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Morphine prevents peroxynitrite-induced death of human neuroblastoma SH-SY5Y cells through a direct scavenging action

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Abstract

N-Ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)-ethanamine (NOC12), a nitric oxide donor, 3-morpholinosydnonimine (SIN-1), a generator of peroxynitrite ($ONOO^-$), and peroxynitrite induced cell death accompanied by DNA fragmentation in human neuroblastoma SH-SY5Y cell cultures. Morphine prevented the cell death induced by SIN-1 or peroxynitrite, but not that induced by NOC12. The protective effect of morphine was concentration-dependent (10–100 μM), but was not antagonized by naloxone. The selective ligands for opioid receptor subtypes, [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO, μ-opioid receptor agonist), [D-Pen^{2,5}]enkephalin (DPDPE, δ-opioid receptor agonist) and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl)benzeneacetamide (U-50488, κ-opioid receptor agonist) even at the concentration of 100 μM did not prevent the cell death induced by SIN-1. From measurement of the absorbance spectrum of peroxynitrite, the decomposition of peroxynitrite in 0.25 M potassium phosphate buffer (pH 7.4) was very rapid and complete within seconds. However, the absorbance was very stable in the presence of morphine. In addition, morphine inhibited peroxynitrite-induced nitration of tyrosine in a concentration-dependent manner. These results indicate that morphine rapidly reacts with peroxynitrite. The present study showed that morphine prevented peroxynitrite-induced cell death through its direct scavenging action, suggesting that morphine can protect cells against damage caused by peroxynitrite.

Keywords: Morphine; Peroxynitrite; Cell death; Scavenging; Neuroblastoma

1. Introduction

Nitric oxide (NO) is synthesized from molecular oxygen and the guanidine group of L-arginine by several isoform of NO synthase, and is an important mediator of cellular communication (Knowles et al., 1989). Peroxynitrite ($ONOO^-$) is a powerful biological oxidant produced by the diffusion-limited reaction of superoxide with NO (Beckman et al., 1990; Huie and Padmaja, 1993). Some reports have shown that both NO and peroxynitrite induce cell death in several cell lines (Dawson et al., 1991; Lipton et al., 1993; Bonfoco et al., 1995). It is well-known that the extracellular concentration of glutamate in the brain markedly increases during cerebral ischemia (Wahl et al., 1994). Glutamate released during ischemia activates NMDA receptors, followed by Ca^{2+} influx into neurons and NO synthase activation (Gunasekar et al., 1995). The production of NO and peroxynitrite induces ischemic neuronal cell death (O'Neill et al., 1996; Szabo, 1996). Antagonists of opioid receptors such as naloxone attenuate ischemic brain damage in animal models of ischemia and in human patients (Baskin and Hosobuchi, 1981; Hosobuchi et al., 1982; Jabaily and Davis, 1984; Wexler, 1984). However, some investigators have failed to observe a protective effect of naloxone in their particular ischemia model (Holaday and D'Amato, 1982; Fallis et al., 1984). There is some evidence that κ-opioid receptor agonists can attenuate post-ischemic neuronal death (Baskin et al., 1984; Tang, 1985; Kusumoto et al., 1992; Mackay et al., 1993; Baskin et al., 1994). Recently, it has been reported that enadoline, a κ-opioid receptor agonist, inhibits glutamate release during ischemia (MacKay et al., 1996). However, the effect of opioids on NO- and peroxynitrite-induced neuronal cell death is largely unknown. In the present study, we examined the effect of morphine on NO- and

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peroxynitrite-induced cell death using a human neuroblastoma SH-SY5Y cell line, which abundantly expresses μ-, δ- and κ-opioid receptors.

