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Kinetic characterization of the nitric oxide toxicity for PC12 cells: effect of half-life time of NO release

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Abstract

We investigated the effects of low concentrations of nitric oxide (NO) on cell viability using NO donors, (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1), (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR2), (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR3) and (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine (NOR4). The half-life times of the NO release from these four NOR analogs, NOR1, NOR2, NOR3 and NOR4, were determined (6.5, 84, 105 and 340 min, respectively) by using 4,5-diaminofluorescein (DAF-2), a newly developed indicator of NO. Exposure of undifferentiated PC12 cells to low concentrations of NO donors, NOR2 or NOR3 (1–100 μM), but not NOR1 nor NOR4, resulted in cell death in a dose- and time-dependent manner, as determined from cell viability assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium (MTT) assay. After 24 h exposure to 50 μM NOR2 or NOR3, more than 90% of PC12 cells had died. Furthermore, while the toxic effect of NOR3 was attenuated by replacing the medium at 20 min, 1 or 2 h after drug addition, it was continued by replacing the medium at 3 h or later after drug addition. The cell death was characterized by DNA degradation, nuclear condensation and fragmentation, suggesting apoptosis-like cell death. Pretreatment with an antioxidant ascorbic acid (0.1–0.5 mM) completely prevented the cell death caused by NOR3, while glutathione (0.1–0.2 mM) and cysteine (0.2–0.4 mM) provided partial protection. These findings suggest that the cell toxicity induced by NO at low concentrations strongly depends upon the duration of expose to NO from NO donors, and these toxic effects are effectively prevented by the antioxidant, ascorbic acid.

Keywords: Apoptosis; NOR3; Nitric oxide (NO); Nitric oxide (NO) donor; DAF-2 (4,5-diaminofluorescein); Ascorbic acid

1. Introduction

Nitric oxide (NO) has been shown to act as a multifunctional gaseous modulator in many cellular events. NO is synthesized from L-arginine by a family of isoforms, constitutive Ca^{2+}-dependent and cytokine-inducible NO synthases (NOS) (Moncada et al., 1991). To date, genes encoding three different isoforms of NOS have been cloned (designated neuronal-, endothelial- and inducible-NOS). At present, NO is thought to have important physiological and pathophysiological roles, including in cell signaling and neurotransmission (Garthwaite, 1991), plasticity and alterations of synaptic functions (Bredt and Snyder, 1992; Bliss and Collingridge, 1993; Lipton et al., 1993), inflammation (Mulligan et al., 1991) and delayed cell death after focal ischemia (Nowicki et al., 1991). Several studies have shown that NO transduces a signal as a physiological modulator by activating soluble guanylate cyclase to increase the production of cGMP (Katsuki et al., 1977; Bredt and Snyder, 1989). Also, NO reacts rapidly with superoxide to form peroxynitrite, a cytotoxic oxidant substance (Beckman, 1990; Huie and Padjama, 1993), which produces cytotoxic effects in many cells (Lipton et al., 1993; Estevez et al., 1995).

There is evidence to suggest that the formation of NO in excess by the stimulation of the inducible isoform of NOS (iNOS) or exogenous treatment with a high concentration of NO donors (more than 0.5–1 mM) is responsible for the cytotoxicity against several type of cells (Brown et al., 1994; Estevez et al., 1995), including macrophages (Sarih et al., 1993), astrocytes (Hu and Van Eldik, 1996),

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differentiated PC12 cells (Heneka et al., 1998) and primary brain cultures (Dawson et al., 1993). In several pathological conditions, cytokines and bacterial lipopolysaccharides increase the expression of iNOS, which produces a large amount of NO and sustained NO release compared with the other isoforms of NOS, which synthesize only small amounts of NO and release it in response to increased intracellular Ca^{2+} (Nathan, 1992). From those findings, NO is considered to be a mediator of apoptotic or non-apoptotic cell death in several cellular systems.

In contrast, others have suggested that NO has neurotrophic and neuroprotective effects on serum-deprived PC12 cells (Farinelli et al., 1996) and cerebellar granule cells (Pantazis et al., 1998). These effects were observed at relatively low concentrations of NO or NO donors (1–100 μM), and could be mediated through a NO–cGMP pathway. Unlike constitutive isoforms of NOS, however, iNOS produces a large amount of NO for a long time, and affects several cellular systems. Such a sustained NO release may produce multiple and complex biological responses mediated via not only the activation of the NO–cGMP pathway, but also the direct modification of proteins, DNA and other cellular components by NO itself.

