Inhibition of the NLRP3 Inflammasome Activation/Assembly through the Activation of the PI3K Pathway by Naloxone Protects Neural Stem Cells from Ischemic Condition

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Research Article

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Abstract

Naloxone is a well-known opioid antagonist and has been suggested to have neuroprotective effects in cerebral ischemia. We investigated whether naloxone exhibits anti-inflammatory and neuroprotective effects in neural stem cells (NSCs) injured by oxygen-glucose deprivation (OGD), whether it affects the NOD-like receptor protein 3 (NLRP3) inflammasome activation/assembly, and whether the role of the phosphatidylinositol 3-kinase (PI3K) pathway is important in the control of NLRP3 inflammasome activation/assembly by naloxone. Primary cultured NSCs were subjected to OGD and treated with different concentrations of naloxone. Cell viability, proliferation, and the intracellular signaling proteins associated with the PI3K pathway and NLRP3 inflammasome activation/assembly were evaluated in OGD-injured NSCs. OGD significantly reduced survival, proliferation, and migration and increased apoptosis of NSCs. However, treatment with naloxone significantly restored survival, proliferation, and migration and decreased apoptosis of NSCs. Moreover, OGD markedly increased NLRP3 inflammasome activation/assembly and cleaved caspase-1 and interleukin-1β levels in NSCs, but naloxone significantly attenuated these effects. These neuroprotective and anti-inflammatory effects of naloxone were eliminated when cells were treated with PI3K inhibitors. Our results suggest that NLRP3 inflammasome is a potential therapeutic target and that naloxone reduces ischemic injury in NSCs by inhibiting NLRP3 inflammasome activation/assembly mediated by the activation of the PI3K signaling pathway.

Introduction

With the increase in stroke incidence and decrease in mortality associated with stroke, patients with long-term neurological complications due stroke are on the rise [1]. Early restoration of perfusion after ischemia can reduce the damage from a stroke, but the efficacy of reperfusion is limited by several secondary injury mechanisms, including inflammation, oxidative stress, and apoptosis [2]. Increasing clinical and experimental evidence suggests that inflammation plays a major role in secondary injuries [3]. Stroke patients with systemic inflammation exhibit poor clinical outcomes [4–6]. Research shows that pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1, and IL-6, appear to exacerbate brain injury after stroke [7]. Recently, several studies have emphasized that the NOD-like receptor protein 3 (NLRP3) inflammasome is responsible for cellular damage and mediates the inflammatory response to aseptic tissue injury following cerebral ischemia [8, 9].

The NLRP3 inflammasome mediates caspase-1 activation and secretion of the pro-inflammatory cytokines IL-1β/IL-18 and is a critical component of the innate immune system [10]. In animal stroke studies, inhibition of the NLRP3 inflammasome reduced pro-inflammatory cytokines, resulting in attenuation of cerebral infarction, edema, hemorrhagic transformation, and functional deficit [11]. Therefore, blocking or inhibiting the NLRP3 inflammasome may offer substantial promise to relieve neurological exacerbation or poor prognosis during ischemic stroke. Endogenous neural stem cells (NSCs), which reside in the central nervous system, have been shown to replace lost neurons in models for numerous disorders, including cerebral ischemia, due to their regenerative capacity [12, 13]. Endogenous NSCs can also be damaged, and loss of their function could occur due to inflammatory

processes after ischemic stroke. Therefore, finding methods to protect NSCs from ischemic injury and associated inflammation is essential to reducing neurological sequelae after ischemic stroke.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway has been extensively studied as one of the major cell survival pathways that can prevent ischemic brain injury [14, 15]. The PI3K/Akt pathway is also important for NSC survival and is known to affect endogenous neurogenesis [16]. Therefore, various drugs have been investigated to upregulate the PI3K pathway and enhance its neuroprotective effects after cerebral infarction [17, 18]. Naloxone is an opioid receptor antagonist that is widely used for treatment in opioid overdose [19]. Previous clinical data has also demonstrated the beneficial effects of naloxone against acute ischemic stroke [20, 21]. While its mechanisms of action are not yet understood, several mechanisms have been proposed and the anti-inflammatory effects of naloxone have been reported in several previous studies, focusing on the mu opioid receptors [22], the L-type calcium channels [23], and the PI3K pathway [24]. A previous *in vitro* study showed that naloxone was involved in the transcription and assembly of the NLRP3 inflammasome in activated THP-1 cells [25]. However, its neuroprotective and anti-inflammatory effects against ischemic injury have not yet been reported in NSCs.

