotes Fe-dependent activation of aconitase. The other tested NO-donors (SIN, NaNO2 and NOC18) promoted activation of m-aconitase in time- and dose-dependent manners in the presence of Fe(II)AS. Also, the promoting effects of the NO-donors on aconitase-activation were prevented by NO-scavengers including heme and PTIO. These findings suggest that NO released from NO-donors promotes Fe-dependent activation of aconitase.

It is important to ask why it has been reported that NO ei-
ther inhibits aconitase activity or has no effect. In the present study, we prepared Fe-free activated aconitase and examined the effects of NO-donors. As shown in Table 1, SIN and NOC18 inhibited aconitase activity, which agreed with in vitro studies previously presented. In addition, the activity of Fe-free aconitase was promoted by addition of Fe(II)AS to each of the NO-donors. These findings indicate that NO induced inhibition or promotion of aconitase activity is depend on the existence of useful Fe(II) ion in the area.

In intact mitochondria, all of the present NO-donors also promoted reactivation of aconitase in a dose-dependent manner in the presence of Fe(II)AS, whereas they did not in its absence. The promoting effects of the NO-donors on the reactivation of aconitase were also prevented by the NO-scavenger, PTIO.

In addition, our preliminary experiments revealed that cytosolic aconitase (c-aconitase), iron regulatory protein 1 (IRP1), after damage with EDTA/Fe(CN)₅ reagents was also reactivated by simultaneous addition of the [Fe(II)(β-CG)] complex with the NO-donors. However, c-aconitase prepared from rat liver cytosol had a relatively stable [4Fe–4S] cluster form, whereas c-aconitase ([3Fe–4S] cluster form) after damage with EDTA/Fe(CN)₅ was unstable due to the process of disassembly in the cluster. Therefore, the activating conditions of c-aconitase were difficult to study in greater detail.

In mixed neuronal and glial cultures, all of the NO-donors except for SNP significantly enhanced cell viability at low concentrations by promoting MTT reduction activity, whereas high concentrations decreased MTT reduction activity. It is unclear why only SNP did not enhance MTT reduction activity. It is known that Fe ion is a potent neurotoxin, though it is also an essential nutrient. This fact may explain the ineffective action of SNP. Another explanation might be the release of cyanide ion from SNP. Roncaroli et al. reported that the release of NO from SNP in biological media does not originate from [Fe(II)(CN)₅NO]³⁻ produced on reduction of SNP but probably proceeds through the release of cyanide and further reactions of the [Fe(II)(CN)₅NO]³⁻ ion. The toxic effects of cyanide have traditionally been attributed to inhibition of cytochrome c oxidase, the terminal enzyme of the respiratory chain, although it was also reported that high levels of exogenous NO attenuated cyanide inhibition of both cytochrome c oxidase and respiration, whereas low-level NO enhanced the cyanide inhibition. The ineffective action of SNP toward MTT reduction may be attributed to Fe ion and/or cyanide ion released from SNP molecule. Taken together, these findings suggest that NO is also an endogenous low molecular weight Fe chaperone for aconitase, although it can take off Fe ion from the enzyme.

It is now clear that NO can either induce necrosis or apoptosis, or even protect cells from death. However, the factors that determine these paradoxical actions are largely unknown. Many of the reported contradictory results regarding damaging vs. protective actions of NO have been due to the redox status of NO in neuronal, prostate carcinoma and erythroleukemia cells, cell type such as macrophages, neuronal cells, or hepatocytes, and the level of NO exposure. However, Fe content in cells is considered to be another key factor for determining the effects of NO on cell viability. Recently, Kim et al. suggested that the hepatic cell content of non-heme Fe determines whether cytotoxic levels of NO result in apoptosis or necrosis. Indeed, NO-donors induced apoptosis in murine macrophages with low non-heme Fe contents, whereas it did not do so in hepatocytes with high non-heme Fe contents. However, pre-treatment of macrophages with FeSO₄ increased intracellular Fe to a level similar to that in hepatocytes and delayed NO-induced cell death. Moreover, NO-mediated DNA damage is known to induce apoptotic cell death in tumor cells. Indeed, simultaneous addition of Fe-containing compounds such as SNP and FeCN protected tumor cells from NO-mediated growth inhibition and apoptosis. Taken together, these findings suggest that elevated intercellular iron rescues macrophages and tumor cells from NO-mediated growth inhibition and apoptosis.

In the present study, SIN and NOC18 enhanced MTT reduction activities in mixed neural and glial cultures, while simultaneous addition of the [Fe(II)(β-CG)] complex significantly enhanced those activities, and neither of those NO-donors had effects in the presence of Fe(II)AS and [Fe(III)(Citrate)] (Fig. 6E). Interestingly, the number of surviving neurons in NOC18-treated wells in the presence of [Fe(II)(β-CG)] was greater than in wells with NOC18 or [Fe(II)(β-CG)] alone (Figs. 7B, E,F), whereas neurons were not found in nearly all wells treated with Fe(II)AS alone or NOC18 with Fe(II)AS (Figs. 7C, D). It was previously reported that NO generated from the NO-donor, SIN or S-nitrosocysteine, led to neurotoxicity in mixed neural and glial cultures similar to the present culture systems, though the concentration of NO-donor used in that study was relatively high. In addition, we previously showed that the [Fe(II)(β-CG)] complex itself can transfer Fe to aconitase ([3Fe–4S] cluster form) after disassembly by APS in vitro experiments including mitochondria and in cultured cells. Therefore, these findings suggest that simultaneous addition of the [Fe(II)(β-CG)] complex is a key to determine the neuroprotective or destructive effects of NO. Taken together, we speculate that NO promotes utilization of the [Fe(II)(β-CG)] complex in cells and increases the number of surviving neurons by increasing mitochondrial functions.

Finally, NO which has a higher affinity to Fe ion, is known to easily reach mitochondria from cytosolic or extracellular sources due to its hydrophobic nature, and form dinitrosyl-iron complexes in vitro, in macrophages and tumor cells. Therefore, since the [Fe(II)(β-CG)] complex have a higher affinity to aconitase in mitochondria and can insert its Fe into the [3Fe–4S] cluster of aconitase, it is considered that NO reacts with the Fe moiety of the [Fe(II)(β-CG)] complex, then forms an [(NO)Fe(II)(β-CG)] complex and reaches to mitochondria, resulting in activation of aconitase. β-CG seems to function as a tag addressed to aconitase in mitochondria, though further investigation is required to fully explain this interesting possibility.

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