The present study was conducted to assess the effects of NO on cell viability by kinetic analysis. For this purpose, we used (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1), (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR2), (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR3) and (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine (NOR4) (NOR analogs) as NO donors since the rate of NO release can be controlled by the choice of appropriate NOR analogs. NOR3 is a novel NO donor, which has been isolated from microbial products and reported to release NO spontaneously and stoichiometrically (1–1.5 mol/ mol of NORs) in physiological pH (Kita et al., 1994). Compared to the other classical NO donors, such as sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine, therefore, NOR analogs are assumed to be superior in the present kinetic studies. In addition, we also measured the NO release from NOR analogs by using a newly developed NO-specific fluorescence indicator, 4,5-diaminofluorescein (DAF-2) (Kojima et al., 1998; Nakatsubo et al., 1998), and calculated the half-life time of NOR analogs. Here, we demonstrate that low concentrations of NO-induced cell death in undifferentiated PC12 cells, and the cell toxicity strongly depended on the duration of NO exposure.

2. Materials and methods

2.1. Materials

NOR1, NOR2, NOR3, NOR4 and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Dojindo Lab. (Kumamoto, Japan) and Hoechst 33,258, 1*H*-(1,2,4)-oxadiazolo-(4,3-*a*)-quinoxalin-1-one (ODQ) and ascorbic acid from Wako (Tokyo, Japan). DAF-2 was obtained from Daiichi Pure Chemicals (Tokyo, Japan). Dibutyryl cGMP, 8-bromo cGMP, cysteine and glutathione were from Sigma (St. Louis, MO). All other chemicals were of the highest commercially available quality from Wako.

2.2. Determination of NO release from NOR analogs by DAF-2

NOR analogs were dissolved in dimethylsulfoxide (DMSO) (40 mM), and then diluted to 10 or 50 μM with a HEPES-buffered salt solution (HBS) containing (in mM): NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.8, glucose 5.5 and HEPES 10 (pH 7.3). DAF-2 was also dissolved in DMSO(1 mg/0.55 ml), and diluted to 10 μM with HBS. The reaction mixture (200 μl) was incubated at 37°C in 96-well black microplates and the fluorescence caused by the reaction of DAF-2 with NO released from NOR analogs was measured with a fluorescence microplate reader (Titertek Fluoroscan II, Flow Laboratory, McLean, VA, USA) calibrated for excitation at 485 nm and fluorescence at 530 nm. The half-life time was calculated by the nonlinear-regression program GraphPad Prism 2.01 (GraphPad Software, USA).

2.3. Cell culture and treatment

Undifferentiated PC12 cells (passage 8–29) were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated horse serum, 5% fetal calf serum, 50 units/ml of penicillin G, and 100 ng/ml of streptomycin. Cultures were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The medium was changed every 3 days. For experiments, the cells were plated onto 35-mm-diameter dishes or 96-well plates (Sumilon, Sumitomo Bakelite, Tokyo, Japan). After replating at a density of $1–5 \times 10^4$ cells/cm^2, the various test drugs described above were added at 5 days after plating.

2.4. Cell viability assay

Cell viability was determined by the method of modified MTT assay (Mosmann, 1983). Briefly, PC12 cells were plated on 96-well microtiter plates, and MTT solution (5 mg/ml in DMEM) was added and allowed to incubate for 1–4 h. After the medium had been discarded, the cell and dye crystals were dissolved by adding 100 μl of DMSO, and absorption was measured at 595 nm (655 nm as a reference) in an EIA plate reader (Bio-Rad). Results were expressed as a percentage of the absorbance in the vehicle-treated control culture wells. Data are presented as means ± S.E.M values, at least four independent experi-

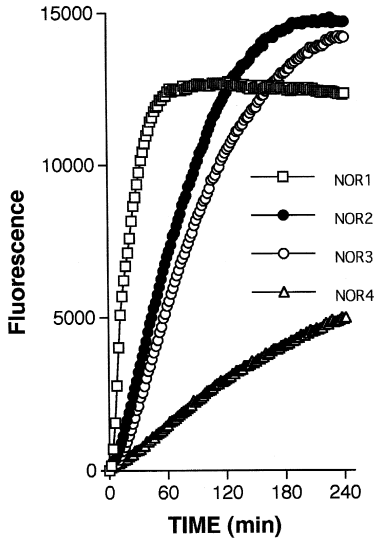

Fig. 1. Measurement of NO release from NOR analogs by DAF-2 fluorescence assay. NOR analogs (5 μM), NOR1 (□), NOR2 (●), NOR3 (○) or NOR4 (△), were incubated in 200 μl of HBS, pH 7.3 at 37°C for up to 240 min. DAF-2 (10 μM final concentration) was added to these mixtures, and the fluorescence intensities from the reaction product, triazolofluorescein, were measured continuously every 2 min with a fluorescence microplate reader. The excitation wavelength was 485 nm and the emission wavelength was 583 nm. The curves shown are representative of kinetic analysis of three independent experiments.

ments were performed, and statistical comparisons were made using an analysis of variance (ANOVA) with the least squares difference post hoc test.

