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Protective effects of morphine in peroxynitrite-induced apoptosis of primary rat neonatal astrocytes: potential involvement of G protein and phosphatidylinositol 3-kinase (PI3 kinase)

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Abstract

Opiates, such as morphine, have been used extensively in the clinical management of pain due to their potent analgesic effect. Astrocytes, representing a major non-neuronal cell population in the CNS, contain opioid receptors that are actively involved in several brain functions. This study was designed to evaluate the effects by which morphine, a preferential mu-opioid receptor agonist, contributes to cytotoxicity of nitric oxide (NO) species, including NO and peroxynitrite ($ONOO^-$), in primary rat neonatal astrocytes. Primary astrocytes isolated from the cerebral cortex of 1- to 2-day-old Sprague–Dawley rats were treated with morphine, naloxone, and 3-morpholinosydnonimine (SIN-1), a donor of peroxynitrite. Morphine significantly protected primary rat astrocytes from apoptosis mediated by sodium nitroprusside, an NO donor, and SIN-1 in a dose-dependent manner, whereas it did not in other types of cells including C6 glioma, RAW 264.7, and HL-60 cells. Moreover, naloxone antagonized the protective effects of morphine on SIN-1-induced apoptosis. Morphine also inhibited the nuclear condensation and fragmentation of SIN-1-treated cells that was antagonized by naloxone pretreatment. The protective role of morphine in SIN-1-induced apoptosis was dependent on an intracellular antioxidant system such as GSH. Furthermore, the effects of morphine on SIN-1-induced cytotoxicity were prohibited by pretreatment with the G_i protein inhibitor, pertussis toxin, and the phosphatidylinositol 3-kinase (PI3 kinase) inhibitors, wortmannin and LY294002. Taken together, these results suggest that morphine may protect primary rat astrocytes from apoptosis by NO species via the signaling cascades that involve both G protein and PI3 kinase.

Keywords: Morphine; Astrocytes; Peroxynitrite; Nitric oxide; Apoptosis; PI3 kinase; Pertussis toxin

1. Introduction

Morphine and endogenous opioid ligands are implicated in diverse functions, from development to immune modulation in the central and peripheral nervous system [1,2]. These functions are mediated mostly via specific opioid receptors, uniquely localized in different regions and types of brain cells of which there are three major types, namely mu, delta, and kappa [3]. These receptors are coupled with *Bordetella pertussis* toxin-sensitive G proteins, which also modulate adenylyl cyclase, the voltage-gated Ca^{2+} channel, and K^+ conductance [4]. Opiates also modulate the viability of neuronal and glial cells via an opioid receptor-mediated mechanism [5]. Mechanisms underlying an effect of opioid on CNS are known to be mediated via immune mediators, such as cytokines, beta-chemokines, reactive oxygen intermediates (ROI), and NO, which are produced by activated glial cells including microglia and astrocytes.

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Abbreviations: $ONOO^-$, peroxynitrite; NO, nitric oxide; PI3 kinase, phosphatidylinositol 3-kinase; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; DMEM, Dulbecco's modified Eagle's medium; MTT, methylthiazol-2-yl-2, 5-diphenyl, tetrazolium bromide; and BSO, L-buthionine-[*S, R*]-sulfoximine.

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Astrocytes regulate synthesis and release of a variety of neuropeptides and growth factor peptides, which in turn function on neural or glial cells. These cells are ubiquitous in the brain and more resistant to oxidative stress than oligodendrocytes or neurons. However, neurons may undergo degenerative changes when astrocytes damaged by oxidative stress do not generate sufficient neuropeptides and nerve growth factors. Recently, it has been reported that longer exposure to peroxynitrite ($ONOO^-$), a reactant of NO with superoxide anion (O_2^-), increases astrocyte death [6]. Hydrogen peroxide (H_2O_2) also induces the apoptosis of cultured primary astrocytes [7]. The cytotoxic effect of NO remains elusive, although it has been postulated that NO cytotoxicity might be mediated by peroxynitrite [8–10]. Peroxynitrite is a strong oxidant that damages subcellular organelles, membranes, and enzymes through nitration of proteins, lipid peroxidation, and direct breakage of DNA. In our experimental model, primary astrocytes underwent nuclear shrinkage, chromatin condensation, and nuclear fragmentation in reactive nitrogen intermediates (RNI)-induced astrocyte death. It has recently been reported that NO is involved in the antinociceptive effects of multiple opioid receptor agonists, which represents a possible interaction between NO and opioid systems [11]. Singhal *et al*. [12] have demonstrated that morphine enhances apoptotic death of macrophages through NO generation. However, Meriney *et al*. [13] have reported the possibility that morphine delays the neuronal cell death of avian ciliary ganglion through an inhibition of neurotransmission. In this study, we examined the effects of morphine and opioids on the free radical-induced death of rat primary astrocytes.