In this study, the effect of oxygen-glucose deprivation, a well-known pathogenic mechanism of cerebral infarction, on NSCs was examined [26]. The neuroprotective and anti-inflammatory effects of naloxone in NSCs damaged by oxygen-glucose deprivation (OGD) were also investigated, as well as whether it has any effect on NLRP3 inflammasome activation/assembly and the PI3K pathway, and what kind of relationship exists between the NLRP3 inflammasome and the PI3K pathway.

Materials And Methods

Culture of NSCs and hNSCs

All animal-related procedures were performed in accordance with the Hanyang University Guidelines for the Care and Use of Laboratory Animals and approved by the Hanyang University Institutional Animal Care and Use Committee (IACUC). Every effort was made to minimize the number of animals used and the pain caused. All animals were used only once.

A previously published protocol was followed for primary culture of embryonic NSCs [27–30]. NSCs were obtained from embryonic rat cortexes on days 13–14. Embryos were transferred to 100-mm petri dishes containing ice-cold Hank's balanced salt solution (HBSS; 0.3 mM Na_2HPO_4, 5.4 mM KCl, 137 mM NaCl, 2.5 mM HEPES, 5.6 mM glucose, and 0.4 mM KH_2PO_4) (Gibco, Frederick, MD, USA) and washed 4–5 times using the same buffer. Single NSCs were isolated from the lateral ganglionic eminence, ventral midbrain, and cerebral cortex of the embryos. The cells were moved to new petri dishes pre-coated with poly-L-ornithine (Sigma, St. Louis, MO, USA) and fibronectin (Gibco) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS, Gibco). The cells were then cultured in N2 medium (DMEM/F-12 (Gibco), 100 mg/L transferrin (Prospec, Rehovot, Israel), 25 mg/L insulin (Gibco), 100 μM putrescine (Sigma), 30 nM selenite

(Sigma), 0.2 mM ascorbic acid (Sigma), 20 nM progesterone (Sigma), 8.6 mM D(+) glucose (Sigma), 2 nM L-glutamine (Sigma), and 20 nM $NaHCO_3$ (Sigma), to which basic fibroblast growth factor (bFGF;10 ng/mL, Gibco) was added.

Human neural stem cells (hNSCs; N7800-100, Gibco) were derived from National Institute of Health (NIH) approved H9 (WA09) human embryonic stem cells (hESCs). hNSCs were cultured in StemPro® NSC SFM complete medium (1× KnockOut™ DMEM/F12 (Gibco), 2 mM GlutaMax™-I supplement (Gibco), 20 ng/mL bFGF (Gibco), 20 ng/mL EGF (Gibco), and 2% StemPro® Neural Supplement (Gibco)). The cells were cultured in plates pre-coated with CeLLstart™ (1:100 dilution in Dulbecco's phosphate-buffered saline (D-PBS, Gibco) containing Ca^{2+}/Mg^{2+}, Gibco).

Culture medium was replaced every 2–3 days and the cultures were maintained at 37°C with 5% CO_2 in a humid environment.

Exposure Of Nscs And Hnscs To Ogd And Treatment With Naloxone

Cells were subjected to OGD to evaluate its effects on their properties. All OGD experiments were performed using a modular incubator chamber (Billups-Rothenberg, Inc., San Diego, CA, USA), and a gas mixture containing 5% CO_2, 5% H_2, and N_2 balance was flushed into the chamber for 0–24 h [31–33].

Naloxone was dissolved in distilled water to obtain a concentration of 10 mM, diluted to 100 μM, and separated into aliquots in sterile microtubes. To determine the effects of naloxone on NSCs, the cells were treated with 0, 0.001, 0.01, 0.1, 1, 10, or 100 μM naloxone for 24 h.