2.5. Measurement of nuclear fragmentation

Chromatin condensation and nuclear fragmentation were determined by Hoechst 33,258 staining. The cells were grown on polyethyleneimine-coated 35-mm-diameter glass-bottom dishes (MatTek, Ashland, MA, USA). At various times after exposure to NOR3 (10–100 μM), the cells were fixed for 30 min with 4% paraformaldehyde in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS), washed with CMF-PBS and then stained for 10 min with 10 μg/ml Hoechst 33,258 in CMF-PBS. Cells were then visualized for nuclear condensation and fragmentation using a Zeiss Axiovert fluorescence microscope.

2.6. DNA agarose gel electrophoresis

For DNA degradation analysis, a pellet of cells from 35-mm-diameter culture dishes were lysed in 100 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 20 mM EDTA, 1% NP-40). After lysis, the DNA fragment was separated from intact chromatin by centrifugation for 5 min at $1600 \times g$. The supernatant was successively treated with 0.5 mg/ml RNase A for 2 h at 56°C and 0.5 mg/ml proteinase K for 4 h at 37°C. The DNA samples were ethanol precipitated and then loaded onto 1.0% agarose gel and separated by electrophoresis in 1 × Tris/borate/EDTA (TBE) running buffer for 40 min at 100 V. The DNA band pattern was visualized by ethidium bromide staining.

2.7. Statistical analysis

The data are presented as the means ± S.E.M., and n represents the number of experiments. Statistical analysis was made by one-way or two-way ANOVA followed by the Bonferroni test for multiple comparisons. A value of $P < 0.01$ was considered significant. The pharmacological response to each NO donor, described in terms of EC_{50}, was calculated using the GraphPad Prism 2.01 software computer program (GraphPad Software).

3. Results

3.1. NO release from NOR analogs

NOR analogs decomposed in HBS (pH 7.3) spontaneously and released NO. The amounts of NO released were measured using DAF-2, since DAF-2 reacts with the oxidized form of NO (Nakatsubo et al., 1998), and the resultant fluorescent triazolofluorescein was relatively stable (data not shown). As shown in Fig. 1, the amount of NO released in the steady state from NOR1, NOR2 and NOR3 was similar, but that from NOR4 was about 20% of the other NOR analogs during 3 h incubation. The half-life time of the NO release from these four NOR analogs was 6.5 (NOR1), 84 (NOR2), 105 (NOR3) and 340 (NOR4) min, respectively.

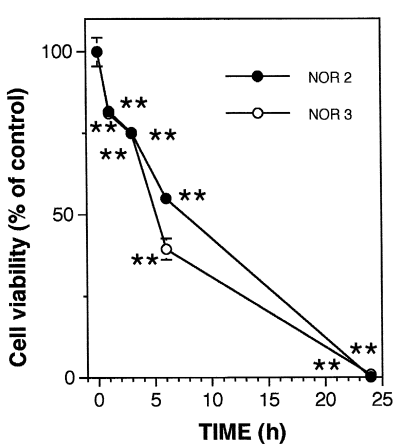

Fig. 2. Time course of the viability of PC12 cells exposed to NOR2 and NOR3 (50 μM). The cells in 96-well microtiter plates were exposed to 50 μM NOR2 (●) or NOR3 (○). The cell viability was determined by MTT assay after treatment with the drugs at the indicated time. Results are expressed as a percentage of the control (i.e., cells without NOR2 or NOR3). Values are the means ± S.E.M. (bars) of five independent experiments (** $P < 0.001$; ANOVA followed by Bonferroni test, $n = 6$).

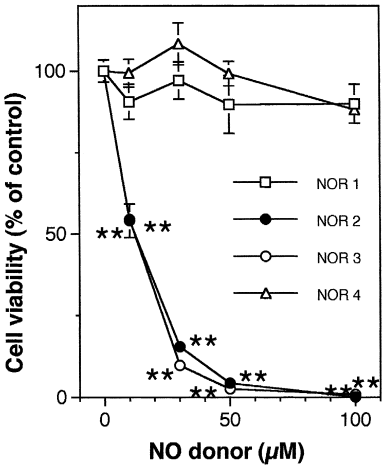

Fig. 3. Effects of NOR analogs, NOR1, NOR2, NOR3 and NOR4, on the viability of PC12 cells. The cells were exposed to the indicated concentrations of each analog, NOR1 (□), NOR2 (●), NOR3 (○) or NOR4 (△). The cell viabilities were determined by MTT assay after 24 h of treatment. Results are expressed as a percentage of the control (i.e., cells without NOR analogs). Values are the means ± S.E.M. (bars) of five independent experiments (** $P < 0.001$ vs. control; ANOVA followed by Bonferroni test, $n = 6$).