2. Materials and methods

2.1. Animals and reagents

Pregnant Sprague–Dawley rats were obtained from the Korean Experimental Animal Center. DMEM, fetal bovine serum, glutamine, gentamycin, penicillin, and streptomycin were purchased from GIBCO BRL. Culture flasks were bought from Falcon Co. and slide chambers were from Nunc Inc. MTT, BSO, SNP, pertussis toxin, and Hoechst 33258 were purchased from the Sigma Chemical Co., while SIN-1 was from Biomol. Sodium peroxynitrite was purchased from Cayman Co. Wortmannin and LY294002 were obtained from Calbiochem Co., morphine–HCl was bought from GeukDong Pharmaceutics Co., and naloxone–HCl was obtained from SangJin Pharmaceutics Co.

2.2. Culture of primary rat astrocytes

Primary rat astrocytes were isolated from the cerebral cortex of 1- to 2-day-old Sprague–Dawley rats as described elsewhere [14,15]. The cerebrum was dissected and stripped of the meninges in calcium-free Hanks' balanced salt solution and transferred into 3 mL of 1% trypsin in PBS for 10 min at room temperature. Brain tissue was dissociated by mechanical chopping for 10–20 min and filtered through nylon meshes to obtain a single-cell suspension. Cells were sedimented at $200 \times g$ and resuspended in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, penicillin G (100 U/mL), and streptomycin (100 mg/mL). Cells were maintained in an atmosphere of 5% CO_2 and 95% humidified air at 37°. Medium was refreshed twice a week. After 10–12 days in culture, cells were isolated from microglia and oligodendrocytes by shaking for 12 hr in an orbital shaker (60 times/min). Shaking was repeated after a gap of one day, and cells in passages 2 and 3 were subjected to the experiments. Cells were plated at a density of 3×10^5 cells/well in poly-L-lysine-precoated 24-well plates to measure cell viability. We also confirmed that were consisted of >95% astroglia, as determined by immunofluorescent staining with antibodies for glial fibrillary acidic protein.

2.3. Cell viability test

Primary rat astrocytes (3×10^5 cells/well) were maintained in serum-free DMEM for 1 hr and pretreated with morphine and naloxone, followed by treatment with SNP or SIN-1 for 24 hr. MTT (5 mg/mL), one-tenth of the original culture volume, was added to each culture, incubated for 3 hr at 37°, 5% CO_2 incubator, and the supernatant then aspirated off. The purple formazan formed by viable cells was lysed by the addition of DMSO and absorbance then measured at 540 nm by using a microplate reader (Titertek Multiskan). Results were expressed as means (%) ± SD of three different experiments.

2.4. Fluorescent staining of nuclei

The nuclei of primary rat astrocytes were stained with chromatin dye, Hoechst dye 33258. Cells were fixed in PBS containing 4% formalin for 30 min at room temperature, washed twice with PBS, and then reacted with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. After three washes, the samples were observed under a fluorescence microscope (Leica MPS 60) with excitation at 360 nm.

2.5. Genomic DNA extraction and electrophoresis

DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis. Genomic DNA was isolated by the Wizard Genomic DNA purification kit (Promega). After ethanol precipitation, 50 μg of DNA in each experimental group was subjected to electrophoresis on 1.5% agarose at 50 V for 3 hr. DNA was visualized by staining with ethidium bromide.

2.6. Statistical analysis

Data collected were expressed as means ± SD (%). Statistical analysis was performed by the Student's *t*-test to express the difference between two groups. * $P < 0.05$, significantly different from the control. Results with $P < 0.01$ were considered as statistically significant.