NSCs and hNSCs were treated with naloxone for 16 h following OGD to evaluate whether different concentrations of naloxone (0, 0.001, 0.01, 0.1, 1, or 10 μM) could rejuvenate NSCs damaged by OGD. In addition, NSCs were treated with naloxone during 1 h of OGD, to determine the early effects of naloxone on the activation of the PI3K pathway (PI3K, pAkt, and pGSK-3β) [34, 35]. To investigate inflammasome signaling, cells were treated with naloxone for 6 h of OGD.

To investigate whether the PI3K pathway is a crucial mediator of the effects of naloxone against OGD in NSCs, cells were also treated with 10 μM LY294002 (Sigma) or 50 nM wortmannin (Sigma), a PI3K inhibitor, 1 h before exposure to various conditions.

Trypan Blue Staining (Tbs) And Lactate Dehydrogenase (Ldh) Assays

NSCs and hNSCs were treated with different doses of naloxone and OGD, briefly washed twice with D-PBS, and replaced with fresh medium. The cells were then stained using a trypan blue solution (Gibco) for 2 min. Following staining, a cell count was carried out using a hemocytometer [36].

Cytotoxicity due to LDH-releasing cells was evaluated using an LDH cytotoxicity detection kit (Takara, Shiga, Japan). NSCs and hNSCs treated with naloxone and OGD were centrifuged at 200 × *g* for 10 min, after which the supernatant was transferred to a new plate. The supernatant was added to colorimetric solutions, and the mixture was incubated in dark according to the manufacturer's instructions. Cytotoxicity was detected using an enzyme-linked immunosorbent assay (ELISA) plate reader following 30 min of incubation (Synergy H1 Hybrid reader, BioTek, Winooski, VT, USA) at 492 nm and 690 nm.

Terminal Deoxynucleotidyl Transferase-mediated Deoxyuridine Triphosphate Nick-end Labeling (Tunel) Staining

Apoptotic cells exposed to naloxone and OGD for 16 h were measured using TUNEL staining (Roche Boehringer-Mannheim, Indianapolis, IN, USA) as per the manufacturer protocol. NSCs treated with OGD and naloxone were fixed with 4% paraformaldehyde in D-PBS for 1 h. Fixed cells were then incubated with 3% H_2O_2 in methanol. After 10 min, the cells were washed with D-PBS and incubated with 0.5% Triton X-100 in 0.1% sodium citrate. After 2 min, the TUNEL-stained cells were washed 2–3 times with D-PBS for 2 min to monitor condensed, intact, and fragmented nuclei. The cells were then mounted with DAPI mounting solution (Vector Laboratories Inc., Burlingame, CA, USA) and observed under a fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan).

Flow Cytometry Using Annexin V And Propidium Iodide (Pi)

To evaluate the degree of necrosis, apoptosis, and dead cells in NSCs, the FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson, Franklin Lakes, NJ, USA) was used. NSCs treated with naloxone and OGD were washed several times with PBS and transferred to microtubes using a cell scraper. Cells were then centrifuged at 300 × *g* for 5 min, and the pellets were saved. The pellets were resuspended in annexin V binding buffer and mixed with annexin V and PI, as per the manufacturer's protocol. Following gentle mixing, the mixture was placed in the dark for 15 min, and annexin V binding buffer was added to each sample. The samples were analyzed using a flow cytometer (Accuri C6 flow cytometer, Becton Dickinson).

Immunostaining For Nestin And Ki67

To measure the effects of naloxone and OGD on the characteristics of NSCs, immunostaining was performed to detect nestin and KI67. NSCs treated with OGD and naloxone were fixed with 2% paraformaldehyde in D-PBS for 15 min. Fixed cells were then treated with 0.5% Triton X-100 in D-PBS for permeabilization. After 5 min, the permeabilized cells were washed several times with D-PBS, and 3% H_2O_2 was added to block endogenous peroxidase activity. After 20 min, the cells were then blocked with 5% normal serum in D-PBS for 1 h and incubated overnight with a specific primary antibody at 4°C. The following antibodies were used in these experiments: anti-nestin (1:100, Abcam, Burlingame, CA, USA)

and KI67 (1:100, Abcam). The cells were washed several times and processed using the appropriate tetramethylrhodamine goat anti-rabbit IgG (H + L) (Life Technologies, Carlsbad, CA, USA) and goat anti-mouse Alexa Fluor 488 (Life Technologies) secondary antibodies for 1 h. The cells were then washed, mounted with DAPI mounting solution (Vector Laboratories Inc.), and analyzed using a fluorescence microscope.