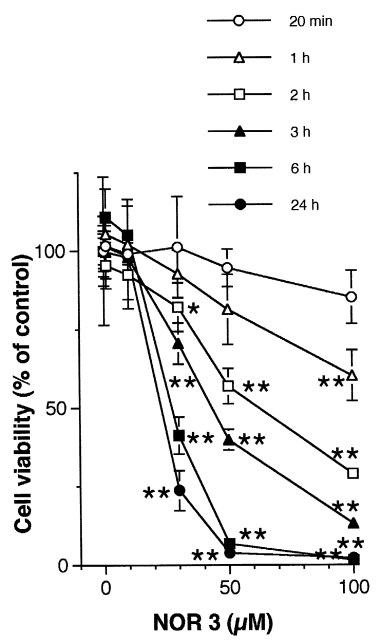

Fig. 4. Effect of NOR3 exposure time on the viability of PC12 cells after 24 h. The cells were exposed with indicated concentrations of NOR3 (50–500 μM) for 20 min (○), 1 h (△), 2 h (□), 3 h (▲), 6 h (■) or 24 h (●). Then, the medium containing the drugs was changed to fresh drug-free medium at each time point, and incubation continued. The cell viability was determined by MTT assay after 24 h. Results are expressed as a percentage of control. Values are the means ± S.E.M. (bars) of five independent experiments. Two-way ANOVA with repeated measures on the data revealed a significant effect of drug (NOR3; $F = 486.9$, $df = 5$; $P < 0.001$) and treatment time ([hour]; $F = 139.0$, $df = 5$; $P < 0.001$), and a significant drug (NOR3)×[hour] interaction ($F = 32.7$, $df = 25$; $P < 0.001$). Post hoc contrasts revealed a significant effect of NOR3 (* $P < 0.01$, ** $P < 0.001$, ANOVA followed by Bonferroni test).

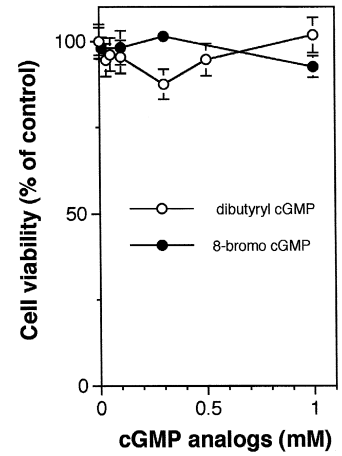

Fig. 5. Effect of analogs of cGMP, dibutyryl cGMP and 8-bromo cGMP, on the viability of PC12 cells. The cell viability was determined by MTT assay in the presence of increasing concentrations of dibutyryl cGMP and 8-bromo cGMP. Results are expressed as a percentage of the control (i.e., cells without cGMP analogs). Values are the means ± S.E.M.(bars) of four independent experiments.

3.2. Effects of NOR analogs on the viability of PC12 cells

The PC12 cells were exposed to NOR2 or NOR3 (both at a concentration of 50 μM) to assess the possible toxic effects of NO on the cell viability. As shown in Fig. 2, both NOR analogs caused a time-dependent decrease in cell viability. After 24 h treatment, 90% of the cells had died and a few viable cells remained. NOR2 or NOR3 (10–100 μM) induced a concentration-dependent cell death (Fig. 3) with an EC_{50} of 7.5 ± 1.4 or 7.0 ± 1.5 μM (means ± S.E.M.), respectively. When PC12 cells were treated with NOR1 or NOR4, having a shorter (6.5 min) or

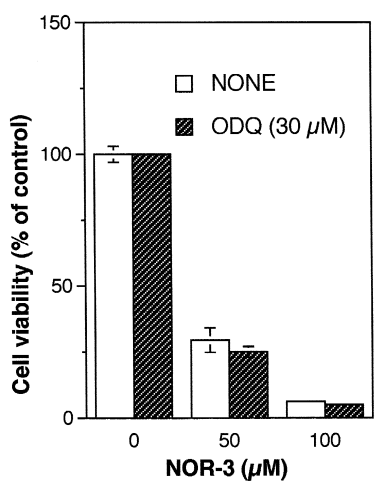

Fig. 6. Effect of ODQ on NOR3-induced PC12 cell toxicity. PC12 cells were exposed to the ODQ for 30 min before NOR3 was added. After 24 h, the cell viability was measured by an MTT assay. All groups were measured with five replicate points of four independent experiments. Data are expressed as % control (vehicle) with error bars representing S.E.M. Differences between cells treated with ODQ and without were analyzed by one-way ANOVA followed Bonferroni test. * $P < 0.01$, ** $P < 0.001$.

longer (340 min) half-life time, respectively, only a small or no effect on the cell viability was observed at 24 h after the drug treatment (Fig. 3). We next examined the effect of the duration of the exposure to NOR3 on the cell viability.