2. Materials and methods

2.1. Cell culture and treatment with drugs

The human neuroblastoma SH-SY5Y cell line was a gift from Dr. Wolfgang Sadee (University of California, San Francisco, USA). Cells were grown in RPMI1640 medium supplemented with 5% fetal bovine serum (HyClone, UT, USA) and 5% newborn calf serum (BioWhitteker, Walkersville, USA) containing 100 μg/ml streptomycin, 100 IU/ml penicillin and 1 μl/ml fungizone (Gibco-BRL, Gaithersburg, USA) in a humidified atmosphere of 95% air–5% CO_2. For experiments, cells were plated at a density of 10^5 on 35-mm diameter dishes. For exposure to *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)-ethanamine (NOC12, Dojindo, Kumamoto, Japan), 3-morpholinosydnonimine (SIN-1, Dojindo) or sodium peroxynitrite (Cayman, Ann Arbor, USA), these drugs were added in concentrated form to the culture medium and mixed extremely gently. Morphine and other opioids were added to the medium 30 min before the addition of these drugs.

2.2. Cell count

To count viable cells, cells were removed from dishes by trituration in their culture medium (to avoid loss of detached cells). Cells were concentrated by brief centrifugation ($1000 \times g$, 5 min), the supernatant was removed, and the cells were resuspended in 100 ml of phosphate buffered saline (PBS) and 100 μl of trypan blue solution (0.4% in PBS). The numbers of both trypan blue-permeable blue cells and viable white cells were counted using a hemocytometer.

2.3. MTT assay

Cell toxicity was determined by MTT assay (Mosmann, 1983). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, USA) is a compound which is taken up by viable cells and converted to an insoluble dye (formazan) via the action of succinate dehydrogenase. Cells were incubated with MTT solution (0.5 mg/ml) for 4 h. The resulting dye was extracted with acidified isopropanol, and the absorbance was measured at 590 nm. MTT reduction was expressed as a percentage of that of the untreated control.

2.4. DNA fragmentation analysis

DNA fragmentation was determined by gel electrophoresis. Culture media and trypsinized cells were combined and centrifuged for 5 min at $200 \times g$, then washed and repelleted once in PBS (pH 7.4). The pellet was gently agitated for 30 min at 37°C in lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.1% sodium dodecylsulfate). After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 45 min at $18{,}000 \times g$. The supernatant were precipitated overnight at −20°C in ethanol containing 0.3 M sodium acetate, and centrifuged for 45 min at $18{,}000 \times g$. The pellets were resuspended in Tris–EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with proteinase K (100 μg/ml, Sigma) and RNase A (100 mg/ml, Sigma), and incubated for 6 h at 37°C. The samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once again with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was recovered by ethanol precipitation at −20°C and centrifugation. The pellet was resuspended in TE buffer and electrophoresed through 1.2% agarose gels containing 0.5 mg/ml ethidium bromide, for 1.5 h at 90 V. Pictures were obtained by UV transillumination.

2.5. Spectrophotometric analysis

Absorbance measurements were performed with a spectrophotometer (Shimazu UV-240, Kyoto, Japan). Absorbance spectra of the reaction mixture in a quartz cuvette (light path 1 cm) were recorded immediately after addition of peroxynitrite against buffer or morphine solution as reference.

2.6. Measurement of tyrosine nitration

A solution of 1 mM L-tyrosine was prepared in 50 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.4). Small aliquots of peroxynitrite (final concentration: 300 μM) were added to the tyrosine solution and rapidly mixed with vortexing. The yield of nitrotyrosine was determined by measuring absorbance at 420 nm (van der Vliet et al., 1994). Morphine was added to the buffer to give the final concentration before the addition of peroxynitrite.

2.7. Statistical evaluation

All values in the figure are expressed as means ± S.E.M. of three or four experiments. Statistical differences between groups were determined using Student's *t*-test.