Brdu Cell Proliferation And Colony-forming Unit (Cfu) Assays

A BrdU labeling and detection kit (Roche Boehringer–Mannheim) was used to assess the effects of naloxone and OGD on the proliferation of NSCs. NSCs treated with naloxone and OGD were labeled with 10 μM BrdU, as per the manufacturer's instructions. Following 16 h of incubation, the cells were fixed with a fixing solution for 30 min. An additional anti-BrdU-POD working solution was added to the cells and incubated for 2 h in dark. The cells were then washed three times with washing solution and reacted with the substrate for 5 min in dark. The proliferation of NSCs was measured using an ELISA plate reader at 370 nm and 492 nm.

In addition, a CFU assay was conducted based on a previously published protocol [37] to confirm the proliferation of NSCs. NSCs were seeded in pre-coated 60-mm plates at 7 x 10^3 cells/cm^2 and treated with different doses of naloxone and subjected to OGD. The treated cells were then washed with D-PBS and transferred to a culture medium. Following culture for 14 days, the cells were washed again and stained with 0.5% crystal violet (Sigma) in methanol for 30 min. After staining, the cells were washed several times with D-PBS for 10 min and allowed to air-dry. About 300–500 stained colonies were counted.

Migration Assay

The QCM Chemotaxis Cell Migration Assay Kit (Chemicon, Temecula, CA, USA) was used to measure cell migratory capacity. The assay was performed using a 24-well plate with an 8 μm pore size with colorimetric detection. NSCs were seeded in the upper chamber, and different concentrations of naloxone were added to the lower chamber. The cells were then exposed to OGD. The cells that had migrated through the polycarbonate membrane were incubated with cell staining solution as per the manufacturer's instructions. The cells were then extracted and detected using an ELISA plate reader at 560 nm.

Western Blotting

The expression levels of key members of the PI3K pathway and the inflammasome in NSCs were analyzed using western blotting. NSCs treated with different doses of naloxone and OGD were washed with D-PBS and lysed with lysis buffer (RIPA II cell lysis buffer with Triton (1×), without EDTA (GenDEPOT, Katy, TX, USA), 1 mM sodium fluoride (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.5%

protease inhibitor cocktail 1× (GenDEPOT), and 1 mM sodium orthovanadate (Sigma)). After incubating on ice for 30 min, the cells were sonicated with Sonoplus (Bandelin Electronics, Berlin, Germany) and incubated on ice for another 30 min. The lysates were then centrifuged at 16,200 × *g* for 15 min, and the supernatant was transferred to a new microtube. The protein concentrations of the supernatant were calculated using a bicinchoninic acid solution (Sigma) and copper (II) sulfate solution (Sigma). Equal amounts of lysate protein were resolved by 4–12% sodium dodecyl sulfate-polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Ponceau S (Sigma) staining was performed for total protein normalization, but all bands looked similar, so normalization was performed using a housekeeping gene, GAPDH, for more accurate results. The membranes were then blocked with 2% skim milk. The blocked membranes were then incubated overnight with a specific primary antibody. The following antibodies were used in these experiments: anti-KI67 (1:200, Abcam), nestin (1:500, Millipore), PI3K (1:500, Cell Signaling Technology, Beverly, MA, USA), pAkt (Ser-473, 1:100, Cell Signaling Technology), Akt (1:1000, Cell Signaling Technology), pGSK-3β (Ser-9, 1:200, Cell Signaling Technology), GSK-3β (1:1000, Cell Signaling Technology), Bcl-2 (1:200, Cell signaling Technology), Bax (1:500, Cell signaling Technology), cleaved caspase-9 (1:200, Cell Signaling Technology), NLRP3 (2 µg/mL, Novus Biologicals, Littleton, CO, USA), cleaved caspase-1 (2 µg/mL, Novus Biologicals), IL-1β (0.4 µg/mL, Abcam), and GAPDH (1:4000, Cell Signaling Technology). The following day, membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the appropriate secondary antibodies: horseradish peroxidase-conjugated anti-rabbit antibody (1:2000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and anti-mouse antibody (1:2000, Jackson ImmunoResearch Laboratories, Inc.) for 1 h. The membranes were washed with TBST buffer, followed by West-Q chemiluminescent substrate plus kit (GenDEPOT) to detect the membranes. Protein bands were visualized using a biomolecular imager (ImageQuant LAS 4000, GE Healthcare, Little Chalfont, UK) and the images were quantified using an image analyzer (ImageQuant TL 7.0 image analysis software, GE Healthcare). Relative levels of pAKT and pGSK3β were normalized to the levels of AKT and GSK3β respectively. The expression levels of other key factors of the PI3K pathway and the inflammasome were normalized to GAPDH expression.