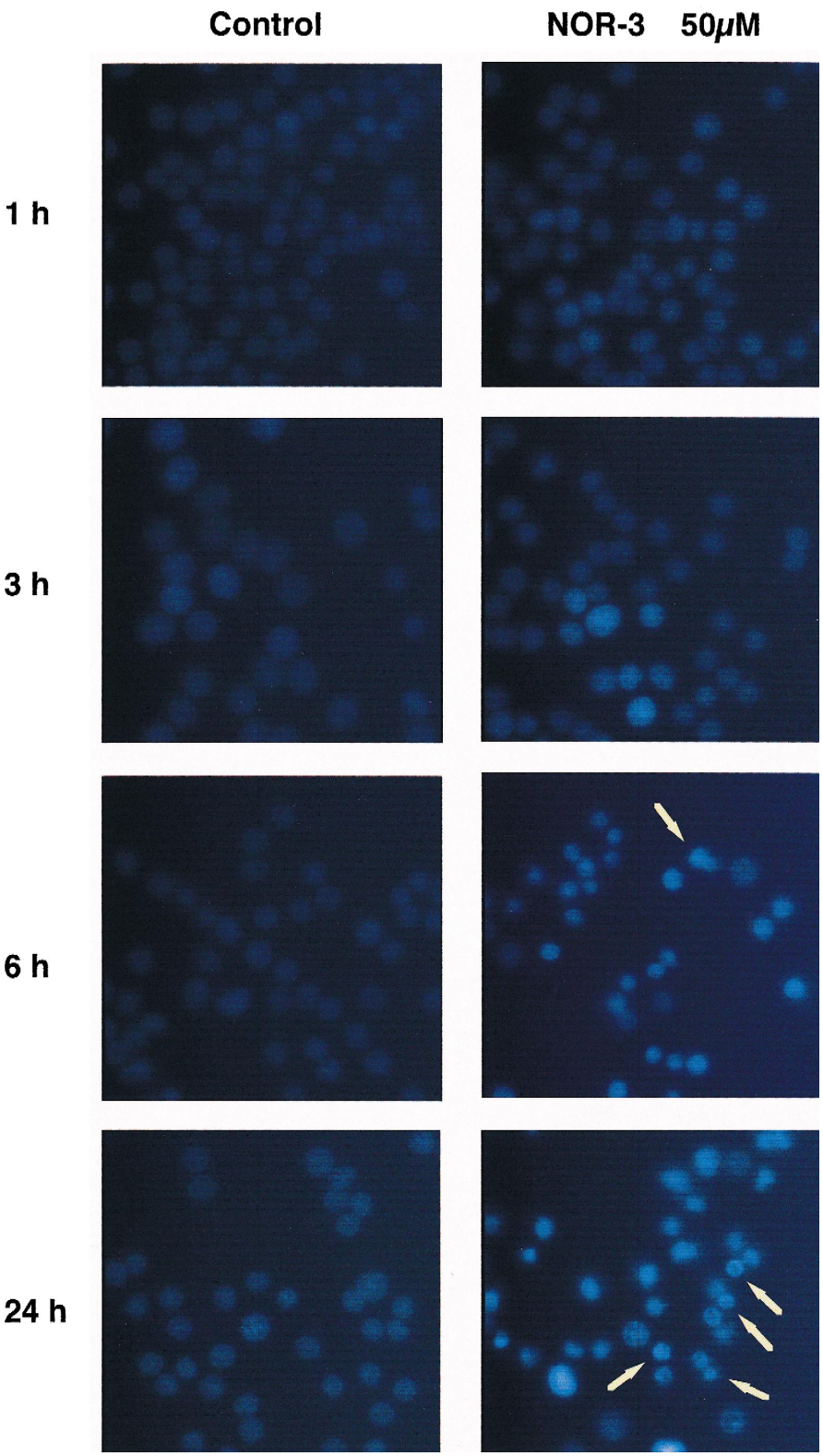

Fig. 7. Time course of morphological changes in PC12 cells as monitored by Hoechst staining. PC12 cells were exposed for 1, 3, 6 h in the absence (left panels) or presence (right panels) of 50 μM NOR3. Cells were fixed, stained with Hoechst 33,258, and observed with a fluorescence microscope for morphological changes associated with apoptosis (chromatin condensation and nuclear fragmentation (arrows)).

Culture medium containing NO donor was removed and replaced with the same fresh medium at 20 min, 1, 2, 3 or 6 h after the addition of NOR3, and then cell viabilities were measured 24 h after the initiation of NOR3 treatment. NOR3-induced cell death was induced by exposure to NOR3 for more than 3 h (Fig. 4). Control experiments showed that decomposed NOR3 had no effect on the cell viability (101.5 ± 2.9% at 100 μM of decomposed NOR3, $n = 5$). Furthermore, oxy-hemoglobin, which is an NO scavenger, significantly attenuated the cell death induced by NOR3 (cell viability in the presence of 50 μM NOR3 was 18.1 ± 2.3 and 61.8 ± 4.6% without and with 100 μM oxy-hemoglobin, respectively, $n = 4$, $P < 0.001$).