3. Results

3.1. Effect of morphine on NO- or peroxynitrite-induced cell death

We estimated the damage to SH-SY5Y cells induced by NOC12, SIN-1 or peroxynitrite using both the trypan blue

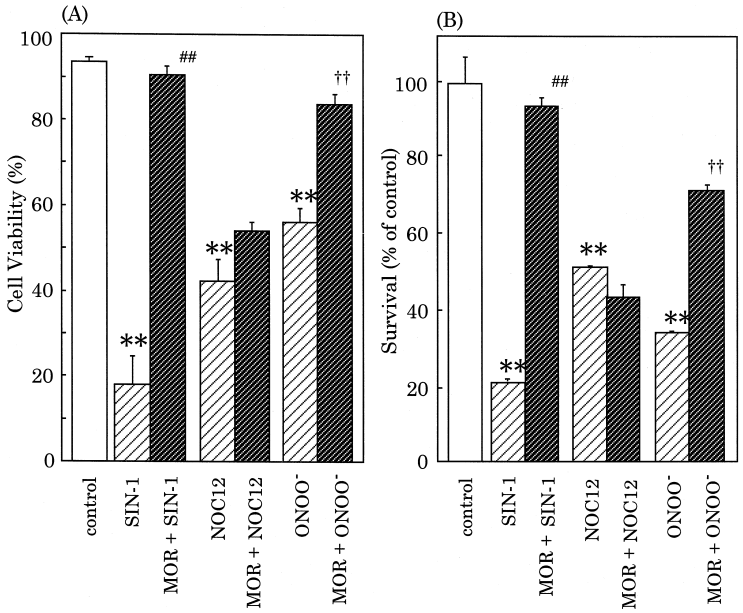

Fig. 1. Effect of morphine on cytotoxicity induced by SIN-1, NOC12, or peroxynitrite in SH-SY5Y cells. Cells were treated with 500 μM SIN-1, 500 μM NOC12 or 10 μM peroxynitrite ($ONOO^-$) for 24 h in the absence or presence of 100 μM morphine (MOR). Cytotoxicity was measured using trypan blue dye exclusion test (A) and the MTT assay (B). Statistically significant differences were estimated by the Student's *t*-test. The column represents the mean ± S.E.M. from three or four experiments. $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with SIN-1; $^{\dagger\dagger}P < 0.01$ compared with $ONOO^-$.

exclusion test (Fig. 1A) and the MTT assay (Fig. 1B). Treatment with 500 μM SIN-1, 500 μM NOC12 or 10 μM sodium peroxynitrite for 24 h caused loss of cell viability. Decomposed peroxynitrite had no significant effect on cell viability (data not shown). Addition of morphine (100 μM) to the culture media 30 min before the treatment with SIN-1 or peroxynitrite prevented cell death (Fig. 1A,B). However, NOC12-induced cell death was not prevented by the addition of morphine. The protective effect of morphine against the cell death induced by SIN-1 was concentration-dependent (10–100 μM) (Fig. 2A). Agarose gel electrophoresis of DNA extracted from SIN-

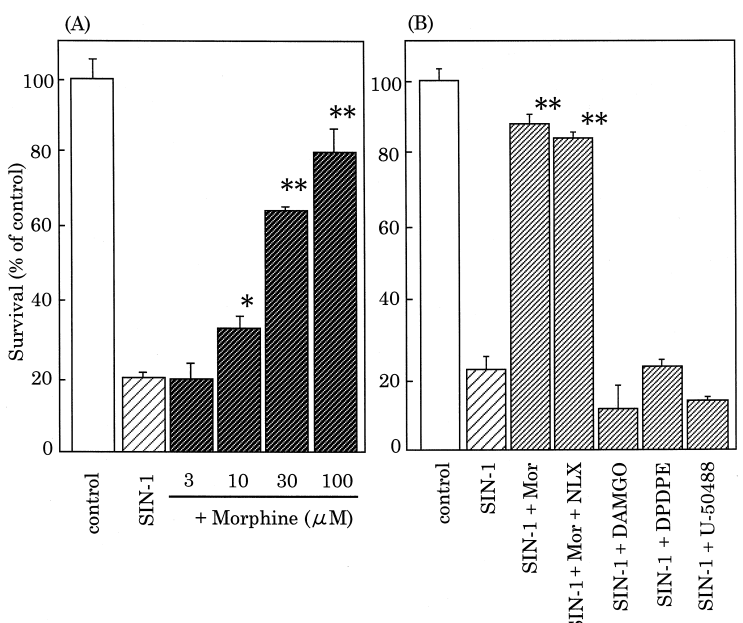

Fig. 2. (A) Dose-dependency of the protective effect of MOR on cytotoxicity induced by 500 μM SIN-1 as detected by the MTT assay. (B) Effect of naloxone (NLX, 10 μM) on the prevention by 100 μM morphine, and effects of various opioids (100 μM) on cytotoxicity induced by 500 μM SIN-1 as detected by the MTT assay. Statistically significant differences were estimated by the Student's *t*-test. The column represents the mean ± S.E.M. from three or four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with SIN-1.