Statistical Analyses

All data is presented as mean ± standard deviation from three or more independent experiments. Statistical analyses of three or more datasets were performed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Two-tailed *p*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS 21.0 software package for Windows (SPSS Inc., Chicago, IL, USA).

Results

Effect of OGD and Naloxone on Viability, Cytotoxicity, and Proliferation of NSCs and hNSCs

NSCs were subjected to OGD for 4, 8, 16, or 24 h. TBS and LDH assays revealed that OGD significantly reduced NSC viability and increased cytotoxicity in a time-dependent manner (Fig. 1A, B). As cell viability was 70% after 16 h of OGD, this duration was considered optimal for subsequent experiments (Fig. 1A). TBS, LDH, and CFU assays revealed that naloxone alone at concentrations up to 100 μM did not influence NSC viability, cytotoxicity, and proliferation (Fig. 1C—F).

The effects of different concentrations of naloxone on NSCs subjected to OGD for 16 h were determined. NSC viability and cytotoxicity were assessed using TBS and LDH assays, respectively. Treatment with naloxone at 0.1 μM and 1 μM significantly increased NSC viability (Fig. 2A) and remarkably reduced cytotoxicity (Fig. 2B) compared to NSCs subjected to OGD alone.

To investigate the effects of naloxone on hNSCs damaged by OGD, hNSCs were subjected to OGD for 16 h and simultaneously treated with 0.001, 0.01, 0.1, and 1 μM naloxone. TBS and LDH assays were performed to evaluate the cell viability and cytotoxicity, respectively. Like the results in NSCs (Fig. 2), viability was increased (Fig. S1A), and cytotoxicity was decreased (Fig. S1B) in hNSCs treated with 0.01–1 μM naloxone compared to hNSCs treated with OGD alone. Thus, naloxone is expected to exhibit similar neuroprotective effects in humans. Since obtaining large amounts of hNSCs to proceed with the experiments was difficult, subsequent experiments were performed using NSCs.

Effect Of Naloxone On Apoptosis And Cell Death Of Nscs Damaged By Ogd

TUNEL staining was performed to evaluate the effect of naloxone on the apoptosis of NSCs. TUNEL-positive cells increased after OGD but were reduced by treatment with naloxone at concentrations up to 10 μM (Fig. 3A, B). Flow cytometry was performed with several antibodies to evaluate whether OGD and naloxone influence NSC death. Necrosis and apoptosis of NSCs were evaluated by flow cytometry, which confirmed that OGD significantly increased NSC death, while 0.1 μM and 1 μM naloxone attenuated OGD-induced NSC death (Fig. 3C, D).

Effect Of Naloxone On The Proliferation And Migration Capacity Of Nscs Injured By Ogd

Proliferation is one of the most important characteristics of NSCs. Intracellular protein levels of KI67 (a well-known proliferation marker) and nestin (a neural stem cell marker) were evaluated by immunocytochemistry. The proliferation of NSCs was confirmed by BrdU and CFU assays. OGD reduced the expression of KI67 and nestin in NSCs, but administration of naloxone restored KI67 and nestin levels

(Fig. 4A). In addition, OGD significantly reduced the BrdU-positive cells and the number of colonies in the BrdU and CFU assays. Treatment with 0.1 μM and 1 μM naloxone significantly increased the number of BrdU-positive cells and the number of colonies (Fig. 4B–D).

Migratory capacity is another important characteristic of stem cells. Migration assay kits were used to evaluate whether OGD and naloxone affected NSC migration. OGD markedly reduced the migratory capacity of NSCs compared with that of control. However, it was restored by treatment with 0.1 μM and 1 μM naloxone (Fig. 4E).