3. Results

3.1. Protective effects of morphine in NO- and peroxynitrite-induced cell death of primary rat neonatal astrocytes

We investigated whether morphine might affect the viability of rat neonatal primary astrocytes via addition of the peroxynitrite donor SIN-1 (Fig. 1A). Cells (3×10^5/well) were maintained in serum-free DMEM for 1 hr and pretreated with various concentrations of morphine for 30 min, followed by treatment with 2.5 mM SIN-1 for 24 hr. Measuring mitochondrial activity in forming purple formazan by MTT determined cell viability. Morphine significantly increased cell viability of control cells from 60% to 92% at 100 μM, whereas SIN-1 decreased viability to 60% of control cells. SIN-1 is known to produce $ONOO^-$ by simultaneously releasing NO and superoxide anion (O_2^-) [16] and has generally been used as an $ONOO^-$ donor [16–18]. In our preliminary study, we confirmed that superoxide dismutase (SOD) and catalase inhibited SIN-1-induced cytotoxicity (from 55% to 85%) (data not shown). It is evident that SIN-1 did not form $ONOO^-$ in the absence of O_2^-. Because peroxynitrite has a short half-life per se and is degraded in a few seconds, we chose SIN-1, which continuously releases $ONOO^-$. We also tested authentic peroxynitrite in primary rat astrocytes. When cells were exposed to sodium peroxynitrite, astrocytes underwent cell death. The apoptotic changes in astrocytes by sodium peroxynitrite became apparent at 72 hr after treatment. Morphine also completely blocked the apoptotic changes in cells by $ONOO^-$ (data not shown). We then tested for the effect of morphine on the NO-mediated death of rat neonatal astrocytes (Fig. 1B). Treatment with the NO donor, SNP, decreased cell viability to 80% of control cells. Since SNP breaks down to NO and $Fe(CN_5)_2^-$, it is possible that SNP kills astrocytes by a process unrelated to NO. To determine whether SNP toxicity could involve $Fe(CN_5)_2^-$, we applied $K_4[(Fe(CN)_6)$ at concentrations up to 3 mM under identical exposure conditions and found no significant cytotoxic effects in astrocytes. Next, we evaluated the effect of morphine on the NO-induced death of primary rat astrocytes. Morphine significantly rescued cells from SNP-induced damage in a dose-dependent manner. The protective effect of morphine reached a maximum at 100 μM, i.e. 93% viability of control in cells treated with SNP. The pure NO releasers *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were also tested for comparison. These donors also decreased viability at a higher concentration (0.4 mM) to 85 and 84%, respectively, while morphine also completely inhibited this cytotoxicity (Fig. 1C). These data indicated that NO and peroxynitrite induced death of rat neonatal astrocytes, a phenomenon which morphine significantly protected against.