3.3. Effects of dibutyryl cGMP, 8-bromo cGMP and ODQ on PC12 cell viability

To determine whether the cell death by NOR2 or NOR3 is coupled with the activation of soluble guanylate cyclase, PC12 cells were exposed to a cGMP analog, dibutyryl cGMP or 8-bromo cGMP (0.1–1 mM), for 24 h. Fig. 5 shows that neither cGMP analog had an effect on the cell viability. Furthermore, ODQ (30 μM), a selective inhibitor of soluble guanylate cyclase, did not affect the NOR3-induced cell toxicity (Fig. 6). These results suggest a lack of participation of the NO–cGMP signal pathway in the NOR3-induced cell death.

3.4. Effects of NOR3 on DNA condensation and fragmentation

To investigate the properties of NOR3-induced cell death, we stained cells with Hoechst 33,258 dye. Hoechst 33,258 specifically stains DNA and is widely used to detect condensation and fragmentation of the nuclei, which are indicative of apoptosis. As shown in Fig. 7, DNA fragmentation was detectable in PC12 cells 6 h after 50 μM NOR3 exposure. These results strongly suggest that apoptosis was induced in PC12 cells by the addition of 50 μM NOR3. We further examined the DNA fragmentation in NOR3 treated PC12 cells by electrophoresis on agarose gels to assess DNA degradation. As shown in Fig. 8, significant degradation of the DNA was detected after NOR3 treatment. The extent of the degradation increased in a concentration dependent manner.

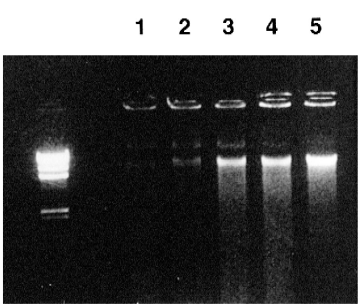

Fig. 8. Effect of NOR3 on the occurrence of internucleosomal DNA fragmentation in PC12 cells. Cells were incubated with NOR3 for 24 h, and cellular DNA was extracted as described in Materials and Methods. DNA was resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. (left lane) l-DNA-*Hin*dIII digest; (lane 1) control cells; (lanes 2, 3, 4 and 5) NOR3 treatment at 10, 30, 50 and 100 μM, respectively. The experiment was performed three times with similar results.

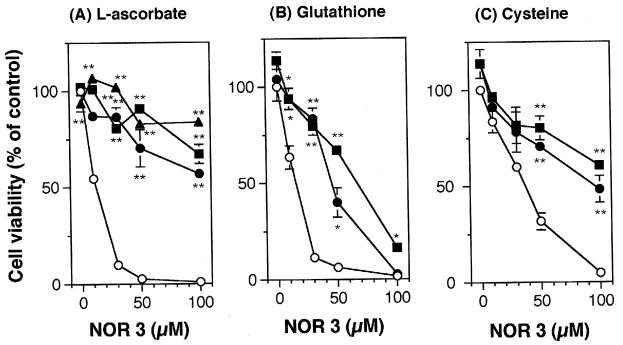

Fig. 9. Effects of antioxidants, (A) ascorbic acid, (B) glutathione and (C) cysteine on NOR3-induced PC12 cell toxicity. PC12 cells were exposed to the antioxidant for 30 min before NOR3 was added. After 24 h, the cell viability was measured by an MTT assay. (A) Ascorbic acid: 100 μM (●), 300 μM (■), 500 μM (▲) and control (○); (B) glutatione: 100 μM (●), 200 μM (■) and control (○); (C) cysteine, 200 μM (●), 400 μM (■) and control (○). All groups were measured with four replicate points of four independent experiments . Data are means ± S.E.M. (bars). Differences between cells pretreated with antioxidants and without (○) were analyzed by one-way ANOVA followed by Bonferroni test, at all doses: $^{*}P < 0.01$, $^{**}P < 0.001$.

3.5. Effects of antioxidants on the NOR3-induced cell death

To determine whether antioxidants would protect PC12 cells from NOR3-induced apoptosis, cells were treated with ascorbic acid (0.1–0.5 mM), reduced glutathione (0.1 and 0.2 mM) or cysteine (0.2 and 0.4 mM) for 30 min before the addition of NOR3 (10–100 mM) to the medium. Twenty-four hours after NOR3 addition, cell viability was measured (Fig. 9). Preincubation with these antioxidants significantly reduced NOR3-induced cell death. Ascorbic acid was the most effective antioxidant to protect the cells from death caused by NOR3. Both glutathione and cysteine partially protected PC12 cells from NOR3-induced cell death.

4. Discussion

Based on the evidence obtained from several studies, the cytotoxic effects producing high concentrations of NO donors are most likely mediated through the formation of peroxynitrite, which is a powerful oxidant resulting from the reaction of NO with superoxide anion (Beckman, 1990; Lipton et al., 1993; Estevez et al., 1995). However, the

effect of NO on the cells at lower concentrations, approximating the physiological condition, has not been fully elucidated. In this study, we demonstrated that a low concentration of NO donors, NOR2 or NOR3, induced apoptosis-like cell death in undifferentiated PC12 cells.