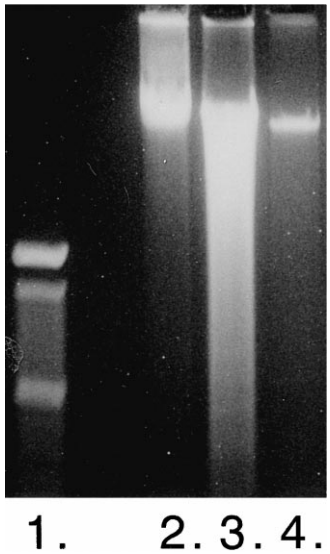

Fig. 3. Agarose gel electrophoresis of DNA fragmentation. SH-SY5Y cells were exposed to 500 μM SIN-1, or to 500 μM SIN-1 plus 100 μM morphine for 24 h. Lane 1, 2-kb DNA marker; Lane 2, control; Lane 3, 500 μM SIN-1; Lane 4, 500 μM SIN-1 plus 100 μM morphine.

1-treated cells showed there to be DNA fragmentation (Fig. 3). The DNA fragmentation was inhibited by morphine. [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO), [D-Pen^{2,5}]enkephalin (DPDPE) and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl)-benzeneacetamide (U-50488), which are selective ligands for μ-, δ- and κ-type opioid receptors, respectively, had no effect on the cell death induced by SIN-1 even at a concentration of 100 μM (Fig. 2B). Naloxone (10 μM) did not antagonize the effect of morphine on cell death induced by SIN-1 (Fig. 2B). When cells were pretreated

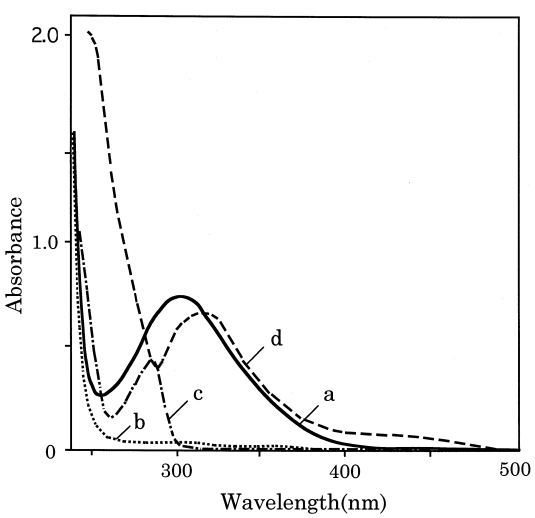

Fig. 4. Absorption spectra from the reaction of morphine with peroxynitrite. Spectrum of 1 mM peroxynitrite solution is shown as **a** (pH 11.0) and **b** (pH 7.4). Spectrum of 0.2 mM morphine solution is shown as **c** (pH 7.4). Spectrum of the mixture of peroxynitrite plus morphine against morphine solution as reference is shown as **d** (pH 7.4).