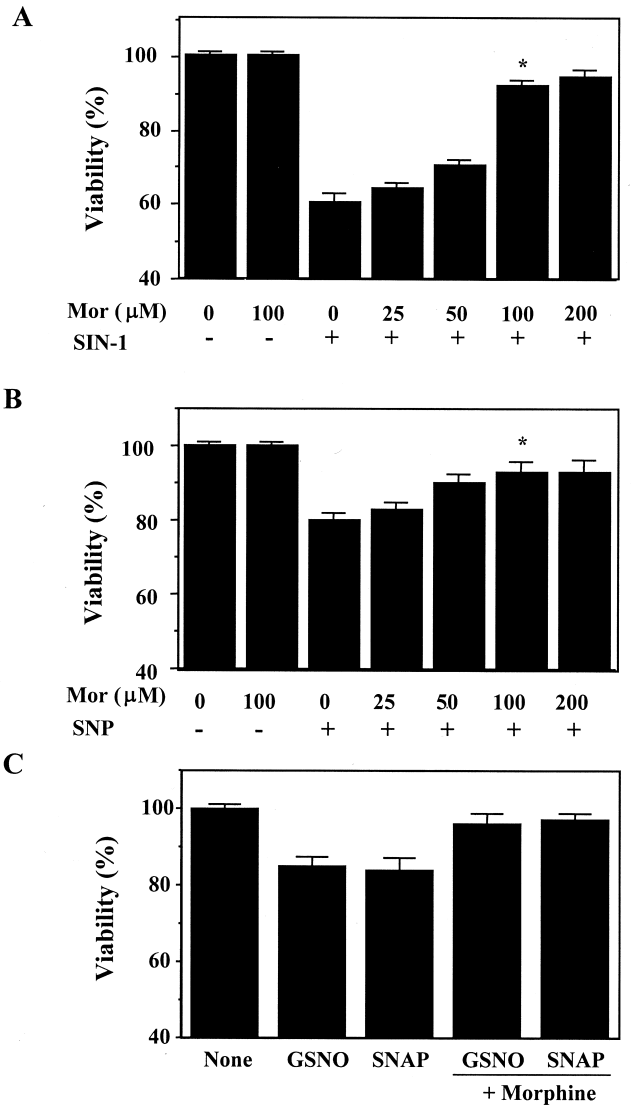

Fig. 1. Morphine protected primary rat astrocytes from NO species-induced death in a dose-dependent manner. Cells (3×10^5 cells/well) were washed, refreshed with serum-free DMEM for 1 hr, and pretreated with various concentrations of morphine (Mor) for 30 min, followed by stimulation with 3 mM SNP (A) and 2.5 mM SIN-1 (B) for 24 hr. Cells were also stimulated with 0.4 mM GSNO and 0.4 mM SNAP (C) for 24 hr after pretreatment with 100 μM morphine for 30 min. Cell viability was measured by the MTT assay. Results are represented as means (%) ± SD of three different experiments. * Statistically significant differences from values of SIN-1 alone and SNP alone at $P < 0.05$.

To further examine the protective effects of morphine on other types of cells, we tested the viability of various cells, including C6 glioma, RAW 264.7, and HL-60 cells after treatments with SNP (1 or 2 mM) and morphine (100 μM)

Table 1
Morphine did not protect against death of various cells, including C6 glioma, RAW 264.7, and HL-60 cells by donors

Cell lines	Viability (%)		
	None	SNP	Mor + SNP
C6 glioma	100 ± 0.5	70 ± 1.4	65 ± 2.1
RAW 264.7	100 ± 0.6	45 ± 1.7	40 ± 2.5
HL-60	100 ± 0.6	60 ± 1.5	50 ± 1.2

Various cells including C6 glioma, RAW 264.7, and HL-60 cells (3×10^5/well) were pretreated with 100 mM morphine for 30 min, followed by treatment with 1 mM SNP in C6 glioma, RAW 264.7, 2 mM SNP in HL-60 cells for 24 hr. The viability of the cells was measured by the MTT assay described previously. Results represented as means (%) ± SD of three different experiments.

for 24 hr. In contrast to the primary rat astrocytes, morphine did not protect SNP-induced cytotoxicity in other cell types (Table 1). For example, in the HL-60 human leukemia cell line, morphine seemed to increase SNP-induced cytotoxicity (2 mM). The viability of PC12 cells, a rat pheochromocytoma cell line, was also not affected by morphine in the presence of SIN-1 (2.5 mM) (data not shown).

3.2. Antagonistic inhibition of naloxone on the protective effects of morphine in SIN-1-induced death of primary rat astrocytes

To investigate whether the protective effect of morphine on SIN-1-induced cytotoxicity is specific to the opioid receptor, we examined the antagonistic effects of naloxone, an antagonist of opioid receptors, in astrocytes (Fig. 2). Naloxone did antagonize the protective effect of morphine on SIN-1-induced cytotoxicity from 92% to 60%, whereas naloxone alone did not affect SIN-1-induced death of primary rat astrocytes. Cell viability was not affected by a single treatment of either morphine or naloxone. In a similar fashion to Fig. 1A, treatment of cells with SIN-1 markedly decreased viability to 58% of control cells, and morphine (100 μM) significantly recovered cells from SIN-1-induced cytotoxicity (92%). Naloxone was able to antagonize the protective effects of morphine in the presence of SIN-1 at 20–50 μM. The antagonistic effect of naloxone reached a maximum at 50 μM.

3.3. Inhibition of chromatin condensation and nuclear fragmentation by morphine in SIN-1-induced apoptosis of primary rat astrocytes

The nature of SIN-1-induced cell death, apoptosis or necrosis, was evaluated as necrotic and apoptotic death by genomic DNA fragmentation and Hoechst staining of the nucleus (Fig. 3). SIN-1 induced the apoptotic DNA fragmentation in a dose-dependent manner (Fig. 3A). Cells cultured on a slide chamber were stained with Hoechst 33258 (10 μM) and observed under a fluorescent microscope. Nuclei of cells were shown to be round and oval-shaped with homogenous intensity of Hoechst dye in control rat neonatal astrocytes (Fig. 3Ba). However, the nuclei of the cells became smaller, and heterogeneous shapes with highly condensed chromatin and few nuclear fragmentations represented a characteristic biochemical feature of apoptosis (Fig. 3Bb). When cells were pretreated with morphine, SIN-1-induced chromatin condensation was not observed, which was consistent with the protective effect of morphine on SIN-1-mediated cytotoxicity represented in Fig. 1B (Fig. 3Bc). Nuclear staining of naloxone-pretreated cells showed that the protective effects of morphine on SIN-1-induced apoptosis of rat neonatal astrocytes were antagonized by naloxone (Fig. 3Bd). Cells were also retarded in terms of outgrowth of cellular processes, with cell shrinkage and detachment from culture dishes undergoing apoptosis of astrocytes by SIN-1 (data not shown).