Effect of OGD and Naloxone on Intracellular Signaling Proteins Related to Proliferation, Survival, and NLRP3 Inflammasome Activation/Assembly in NSCs

Western blotting was performed to measure levels of intracellular signaling proteins affected by OGD and naloxone in NSCs. The expression levels of KI67 and nestin were markedly reduced in NSCs subjected to OGD for 16 h but significantly increased after naloxone treatment in a concentration-dependent manner up to 1 μM (Fig. 5A, B). The expression levels of PI3K, pAkt (Ser473), and pGSK-3β (Ser 9), which are associated with survival signals, were also reduced in NSCs after 1 h of OGD but were restored by naloxone (Fig. 5C, D). Bcl-2 level was reduced after 16 h of OGD but increased with naloxone treatment (Fig. 5E, F). In contrast, the levels of Bax and cleaved caspase-9, which are associated with cell death signals, increased after 16 h of OGD but reduced after naloxone treatment (Fig. 5E, F). In addition, levels of NLRP3 inflammasome signal-related proteins, such as NLRP3, cleaved caspase-1, and IL-1β, were markedly increased in NSCs subjected to OGD for 6 h but recovered after naloxone treatment (Fig. 5G, H). NSCs were subjected to OGD for 2–16 h to investigate alterations in the expression levels of inflammasome factors. As a result, 6 h of OGD was confirmed to cause the greatest change in the levels of inflammasome-associated proteins (Fig. S2A, B). Protein expression was normalized against GAPDH (Fig. S3J). Figure S3A–I show non-cutting gels to demonstrate that the antibodies were specific. The specificity of antibodies was confirmed using only the secondary antibody without the corresponding primary antibody in western blotting analysis (Fig. S3K). Protein markers were loaded to identify the molecular weight of each protein band, but markers were not detected in the chemical reaction (Fig. S4). Therefore, the exact molecular weight and arrows for each protein band are shown in Fig. 5A and Fig. S3.

Role of the PI3K pathway in the Naloxone-Induced Recovery of OGD-Damaged NSCs via Inhibition of NLRP3 Inflammasome Activation/Assembly

Survival and proliferation of NSCs were impaired by OGD. However, naloxone effectively rescued these properties. NLRP3 inflammasome activation/assembly and the PI3K pathway were strongly affected by

OGD but were restored to normal levels by naloxone. To investigate the relationship between NLRP3 inflammasome activation/assembly and the PI3K pathway in the rejuvenation of NSCs by naloxone, NSCs were pretreated with a PI3K inhibitor (LY294002 and wortmannin) prior to OGD and naloxone treatment. Through trypan blue staining and western blotting, it was confirmed that the PI3K inhibitor blocked the effect of naloxone on NSCs injured by OGD; therefore, the viability of these cells was significantly decreased compared to that of the NSCs not pretreated with the PI3K inhibitor (Fig. 6A, D). The inhibition of NLRP3 inflammasome activation/assembly by naloxone was abolished by treatment with a PI3K inhibitor. The expression levels of NLRP3 inflammasome signaling-associated proteins decreased in response to naloxone treatment in NSCs injured by OGD but were significantly increased when cells were pretreated with the PI3K inhibitor (Fig. 6B, C, E, F).