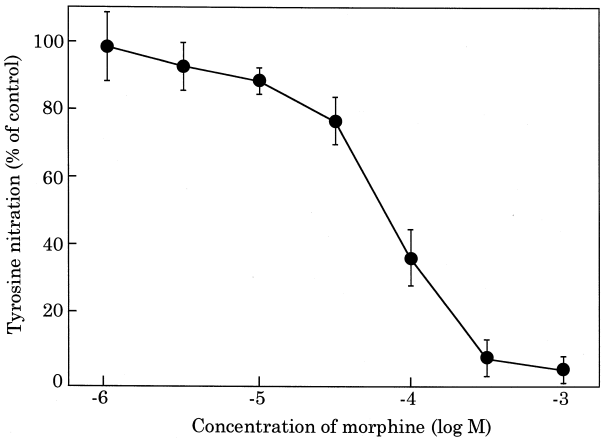

Fig. 5. Effect of morphine on nitration of 1 mM tyrosine by 300 μM peroxynitrite. The yield of nitrotyrosine was measured by the absorbance at 420 nm. A total of 7.9% of tyrosine was nitrated by peroxynitrite in the absence of morphine (control).

with morphine for 30 min followed by its removal prior to the application of SIN-1, cell death was not prevented (data not shown). Furthermore, addition of morphine with SIN-1 had the same effect as the addition of morphine 30 min before SIN-1 exposure (data not shown).

3.2. Interaction of peroxynitrite and morphine

We spectrophotometrically followed the reaction between peroxynitrite and morphine in 0.25 M potassium phosphate buffer (pH 7.4 and 11.0) at room temperature. The spectrum of 1 mM sodium peroxynitrite solution at pH 11.0 had a maximum absorbance at 302 nm (**a** in Fig. 4). When the pH of the solution was lowered from 11.0 to 7.4, the peak of the absorbance at 302 nm disappeared within seconds (**b** in Fig. 4). When morphine (0.2 mM) was present in the peroxynitrite solution, the spectrum of the mixture, measured against morphine solution as reference, at pH 7.4 had an absorbance maximum at 315 nm (**d** in Fig. 4). Since it is well-known that peroxynitrite nitrates tyrosine residues in proteins, the effect of morphine on tyrosine nitration induced by peroxynitrite was examined. Morphine (1 μM–1 mM) reduced the production of nitrotyrosine induced by peroxynitrite (300 μM) in a concentration-dependent manner (Fig. 5).

4. Discussion

SIN-1 has been shown to produce NO and superoxide, resulting in peroxynitrite formation (Hogg et al., 1992). In the present study, morphine prevented the cell death and DNA fragmentation induced by SIN-1 and sodium peroxynitrite in SH-SY5Y cells, but not that induced by NOC12, a NO donor. The selective agonists for μ-, δ- or κ-opioid receptors had no effect on cell death, and the protective effect of morphine was not antagonized by naloxone.

These results suggest that morphine prevents peroxynitrite-induced cell death not via opioid receptors. Peroxynitrite is a strong and versatile oxidant that directly attacks many biological targets (Radi et al., 1991; Castro et al., 1994; Crow et al., 1995), and has been shown to react with methionine (Pryor et al., 1994), ascorbic acid (Bartlett et al., 1995) and ebselen (Masumoto and Sies, 1996). Thus, it is conceivable that the protection by morphine against peroxynitrite-induced cell death may result from a direct interaction with this compound. Therefore, we spectrophotometrically investigated the reaction between peroxynitrite and morphine. Peroxynitrite was stable in alkaline solution (pH 11.0), but it decomposed within seconds at physiological pH (pH 7.4). The result is coincident with some reports that apparent half-life of peroxynitrite, $t_{1/2}$, is 0.1–1 s at pH 7.4 and 37°C (Beckman et al., 1990; Pryor et al., 1995). In the presence of morphine, the absorbance peak did not disappear at pH 7.4, though it shifted from 302 nm to 315 nm. Furthermore, morphine inhibited nitration of tyrosine induced by peroxynitrite. These results suggest that morphine rapidly reacts with peroxynitrite. Peroxynitrite is a toxic oxidant species that has greater cytotoxic potential than NO. Recently, several potential sites for peroxynitrite-mediated cytotoxicity in cells have been revealed, many of which were previously thought to be sites for NO-mediated cytotoxicity (Darley-Usmar et al., 1995). In the present study, we demonstrate for the first time that morphine prevents peroxynitrite-induced cell death through its direct scavenging action, suggesting that morphine can protect cells from damage caused by peroxynitrite.

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