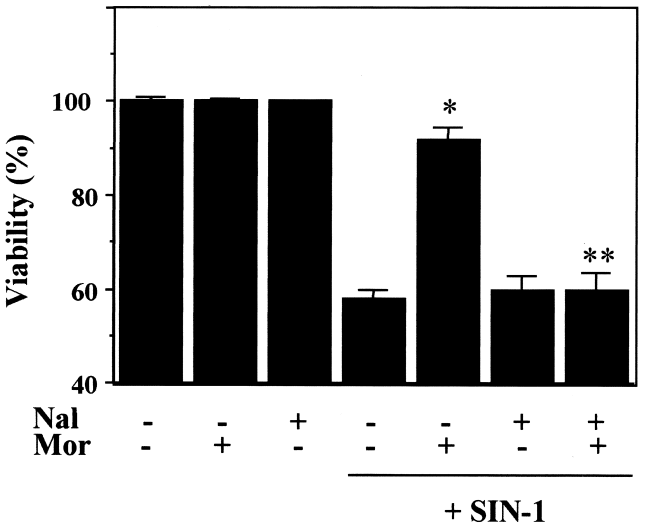

Fig. 2. Naloxone antagonized the protective effect of morphine on the SIN-1-induced death of primary rat astrocytes. Cells were pretreated with 50 μM naloxone (Nal) for 10 min before the addition of 100 μM morphine (Mor) and 2.5 mM SIN-1 for 24 hr. The MTT assay was performed as described previously. Results are presented as means (%) ± SD of three different experiments. Significance of the difference from the values of morphine and SIN-1 alone: *, $P < 0.01$ significantly different from SIN-1 alone; **, $P < 0.05$ compared with the morphine- and SIN-1-treated group.

3.4. Potential involvement of G protein, PI3 kinase, and GSH on the protective mechanism of morphine

To evaluate the protective mechanism of morphine on SIN-1-induced apoptosis of primary rat astrocytes, we examined the influence of GSH, a potent antioxidant. BSO is a potent and selective inhibitor of glutamyl cysteinyl ligase, the rate-limiting enzyme in the synthesis of GSH. Cells were treated with 100 μg/mL of BSO for 24 hr. After a refreshment of the medium, cells were further treated with morphine (100 μM) for 30 min before incubation with or