Interestingly, cell toxic effects of NOR analogs were limited to these two derivatives, NOR2 and NOR3. NOR2 and NOR3, which have a relatively long half-life time of NO release (84 and 105 min, respectively), produced a marked decrease in viability of PC12 cells at 24 h after exposure to drugs. The cytotoxic effect of NOR3 was significantly inhibited by oxy-hemoglobin, an NO scavenger. In addition, the decomposed NOR3 did not induce cell death. These findings strongly suggest that NOR3-induced cell death is mediated by NO. Using NOR analogs, the toxic effects were remarkable even at low doses, in the 10–100 μM range, with EC_{50} values approximately 10 μM. Unlike NOR2 and NOR3, NOR1 did not induce cell death, even though NOR1 released NO in similar amounts to NOR2 and NOR3 as determined with a DAF-2 fluorescence indicator. NOR1 released NO rapidly with a half-life time of 6.5 min, indicating that most of NOR1 decomposed within an hour after addition to the cells. These results indicate that exposure of the cells to NO for longer than 1 h is necessary for NO toxicity. Indeed, the cell toxicity of NOR3 was lost on removal of the drug by replacing the medium within the first 1 h. As the time the cells were exposed to NOR3 increased to over 2 h, the toxic effect of NOR3 on the cells became significant. Thus, the toxic effect of NO is strongly dependent on the length of exposure to NO. On the other hand, a longer half-life time analog, NOR4 ($T_{1/2} = 340$ min), had no significant effect on the cell viability. This may be because the levels of free NO are below the threshold to accumulate under these conditions. Unlike NOR4, both NOR2 and NOR3 released too much NO to be metabolized in a short time and thus free NO will accumulate to induce the cell death. Indeed, the half-life of NO in biological systems has been reported as 3–5 s, and the concentrations of free NO observed in the steady state physiological conditions are very low (Czapski and Goldstein, 1995). It has been assumed that the low concentrations of free NO in biological systems are due to its rapid metabolism by biological molecules, including superoxide anion. For example, NO rapidly interacts with mammalian 15-lipoxygenase (ferrous Fe(II) form of the enzyme) and the dissociation constant of the Fe(II)–NO complex amounts to 2.5 μM (Holzhutter et al., 1997). The second-order rate constants of the reactions of NO with ubiquinol-0 and ubiquinol-2, analogs of coenzyme Q, were 0.49 and 1.6×10^4 M^{-1} s^{-1}, respectively (Poderoso et al., 1999). Furthermore, NO reacts with ferrocytochrome *c* and xanthin oxidase at a rate of 200 and 14.8 M^{-1} s^{-1}, respectively (Sharpe and Cooper, 1998; Ichimori et al., 1999). Eiserich et al. (1995) reported that NO was highly reactive with tyrosine and tryptophan radicals in amino acids, peptides and proteins at the rate constants of $1–2 \times 10^9$ M^{-1} s^{-1}. Therefore, NO might be metabolized by those biological systems according to their rate constants. These differences in the rate constants of the reaction with NO may reflect the differential effects of NO in several tissues. In the present study, NOR4 did not induce cell death because it released NO too slowly and the NO reacted with highly reactive substances, not the cell components relating to the cell death pathway. Of course, NOR4 at a concentration higher than 1 mM induced cell death in our preliminary experiments (unpublished observations). Thus, if we examine the effect of low concentrations of NO on the cell viability, NOR2 and NOR3 are the most suitable supply of NO. In the present study, it was revealed that both the rate of NO production and the duration of NO exposure are important factors to the cell toxicity produced by low concentrations of NO donors.

Recently, NO has also been proposed to have a neuroprotective effect at low concentrations (Farinelli et al., 1996; Pantazis et al., 1998). Farinelli et al. (1996) have reported that NO donors, such as *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside, promote short-term survival in serum-deprived PC12 cells at concentrations less than 0.5 mM. Furthermore, Estevez et al. (1998b) have shown that NO produced by the endothelial NOS (eNOS) contributes to the survival of motor neurons cultured with trophic factor. These protective effects of NO are likely to be mediated via the activation of guanylate cyclase and subsequent increase in intracellular cGMP. However, our results have shown that NO produced from the NO donors, NOR2 and NOR3 at low concentrations (1–100 μM), could be toxic to undifferentiated PC12 cells in serum-supplemented medium. Moreover, cell-permeable cGMP analogs dibutyryl cGMP and 8-bromo cGMP had no effect on the cell viability up to a concentration of 1 mM. This is consistent with previous reports that cGMP analogs 8-bromo cGMP and dibutyryl cGMP had no effect on cell viability in primary cultures of rat cerebral cortical neurons (Lustig et al., 1992) and cortical, striatal and hippocampal neurons (Dawson et al., 1993). Furthermore, guanylate cyclase inhibitor, ODQ, did not inhibit the toxic effect induced by NOR3. Thus, cGMP is unlikely to participate in the toxic effect, and a short or transient NO production may affect cell survival through the cGMP pathway, whereas a longer lasting NO production even at low concentration might cause cell damage not through the activation of guanylate cyclase.

