The Nitric Oxide Donor NOC12 Protects Cultured Astrocytes Against Apoptosis via a cGMP-Dependent Mechanism

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Received November 26, 2001 Accepted February 12, 2002

ABSTRACT—We examined the effect of 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC12), a nitric oxide (NO) donor, on apoptosis in cultured astrocytes. Reperfusion after hydrogen peroxide (H₂O₂) exposure caused a decrease in cell viability, loss of mitochondrial membrane potential, caspase-3 activation, DNA ladder formation, and nuclear condensation. NOC12 at 10 – 100 μM significantly attenuated these apoptotic changes, while the NO donor at 1 mM caused cell injury and exacerbated the H₂O₂-induced cell injury. NOC12 increased intracellular cGMP levels in a dose dependent manner with the maximal effect at 100 μM. The protective effect of NOC12 was mimicked by the NO-independent guanylate cyclase activator 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole, and was attenuated by the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and the cGMP-dependent protein kinase inhibitor KT5823. ODQ and KT5823 did not block but rather exacerbated the cytotoxic effect of NOC12 at 1 mM. These findings demonstrate that lower concentrations of NOC12 inhibit the H₂O₂-induced apoptosis of astrocytes in a cGMP-dependent way, but higher concentrations of NOC12 show a toxic effect on astrocytes in a cGMP-independent way.

Keywords: Nitric oxide, NOC12, Apoptosis, cGMP, Astrocyte

Nitric oxide (NO), a highly diffusible and short-lived free radical, has been proposed to act as a crucial signaling molecule under various physiological and pathological conditions in the central nervous system (1 – 4). NO induced apoptosis (5, 6), and conversely, it prevents apoptosis by inhibiting caspase proteolytic activation (7, 8). Although astrocytic apoptosis may play a role in brain injuries such as spinal trauma and cerebral ischemia (9 – 13), there is little information about the effect of NO on astrocyte injury.

We previously showed that incubation of cultured rat astrocytes with Ca²⁺-containing medium after exposure to Ca²⁺-free medium caused an increase in intracellular Ca²⁺ concentration followed by delayed cell death, including apoptosis (14 – 16). This injury is considered to be an in vitro model of ischemia/reperfusion injury, because a similar paradoxical change in extracellular Ca²⁺ concentration is reported in ischemic brain tissue (17 – 19). Subsequently, we have found that the Ca²⁺ reperfusion injury was mimicked by reperfusion after exposure to hydrogen peroxide (H₂O₂) (16). The reperfusion injury models using Ca²⁺ depletion and H₂O₂ exposure may contribute to clarify the apoptotic signal cascade of astrocytes, which is associated with ischemia/reperfusion-induced brain dysfunction.

In this paper, we examined the effects of NO donors on reperfusion injury after exposure to H₂O₂-containing medium in cultured rat astrocytes and studied the mechanism underlying the effect of NO. The present study demonstrates that low levels of NO inhibit apoptosis in astrocytes by inhibiting the loss of mitochondrial membrane potential and caspase-3 activation through cGMP production, while high levels of NO show cytotoxicity through a cGMP-independent pathway.
MATERIALS AND METHODS