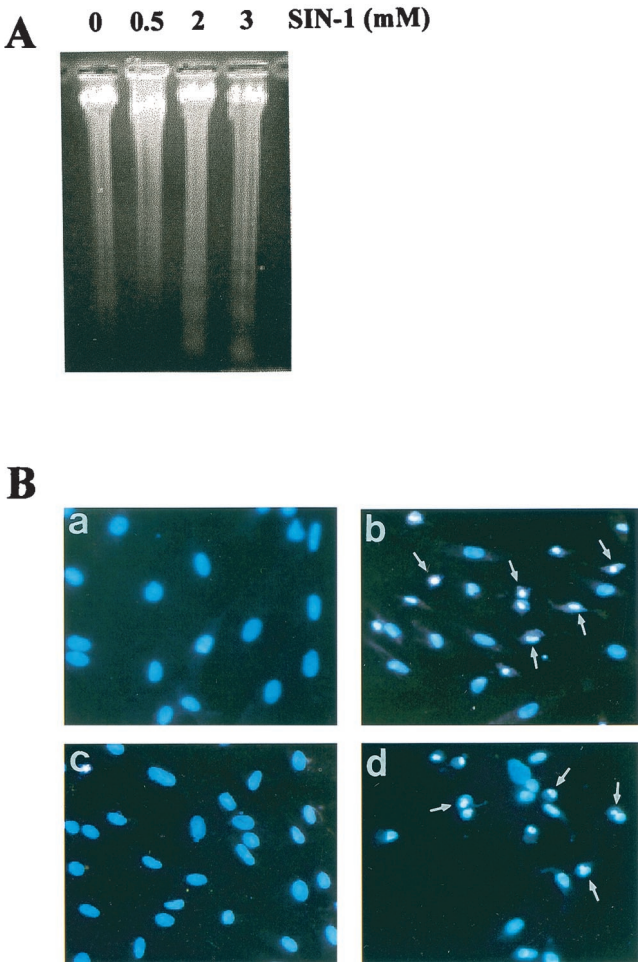

Fig. 3. Morphine inhibited the SIN-1-induced DNA fragmentation and chromatin condensation of primary rat astrocytes. (A) Cells (2×10^7) were treated with various concentrations of SIN-1 for 24 hr at 37° in a humidified incubator with 5% CO_2. DNAs (50 μg/each lane) were isolated from astrocytes and separated on 1.5% agarose gel. (B) Control cells (a) were treated with 2.5 mM SIN-1 for 20 hr (b) and stained with Hoechst 33258 for observation under a fluorescent microscope (100 ×). Preincubation of SIN-1-treated cells with 100 μM morphine demonstrated the nuclear pattern of control cells by the roundness and homogenous intensity of fluorescent-stained chromatin (c). Naloxone (50 μM) was added for 10 min before treatment with morphine and SIN-1 (d). Arrows indicate fragmented cells.

without 2.5 mM SIN-1 for 24 hr (Fig. 4). BSO alone did not affect the viability of primary rat astrocytes, although the combination of BSO and SIN-1 severely damaged cells, viability being less than half in SIN-1-only-treated cells. Morphine attenuated SIN-1-induced apoptosis from 60% to 90% of control cells. Morphine also recovered the viability of SIN-1-treated cells from 40 to 85%, even though an intracellular antioxidant, GSH, was depleted by the addition of BSO.

We also tested the possible involvement of PI3 kinase and G protein in the signaling pathway of morphine-mediated protection from SIN-1-induced cytotoxicity in primary rat astrocytes (Fig. 5). When cells were pretreated with 100 ng/mL of pertussis toxin, a G_i protein inhibitor, for 20 hr,

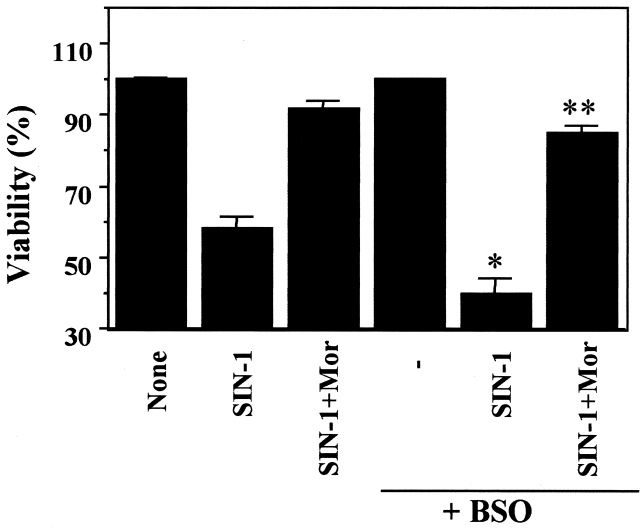

Fig. 4. Morphine rescued GSH-deprived primary rat astrocytes from SIN-1. Cells were pretreated with 100 μg/mL of BSO for 24 hr, washed twice with serum-free DMEM, and treated with 100 μM morphine (Mor) for 30 min before incubating with or without 2.5 mM SIN-1 for 24 hr. The MTT assay was performed to measure cell viability as described previously. Results are presented as means (%) ± SD of three different experiments. *, $P < 0.01$, **, $P < 0.05$ compared with the SIN-1-treated group.

the protective effect of morphine (92%) was significantly inhibited by pertussis toxin (74%). Treatment of cells with pertussis toxin alone did not affect the viability of cells. Next, we assessed the role of specific PI3 kinase inhibitors, wortmannin (100 nM) and LY294002 (10 μM), on the protective effect of morphine on SIN-1-induced apoptosis