Discussion

In the present study, it was demonstrated that naloxone improved the viability, proliferation, and migration of NSCs injured by OGD and exhibited neuroprotective effects by inhibiting NLRP3 inflammasome activation/assembly via activation of PI3K signaling. The viability of OGD-injured NSCs significantly increased, and cytotoxicity, apoptosis, and cell death significantly decreased after treatment with 0.1 μM and 1 μM naloxone (Fig. 2 and Fig. 3). Moreover, the proliferation and migratory capacity of NSCs injured by OGD significantly decreased and recovered after treatment with 0.1 μM and 1 μM naloxone (Fig. 4). Western blotting analysis revealed that the expression of proliferation markers (KI67 and nestin) and survival-related proteins (PI3K, pAkt, pGSK3β, and Bcl-2) decreased in OGD-injured NSCs but recovered after naloxone treatment (Fig. 5A—D). Cell death-related proteins (Bax and cleaved caspase-9) were upregulated in NSCs after OGD but decreased with naloxone treatment (Fig. 5E, F). Pre-treatment with the PI3K inhibitors LY294002 (Fig. 6A) or wortmannin (Fig. 6D) abrogated the neuroprotective effects of naloxone on OGD-injured NSCs, indicating that the neuroprotective effects of naloxone are PI3K-dependent. In the present study, the effects of naloxone on the expression of the NLRP3 inflammasome and related proteins were also evaluated in relation to the PI3K pathway. Marked activation of the NLRP3 inflammasome and increase in the cleaved caspase-1 and IL-1β levels were observed in OGD-injured NSCs, and the effect of OGD was attenuated by naloxone treatment in a dose-dependent manner (Fig. 5G, H). Pre-treatment with LY294002 (Fig. 6B, C) or wortmannin (Fig. 6E, F) eliminated the effects of naloxone on the NLRP3 inflammasome activation/assembly and its signal-related proteins, suggesting that inhibitory effects of naloxone may be related to the PI3K pathway. However, in the present study, naloxone did not exhibit neuroprotective properties at concentrations greater than 10 μM. Some studies have suggested that naloxone exhibits anticancer properties through various mechanisms [38, 39], so naloxone could be toxic to cells at high concentrations. Although there is no direct evidence that naloxone is toxic to NSCs, Cheng et al. [40] showed that the protective properties of naloxone peaked at a concentration of 1.0 μM in BV2 mouse microglial cells and decreased at 2.0 μM. Therefore, naloxone may be toxic to NSCs at concentrations greater than 10 μM, as shown in the present study.

In central nervous system diseases, it is important to consider species differences. Therefore, we also investigated the effects of OGD and naloxone on the viability and cytotoxicity of hNSCs. OGD reduced hNSC viability and induced cytotoxicity. Treatment with 0.01–1 μM naloxone significantly increased viability and decreased cytotoxicity in hNSCs damaged by OGD (Fig. S1). From these results, the neuroprotective effects of naloxone in hNSCs are similar to those of NSCs under ischemic conditions. However, since obtaining a large amount of hNSCs to proceed with the following experiments was difficult, the subsequent experiments were performed using NSCs.

Recently, increasing evidence has implicated that NLRP3 inflammasome signaling and its activation/release products, apoptosis-associated speck-like protein (ASC), caspase-1, and IL-1β, are essential for mediating inflammatory responses in aseptic tissue injury during ischemic stroke [9, 41] and other neurodegenerative diseases [42–45]. Several previous animal studies have indicated that inhibition of NLRP3 inflammasome activation/assembly mitigates ischemic damage and neurovascular complications in stroke models [46–48]. Toll-like receptor 4 (TLR4) is a transmembrane protein whose activation leads to the production of inflammatory cytokines, which play a central role in activating the innate immune system [49]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a key downstream factor of TLR4 signaling and a central mediator of the priming signal of NLRP3 inflammasome activation and induces transcription of NLRP3 and pro-IL-1β [50, 51]. Previous studies have reported that TLR4 expression is elevated [52], and NF-kB is activated [53] following cerebral ischemia, promoting inflammatory reactions that further aggravate ischemic brain injury. In addition, the degree of ischemic brain injury and neuroinflammation was significantly lower in TLR4-deficient mice when compared to that in wild-type mice [54]. Naloxone is a well-known opioid antagonist and, interestingly, acts as a TLR4 antagonist, inhibiting opioid- or lipopolysaccharide (LPS)-induced TLR4 signaling [55]. It has been suggested to have neuroprotective and anti-inflammatory effects in cerebral ischemic conditions. A previous *in vivo* study demonstrated the neuroprotective effects of intranasal naloxone, which reduced microglial activation and improved behavioral recovery caused by ischemic injury in rats that suffered from cortical stroke [56]. There are also few reports on the anti-inflammatory effect of naloxone that focus on the NLRP3 inflammasome and related signals in THP-1 human monocytic leukemia cells [25] and BV-2 murine microglial cells [57]. However, the neuroprotective effects of naloxone on NSCs with ischemic injury in association with the NLRP3 inflammasome and its related proteins have not been investigated. We could postulate from our data that naloxone, as a TLR4 antagonist, binds to TLR4 and attenuates NF-κB activation, which leads to diminished NLRP3 inflammasome activation/assembly and cleaved caspase-1 and pro-inflammatory cytokine synthesis in OGD-injured NSCs.