Materials

Drugs were obtained from the following sources: mouse anti-glial fibrillar acidic protein antiserum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), isoleucin B (biotin-labeled) (Sigma Chemical Co., St. Louis, MO, USA); 10-methoxy-10-methoxycarbonyl-9,10,11,12-tetrahydro-9,12-epoxy-(1H)-dinitidolo[1,2,3,4′-k:ℓ]-pyrrolidino-[3,4-][1,6]-benzdiazocin-2-methyl-1-oxo (KT5823) (Calbiochem, La Jolla, CA, USA); S-nitroso-N-acetylenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), 1-hydroxy-3-methyl-3-(methylamino)prolyl-2-oxo-1-triazene (NOC7), 3-ethyl-3-(ethylaminoethy1)-1-hydroxy-2-oxo-1-triazene (NOC7), 3,3-bis(amin ethanol)-1-hydroxy-2-oxo-1-triazene (NOC18) (Nacalai Tesque, Inc., Kyoto); BIOTRAK cGMP enzymimunoassay (EIA) system, horseradish peroxidase-labeled anti-mouse Ig (Amersham Pharmacia Biotech. UK, Ltd., Buckinghamshire, UK); 3,3′-dihexyloxacarbocyanine (DiOC6(3)), Hoechst 33342 (Molecular Probes, Inc., Eugene, OR, USA); 7-amino-4-methyl-coumarin (AMC), acetyl-l-aspartyl-l-glutamyl-l-valyl-l-aspartic acid α-(4-methylcoumaryl-7-amide) (Ac-DEVD-MCA) (Peptide Institute, St. Louis, MO, USA); 10-methoxy-10-methoxycarbonyl-9,10,11,12-tetrahydro-9,12-epoxy-(1H)-dinitidolo[1,2,3,4′-k:ℓ]-pyrrolidino-[3,4-][1,6]-benzdiazocin-2-methyl-1-oxo (KT5823) (Calbiochem, La Jolla, CA, USA); S-nitroso-N-acetylenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (Tocris Cookson, Inc., Ballwin, MO, USA); (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexena mide (NOR-3), 1-hydroxy-3-methyl-3-(methylaminopropyl)-2-oxo-1-triazene (NOC7), 3-ethyl-3-(ethylamin ethanolyl)-1-hydroxy-2-oxo-1-triazene (NOC12), 3,3-bis(amin ethanolyl)-1-hydroxy-2-oxo-1-triazene (NOC18) (Nacalai Tesque, Inc., Kyoto); BIOTRAK cGMP enzymimunoassay (EIA) system, horseradish peroxidase-labeled anti-mouse Ig (Amersham Pharmacia Biotech. UK, Ltd., Buckinghamshire, UK); 3,3′-dihexyloxacarbocyanine (DiOC6(3)), Hoechst 33342 (Molecular Probes, Inc., Eugene, OR, USA); 7-amino-4-methyl-coumarin (AMC), acetyl-l-aspartyl-l-glutamyl-l-valyl-l-aspartic acid α-(4-methylcoumaryl-7-amide) (Ac-DEVD-MCA) (Peptide Institute, Inc., Osaka); Eagle’s minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo); tissue culture ware (Iwaki Glass Co., Ltd., Tokyo). All other chemicals used were of the highest purity commercially available.

Cell culture

Astrocytes were isolated from cerebral cortices of 1-day-old Wistar rats as previously reported (14, 16). Briefly, tissue was dissociated with dispase and cultured in minimum essential medium containing 10% fetal calf serum and 2 mM of glutamine. Cells were plated in 75-ml tissue culture flasks, split once upon confluence, and plated in 24-well (for MTT assay) and 96-well (for mitochondria energization, cyclic GMP and caspase assays) plastic tissue culture plates and 60-mm (for DNA ladder) plastic tissue culture dishes. The second cultures were grown for 14–20 days in all experiments. The cells were routinely >95% positive for glial fibrillary acidic protein, and approximately 2% of the cells were microglia, based on positive isolectin B staining.

Cell viability

Reperfusion experiments were carried out using confluent astrocytes in fetal calf serum-free medium as previously reported (16). Cells were exposed to H2O2 (100 μM)-containing Earle’s solution for 30 min and then incubated with normal Earle’s solution for the indicated times. MTT reduction activity was measured by a colorimetric assay as reported previously (14, 16). MTT reduction activity is expressed as a percentage of the control.

Measurement of caspase activity

The activity of caspase-3-like protease in cell lysates was measured using the fluogenic substrate Ac-DEVD-MCA (20). After treatment, the cells were washed twice with phosphate-buffered saline and lysed in 52.5 μl of buffer A (10 mM HEPES pH 7.4, 42 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM ethylene glycol bis(β-aminoethy1 ether)N,N,N′,N′-tetraacetic acid, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml aprotinin and 1 μg/ml leupeptin). Then, 50 μl of the lysates were incubated with 150 μl of buffer B (25 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM dithiothreitol) containing 25 mM Ac-DEVD-MCA at 37°C for 1 h. The released AMC levels were measured with excitation at 355 nm and emission at 460 nm using a Wallac Multilabel counter.

Measurement of mitochondrial energization

Mitochondria energization was determined as the retention of the mitochondria specific dye DiOC6(3) (21). Cells were loaded with 100 nM DiOC6(3) during the last 30 min of treatment. After removing the medium, the cells were washed twice with PBS and then lysed by the addition of 100 μl of deionized water. The concentration of retained DiOC6(3) was measured with excitation at 485 nm and emission at 510 nm using a Wallac Multilabel counter.