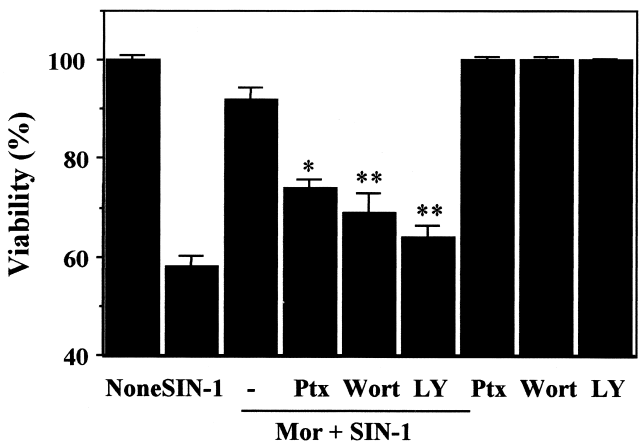

Fig. 5. The protective mechanism of morphine was mediated by the signaling pathway of G protein and PI3 kinases in SIN-1-induced death of primary rat astrocytes. Cells (3×10^5/well) were maintained in serum-free DMEM for 1 hr and pretreated with PI3 kinase inhibitors, including 100 nM wortmannin (Wort) and 10 μM LY294002 (LY) for 30 min, followed by treatment with 100 μM morphine and 2.5 mM SIN-1 for 24 hr. Pertussis toxin (Ptx, 100 ng/mL) was pretreated for 20 hr before the addition of morphine and SIN-1. The viability of cells was measured by the MTT assay described previously. Results are presented as means (%) ± SD of three different experiments. *, $P < 0.01$, **, $P < 0.05$ compared with the SIN-1- and morphine-treated group.

of cells pretreated with PI3 kinase inhibitors for 30 min, followed by treatment with 100 μM morphine and 2.5 mM SIN-1. As shown in Fig. 5, two kinds of PI3 kinase inhibitors markedly abrogated the protective effect of morphine on SIN-1-induced cell deaths. From these results, it could be postulated that morphine rescued primary rat astrocytes from SIN-1-induced cytotoxicity by a signaling mechanism involving both G protein and PI3 kinase.

4. Discussion

We have demonstrated that morphine protected against the death of primary rat neonatal astrocytes by NO-related free radicals, including NO and peroxynitrite (Fig. 1, A and B). Astrocytes are known to be less susceptible than neuronal cells to the cytotoxic effect of peroxynitrite. However, longer exposure to peroxynitrite leads to the death of primary rat astrocytes [6]. In our experimental model, primary rat astrocytes underwent DNA fragmentation, nuclear shrinkage, and chromatin condensation upon exposure to SIN-1 (Fig. 3). A high concentration of NO also induced apoptotic fragmentation. SIN-1-induced apoptosis of primary rat astrocytes was significantly protected by a high concentration of morphine (100 μM). This protective effect of morphine was completely blocked by the addition of naloxone, an opioid antagonist (Fig. 2). However, the effect of morphine was not observed in other cell types, including C6 glioma, RAW 264.7, and HL-60 cells (Table 1), as well as PC12 cells (data not shown). The fact that astrocytes and other cell types commonly expressed the mu (3) receptor of morphine indicates that the protective effect of morphine may not be mediated by mu (3) receptors. However, other types of mu-opioid receptors are needed to clarify whether they are different from other mu (3) receptors in mediating signals of cell survival. Morphine enhances apoptosis of various cells including macrophages [12], splenocytes [19], and kidney fibroblasts [20]. These results were consistent with our results for C6 glioma, RAW 264.7, and HL-60 cells. In human neuroblastoma SK-N-SH cells [21], etrophine, a wide-spectrum and potent agonist of opioid receptors, induced apoptosis, whereas other agonists of opioid receptors such as morphine, [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAGO), dynorphin A, nociceptin/orphanin FQ, and [D-Pen2, D-Pen5]-enkephalin (DPDPE) did not. It has been suggested that the effect of opioid agonists on the viability of brain cells is dependent on their types, because even though the same opioid receptor system is used, various opioid agonists may not use the same opioid receptor system to mediate the signals affecting cell viability. On the other hand, Hauser *et al*. [22] have reported that morphine inhibits astroglial growth by activating mu-opioid receptors. However, they treated the cells with 1 μM morphine for 72 hr, whereas the concentration of morphine and the duration of treatment were different in our experimental model. In this study, a low concentration of morphine (1 μM) did not protect astrocytes against peroxynitrite and NO (data not shown). The concentration of morphine (25–200 μM) that protected astrocytes in this experiment is outside the clinically important range. However, high concentrations of opioid are obtained in cerebrospinal fluid (CSF) after epidural or intrathecal administration of opioids in clinical fields, whereas plasma concentration obtained after systemic administration of morphine is at most 1–3 μM [23,24]. The initial CSF concentrations of morphine following intrathecal administration are high in the micromolar range, and those after epidural administration are about 10 μM. Based on these results, a high concentration of morphine may affect the various functions, including viability, of cells in an *in vivo* system. In addition, Meriney *et al*. reported that morphine delayed neuronal cell death of avian ciliary ganglion through a competitive inhibition of neurotransmission of exogenous and endogenous opioid peptides [25].