In this study, it was demonstrated that NO toxicity induced by a low concentration of NOR3 characterized apoptosis-like cell death. Morphologically, chromatin condensation and nuclear fragmentation were observed. In addition, DNA degradation was found at 24 h after NOR3 (50 μM) exposure. Although a typical apoptotic pattern of DNA (DNA-ladder) could not be detected, a specific nuclear fragmentation and chromatin condensation have been interpreted as indicative of apoptosis-like cell death. There

is increasing evidence that, in some cells, apoptosis can be observed in the absence of oligonucleosomal fragmentations (Oberhammer et al., 1993; Saura et al., 1997). Therefore, NOR3 induced cell death of a type thought to be ''apoptosis'' related.

Although the mechanism underlying NO-induced cytotoxicity at low concentrations is unknown, peroxynitrite might be involved as mentioned above. Recently, Estevez et al. (1998a) have reported that primary cultures of rat embryonic motor neurons deprived of trophic factor induce neuronal NOS and undergo cell death. NOS inhibitors protect the motor neurons from the cell death induced by the deprivation of trophic factor, and the protection is reversed by generating NO with a low concentration (20 μM) of exogenous NO donor (DETA-NONOate) . The toxicity of NO in trophic factor-deprived motor neurons is thought to be resulted from the production of peroxynitrite (Estevez et al., 1998a). It is well known that the reaction of superoxide and NO rapidly results in the formation of peroxynitrite, a highly reactive oxidant species (Beckman, 1992; Pryor and Squadrito, 1995). Thus, the cytotoxicity induced by NO results from the formation of peroxynitrite derived from the reaction with superoxide. At present, it is thought that peroxynitrite participates in the nitration of tyrosine residues in amino acids, peptides and proteins or the oxidation of thiol compounds such as cysteine and glutathione (Moro et al., 1994; Soszynski and Bartosz, 1996). However, our observation, that both glutathione and cysteine partially protected against NO-induced cell toxicity, indicates that peroxynitrite formation is not fully responsible for the cell toxicity. In our preliminary study, 3-(4-morpholinyl)-sydnonimine (SIN-1), which rapidly generates peroxynitrite after decomposition of SIN-1 to NO and superoxide anion, and peroxynitrite-solution itself did not have significant effects on cell viability even at a concentration of 0.5 mM (unpublished observation). These findings were consistent with a previous report that the concentration of peroxynitrite required to cause cell death in undifferentiated PC12 cells is more than 1 mM after 24 h treatment (Estevez et al., 1995). Based on the present findings, the concentration of peroxynitrite would not seem to be high enough to produce cell toxicity on exposure to 10–100 μM of NOR2 or NOR3. Furthermore, this notion is also supported by the preliminary finding that tyrosine and the analogs, which are highly react with peroxynitrite, could not protect against NOR3-induced cell death (unpublished observation). Therefore, it is unlikely that the toxicity of NOR3 at low concentrations (lower than 100 μM) in PC12 cells is mediated through the production of peroxynitrite. In spite of these findings, however, we cannot rule out the possible involvement of peroxynitrite formation in the cell toxicity induced by low concentrations of NOR3 (< 100 μM).

The antioxidants ascorbic acid, glutathione and cysteine all protected PC12 cells from NOR3-induced cell death. It is noteworthy that ascorbic acid more potently protected against NOR3-induced cell toxicity than glutathione or cysteine. Recently, Desole et al. (1998) reported that ascorbic acid (0.1 mM) did not antagonize sodium nitroprusside (0.5–0.75 mM)-induced apoptosis. Interestingly, they have also shown that manganese induces apoptosis in PC12 cells, and these effects were strongly inhibited by ascorbic acid (Desole et al., 1996). This protective mechanism is related to an inhibition of oxidative stress induced by manganese. Similarly, in the present study, ascorbic acid (0.1–0.5 mM) near completely antagonized NOR3 induced cell death, indicating that the cell death induced by low concentration of NOR3 is due to oxidative stress. Further studies are in progress to clarify the exact mechanism of this toxicity.

In conclusion, our study indicates that at low concentration, NOR analogs produced apoptosis-like cell toxicity within the first 24 h of exposure to drugs. The apoptosis-like cell death induced by NOR analogs was strongly dependent on the half-life time of NO release from the analogs, and was not mediated through the NO–cGMP pathway. Moreover, NOR3-induced cytotoxicity may be mediated by oxidative stress, against which ascorbic acid provides protection. Although more detailed studies of half-life time specific NO toxicity will be needed to conclusively identify the mechanisms involved, the model system reflected a sustained production of NO by NOS in the pathophysiological condition.

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