Measurement of DNA ladder formation and Hoechst 33342 staining

DNA was extracted and subjected to 1.8% agarose gel electrophoresis as previously reported (16, 20). DNA in the gel was stained with ethidium bromide and photographed with the Polaroid instant films (type 667) under UV light. To observe individual nuclei, the cells plated on a chamber slide were fixed with 4% formaldehyde and stained with Hoechst 33342 as previously reported (16, 22). An Olympus IX70 inverted fluorescence microscope was equipped with a cooled CCD camera system (Nippon Roper, Chiba) to scan the staining nuclear images.

Measurement of cGMP levels

After treatments with various drugs, lysis reagent containing 0.5% dodecyltrimethylammonium bromide was added to extract intracellular cyclic GMP. The intracellular cGMP levels were determined by a competitive EIA system as previously reported (20). Data are expressed as fmol of cGMP per well.
Statistics
Statistical analysis of the experimental data was carried out by the Student-Newman-Keuls test, Dunnett’s t-test or Tukey HSD test, using a software package (Stat View 5.0) for the Apple Macintosh.

RESULTS
Incubation after exposure of astrocytes to H\textsubscript{2}O\textsubscript{2}-containing medium causes a significant decrease in MTT reduction activity (16). Figure 1 shows the effects of varying concentrations of NOC12 on the H\textsubscript{2}O\textsubscript{2}-induced changes in MTT reduction activity, mitochondrial membrane potential and caspase-3 like protease activity and on cGMP levels in cultured astrocytes. NOC12 attenuated significantly the H\textsubscript{2}O\textsubscript{2}-induced changes in MTT reduction activity, mitochondrial membrane potential, and caspase-3-like protease activity in a dose-dependent manner at the concentrations up to 100 \(\mu\)M. The protection by NOC12 at 100 \(\mu\)M of mitochondrial membrane potential and caspase-3 like protease activity was almost complete, although that of MTT reduction activity was partial. In contrast to these effects of the low concentrations, NOC12 at 1 mM showed cell toxicity and exacerbated the H\textsubscript{2}O\textsubscript{2}-induced changes. NOC12 increased intracellular cGMP levels in astrocytes in a dose dependent manner with a maximal stimulation at

![Fig. 1. Effect of NOC12 on the H\textsubscript{2}O\textsubscript{2}-induced changes in cell viability, mitochondrial membrane potential and DEVDase activity, and on cGMP levels in cultured rat astrocytes. A, B, C and D show the effects on MTT reduction activity, mitochondrial membrane potential, DEVDase activity and cGMP levels, respectively. In A – C, cells were exposed to normal (open circle) or 100 \(\mu\)M H\textsubscript{2}O\textsubscript{2}-containing medium (closed circle) for 30 min and then incubated with Earle’s solution for 23.5 h. The indicated concentrations of NOC12 were added 30 min before \(\text{H}_2\text{O}_2\) exposure and were present until assay. Results are given as the means ± S.E.M. for 8 – 12 wells and were obtained from 4 – 6 separate experiments. \(*P<0.01\), significantly different from the control (Student-Newman-Keuls test); \(\dagger P<0.01\), significantly different from the values without NOC12 (Dunnett’s t-test). In D, cells were treated for 30 min with the indicated concentrations of NOC12. Results are given as the means ± S.E.M. for 5 wells and were obtained from 2 separate experiments. \(\dagger P<0.01\), significantly different from the values without NOC12 (Dunnett’s t-test).]
100 μM. Figure 2 shows the effects of varying concentrations of NOC12 on the H$_2$O$_2$-induced DNA ladder formation and nuclear condensation. NOC12 alone at 1 mM induced DNA ladder formation and nuclear condensation, while it alone at the concentrations up to 100 μM did not. The H$_2$O$_2$-induced DNA ladder formation and nuclear condensation were almost completely blocked by NOC12 at 10−100 μM, but not at 1 mM. The effect of NOC-12 at

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**Fig. 2.** Effect of NOC12 on the H$_2$O$_2$-induced decrease DNA ladder formation and nuclear condensation in cultured rat astrocytes. A: Cells were exposed to normal (control) or 100 μM H$_2$O$_2$-containing medium for 30 min and then incubated with Earle’s solution for 5 days. NOC12 was added 30 min before H$_2$O$_2$ exposure and was present until assay. The typical result of three independent experiments is shown (M: 100 bp marker). B: Cells were exposed to normal (a, b, c) or 100 μM H$_2$O$_2$-containing medium (d, e, f) for 30 min and then incubated with Earle’s solution for 3 days. NOC12 at 100 μM (b, c) and 1 mM (c, f) was added 30 min before H$_2$O$_2$ exposure and was present until assay.
The guanylate cyclase inhibitor ODQ at 20 μM completely inhibited the NOC-12-stimulated cGMP formation, although it did not affect basal levels of cGMP: The levels (pg/ml, means ± S.E.M. of 5 wells) in control cells were 8.9 ± 1.0 (none) and 6.0 ± 0.9 (20 μM ODQ), and those in cells exposed to 100 μM NOC12 were 315.7 ± 38.5 (none) and 7.4 ± 1.1 (20 μM ODQ). The NO-independent guanylate cyclase activator YC-1 attenuated the H₂O₂-induced decrease in cell viability in a dose-dependent way (Fig. 4A).