Astrocytes are known to utilize an effective antioxidant system to protect themselves and other adjacent brain cells against injury from reactive free radicals and oxidants. In particular, GSH is generated at higher levels in astrocytes than in neurons [26] or oligodendrocytes [27] in the adult brain. Depletion of glutathione by BSO, an inhibitor of γ-glutamylcysteine synthetase, in astrocytes increased susceptibility to injury by peroxynitrite (Fig. 4). BSO, a relatively specific inhibitor of glutathione biosynthesis [28], has frequently been used to manipulate the level of glutathione both *in vitro* and *in vivo* [29]. Pretreatment with 100 μM BSO almost completely depleted the GSH of astrocytes to below 20% [18]. However, morphine did rescue astrocytes from peroxynitrite-induced cell death under the depletion of the intracellular antioxidant, GSH (Fig. 5). It has been reported that intracellular GSH content can be modulated by morphine [30]. Systemic morphine had no effect on GSH concentrations in selected brain areas, whereas intracerebroventicular (ICV) morphine resulted in acute GSH depletion in the cerebrospinal fluid of cancer patients [30]. In our preliminary study, we also found that antioxidants, 5 mM GSH and *N*-acetylcysteine, inhibited the SIN-1-induced cytotoxicity of rat primary astrocytes from 55% to 78% viability, even though GSH was depleted by BSO. Morphine also inhibited the cytotoxicity of $ONOO^-$ in GSH-depleted cells to 85%. From these results, we suggest that morphine might compensate for GSH depletion, as like other antioxidant system. Moreover, GSH contents were maintained in the presence of morphine with SIN-1, consistent with the trend in viability.[1]

Opioid receptors have recently been cloned, sequenced, and found to encode highly homologous proteins with a primary structure typical of G protein-coupled membrane receptors [31–33]. G protein is involved in receptor–second messenger coupling [34] and plays an important role in clinical and pharmacological research. Pertussis toxin sta-

[1]Kim MS, Lee KM, Cheong YP, Son Y, Park RK. Unpublished data.

bilizes G_i protein in an inactive state by blocking its coupling with receptors. The protective effect of morphine on SIN-1-induced cytotoxicity was inhibited by pertussis toxin (Fig. 5). Our data are consistent with the report of Childers *et al*. [35], which demonstrated that opioid agonist inhibits adenylyl cyclase through the coupling of the receptor to a $G_{0/i}$ pertussis-sensitive protein.

Pretreatment of astrocytes with PI3 kinase inhibitors, including wortmannin and LY294002, abrogated the effect of morphine on SIN-1-induced cell death, indicating that the effects of morphine were in part mediated by PI3 kinase. The $G\beta\gamma$ subunit of G protein served as an independent activator of different effector pathways that require a PI3 kinase activity as an early step in the signaling cascade [36–38]. Recently, it was suggested that PI3 kinase is critical for the survival of several cell types including neurons, fibroblasts, and oligodendrocytes [39–42].

Recently, Franke and Cantley [43] suggested that Akt is a Bad kinase, stimulating explanations for the role of PI3K and Akt in cell survival. Activated Akt phosphorylated the proapoptotic factor Bad on a serine residue, resulting in its dissociation from Bcl-X_L and released Bcl-X_L, which was then capable of suppressing death pathways in cells. It was also reported that astrocytes over-expressing bcl-2 had elevated glutathione levels and activities of superoxide dismutase as well as glutathione peroxidase. Another possibility which might affect the survival signaling of morphine is that some effects of opioids involve cytokine-like interactions with glial cells in the CNS. Cytokine-like effects of opioid may transmit the survival-signaling glial cells [44]. Recently, Kanesaki *et al*. [45] have shown that morphine prevents peroxynitrite-induced cell death through its direct scavenging action. Our results suggested that morphine protects primary rat astrocytes from oxidative stress of NO-related species via intracellular signaling cascades involving G protein and PI3 kinase. Work is currently in progress to investigate the actual mechanism of the protective roles of morphine in the apoptotic signaling pathway.

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