One of the downstream signal of cGMP is the activation of cGMP-dependent protein kinase. Therefore, we examined the effect of the cGMP-dependent protein kinase inhibitor KT5823 on the protection provided by NO/cGMP signals against cell injury induced by reperfusion after exposure to H₂O₂. KT5823 (2 μM) completely blocked the protective effects of NOC12 and YC-1 on the H₂O₂-induced decrease in MTT reduction activity (Fig. 4B and 5A). Furthermore, KT5823 blocked significantly the inhibitory effect of NOC12 on caspase-3-like protease activity (Fig. 5B).

**DISCUSSION**

We have previously found that reperfusion after exposure of astrocytes to H₂O₂ caused a decrease in MTT reduction activity with apoptotic changes such as a loss of mitochondrial membrane potential, cytochrome c release from mitochondria, caspase-3 activation, DNA ladder formation, and nuclear condensation (14, 16). Furthermore, we have demonstrated that calcineurin (23), the mitogen-activated protein/extracellular signal-regulated kinase kinase (24), phosphatidylinositol 3-kinase (22, 24), and cGMP phosphodiesterase (20) are possible target molecules for prevention against apoptosis in this reperfusion model. In the present study, we found that low concentrations of NOC12, an NO donor, attenuated the H₂O₂-induced astrocytic injury. NOC12 at lower concentrations (up to 100 μM) inhibited the H₂O₂-induced loss of mitochondrial membrane potential, caspase-3 activation, DNA ladder formation, and nuclear condensation. The protection of the apoptosis-related changes was almost complete, while that of MTT reduction activity was partial. The difference suggests that the low concentrations of NOC12 blocks preferentially apoptosis, although the H₂O₂-induced astrocytic injury is induced by not only apoptosis but also necrosis (14, 16). NO is known to activate potent soluble guanylate cyclase, which increases intracellular levels of cGMP (25). Previous studies show that the anti-apoptotic effect of NO is dependent on the production of cGMP (26–29), but a recent study provides evidence against the cGMP-dependent mechanism (30). The present study demonstrated that NOC12 increased cGMP levels in astrocytes with a maximal effect at 100 μM. The dose-response for the protective effect is similar to that for the effect on the cGMP levels. Furthermore, the protective effect of NOC12 was attenuated by the guanylate cyclase inhibitor ODQ and the cGMP-dependent protein kinase inhibitor KT5823 and mimicked by the NO-independent guanylate cyclase activator YC-1 (31). These findings suggest that the effect of NOC12 at lower concentration is mediated by a cGMP-dependent mechanism that may implicate activation of cGMP-dependent protein kinase. On the other hand, we found that NOC12 at 1 mM caused cell injury. NOC12 at 1 mM enhanced the H₂O₂-induced changes in MTT reduction activity, mitochondrial membrane potential and caspase-3 activity, but it did not increase the H₂O₂-induced DNA ladder formation. The apparent discrepancy suggests that cell toxicity by the high concentration of NOC12 is
Fig. 4. Effects of YC-1 and KT5823 on the H$_2$O$_2$-induced decrease in cell viability in cultured rat astrocytes. On the left, cells were exposed to normal (open circle) or 100 μM H$_2$O$_2$-containing medium (closed circle) for 30 min and then incubated with Earle’s solution for 23.5 h. The indicated concentrations of YC-1 were added 30 min before H$_2$O$_2$ exposure and were present until assay. Results are given as the means ± S.E.M. for 6 wells and were obtained from 3 separate experiments. *P<0.01, significantly different from the control (Student-Newman-Keuls test); †P<0.01, significantly different from the values without YC-1 (Dunnett’s t-test). On the right, cells were exposed to normal (open columns) or 100 μM H$_2$O$_2$-containing medium (hatched columns) for 30 min and then incubated with Earle’s solution for 23.5 h. KT5823 (2 μM) was added 60 min before H$_2$O$_2$ exposure and was present until assay. Results are given as the means ± S.E.M. for 10 wells and were obtained from 5 separate experiments. *P<0.01, significantly different from the values without drugs (Tukey-HSD test); †P<0.01, significantly different from the values without KT5823 (Tukey-HSD test).

Fig. 5. Effect of KT5823 on the protection provided by NOC12 against the H$_2$O$_2$-induced changes in MTT reduction activity and DEVDase activity in cultured rat astrocytes. Cells were exposed to normal (open columns) or 100 μM H$_2$O$_2$-containing medium (hatched columns) for 30 min and then incubated with Earle’s solution for 23.5 h. NOC12 (100 μM) was added 30 min before H$_2$O$_2$ exposure and was present until assay. KT5823 (2 μM) was added 60 min before H$_2$O$_2$ exposure and was present until assay. Results are given as the means ± S.E.M. for 6–10 wells and were obtained from 3–4 separate experiments. *P<0.01, significantly different from the values without KT5823 (Tukey-HSD test); †P<0.01, significantly different from 100 μM NOC12 (Tukey-HSD test).
complex. The effect of NOC12 at 1 mM on cell viability was not affected by ODQ and the cGMP-dependent protein kinase inhibitor KT5823. These findings suggest that the effect of NOC12 at 1 mM is independent on cGMP. In this study, we did not examine further the mechanism for the cytotoxic effect of NOC12. The cytotoxic effect of NO donors is generally considered to be mediated partly by a formation of highly reactive peroxynitrite (32–34), and an activation of poly(ADP-ribose) polymerase (35).

NO donors exert their effects through the release of NO. In most cases NO is toxic, but in some cases it reduces neuronal death resulting from excitotoxicity or serum deprivation (29). The differential effects of NO are partly due to the type of NO donors and cells used. In the present study, NOC12 is used as an NO donor, because it releases NO without co-factors or enzymatic metabolism and without the production of toxic metabolites and has a long half-life (327 min). We observed that NOC-18 (half life = 3400 min), like NOC12, also showed the dual effects, whereas NOC7 (half life = 10 min), NOR3 (half life = 30 min), SIN-1, sodium nitroprusside and SNAP showed only the toxic effect. SIN-1 produces peroxynitrite and sodium nitroprusside simultaneously releases both NO and cyanide that has cytotoxicity. The half-lives of SIN-1, sodium nitroprusside and SNAP are not well understood. Taken together, it is likely that persistent spontaneous release of NO is necessary for the protective effect and that peroxynitrite and cyanide contribute to the cytotoxic effect of NO donors.

With respect to dual effects of an NO donor, Canals et al. (30) have recently reported that diethylamine-nitric oxide complex sodium (DEA-NO), an NO donor, exerted neurotoxic effects on dopamine cells at doses of 25 and 50 μM, whereas it at higher doses showed a neurotoxic effect. In contrast to our study, they showed that the effect of DEA-NO at low concentrations was not reversed by ODQ, although the NO donor increased cGMP levels. They suggest that the neuroprotective effect of DEA-NO may be due to up-regulation of glutathione (30). That is, the effect of low concentrations of DEA-NO is mediated by a cGMP-independent mechanism.

The present study shows that NOC12 at low doses protects astrocytes against reperfusion injury via a cGMP signal that activates cGMP-dependent protein kinase, whereas NOC12 at higher doses exacerbated the reperfusion injury via a cGMP-independent mechanism. The involvement of cGMP in the effect of NO donors is also reported in B lymphocytes (26), T lymphocytes (32), eosinophils (33), motor and sympathetic neurons (28, 33), hepatocytes (25, 29), PC12 cells (29, 33), and ovarian follicles (34), but the detailed mechanism is not known. Known substrates for PKG include inositol 1,4,5-triphosphate receptor (36), dopamine- and cAMP-regulated phosphoprotein (37), and cGMP-binding phosphodiesterase (38) in the central nervous system. Schlossmann et al. (39) have recently identified a PKG substrate protein in smooth muscle. However, the role of these substrates in the effect of cGMP is not clear. Our recent study shows that cGMP inhibits the mitochondrial permeable transition pore via the activation of cGMP-dependent protein kinase, and the prevention of mitochondrial dysfunction contributes to its anti-apoptotic effect (40).

Acknowledgments
This research was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; The Science Research Fund of The Japan Private School Promotional Foundation; Uehara Memorial Foundation; and Hyogo Science and Technology Association, Joint Research (B) of Kobe Gakuin University.

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