Several recent studies have reported that TLR4 and PI3K signaling pathways counter-regulate each other [58–60]. Many studies have demonstrated crosstalk between TLR signaling and the PI3K pathway underlying the myocardial protection against ischemia/reperfusion (I/R) injury [61–63]. Li *et al.* reported that activation of PI3K signaling is associated with decreased myocardial ischemic injury through modulation of TLR4-mediated signaling [64]. They reported that glucan phosphate, a ligand of TLR4, may shift TLR4 signaling pathways from a predominant NF-κB pathway to the PI3K/Akt signaling pathway,

which has a protective role in myocardial I/R injury. Their results suggested a possible reciprocal regulation between the NF-κB and PI3K/Akt signaling pathways during myocardial I/R injury. Most studies on the crosstalk between TLR signaling and the PI3K pathway are on myocardial I/R injury, but brain ischemia-related studies are limited. In this study, the levels of the NLRP3 inflammasome, cleaved caspase-1, and pro-inflammatory cytokine IL-1β were elevated in NSCs damaged by OGD and were decreased by naloxone, a TLR4 antagonist. However, this effect was attenuated by the PI3K inhibitors LY294002 and wortmannin, suggesting that PI3K pathway is related to inhibitory effect of naloxone on the NLRP3 inflammasome signaling pathway in NSCs. TLR4-mediated signaling modulation by naloxone may be a potential therapeutic target that can ultimately reduce ischemic injury in NSCs through activation of the PI3K pathway and inhibition of NLRP3 inflammasome signaling pathway.

Several other reports highlight the relationship between the PI3K pathway and the NLRP3 inflammasome activation/assembly. Our results are consistent with several previous reports suggesting that PI3K pathway might have an inhibitory effect on the activation/assembly of the NLRP3 inflammasome [35, 65]. In contrast, few reports indicate that the PI3K pathway can activate the NLRP3 inflammasome [66, 67]. The relationship between the PI3K pathway and NLRP3 inflammasome activation/assembly is complex and may vary depending on the cell line and other conditions. However, to the best of our knowledge, this is the first study to show the inhibitory effects of naloxone on NLRP3 inflammasome activation/assembly in relation to the PI3K pathway in NSCs injured by OGD.

Our study has several limitations. Firstly, although we investigated the effects of OGD and naloxone on the viability and cytotoxicity of hNSCs, it was difficult to secure a large amount of hNSCs for subsequent experiments. Therefore, we conducted most of the experiments using embryonic NSCs from rodents. As OGD usually occurs in the adult brain with ischemic stroke, it might be more reasonable to evaluate this experiment using adult NSCs. However, as most adult NSCs proliferate slowly, *in vitro* expansion may have been insufficient for preclinical studies [68]. Secondly, we performed *in vitro* experiments, which might vary from *in vivo* conditions. Therefore, we cannot guarantee that OGD and naloxone will have similar effects on NSCs under *in vivo* conditions. Thirdly, NLRP3 inflammasome activation/assembly seems to involve many combinations of conditions and processes, as well as the PI3K pathway. Verifying the exact relationship between the PI3K pathway and NLRP3 inflammasome activation/assembly requires further study. However, this is the first study to demonstrate the neuroprotective and anti-inflammatory effects of naloxone on NSCs injured by OGD and its inhibitory effect on NLRP3 inflammasome activation/assembly in relation to the activation of the PI3K pathway. Targeting neuroprotective and anti-inflammatory aspects can improve therapeutic efficacy and prognosis after post-stroke brain injury.

In conclusion, naloxone improved the survival, proliferation, and migration of NSCs and reduced cytotoxicity and apoptosis in NSCs in OGD environments. In addition, naloxone also exhibited anti-inflammatory effects by inhibiting NLRP3 inflammasome activation/assembly in relation to the activation of the PI3K pathway.

Declarations

Supplementary Information The online version contains supplementary material available at https://doi.org/

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Author Contributions J.Y.K., N.Y.C., M.H., and S.H.K. designed the study, N.Y.C. and M.H. performed experiments, J.Y.K. and S.H.K. analyzed and interpreted data, N.Y.C. and M.H. generated figures, J.Y.K. and M.H. wrote original draft, S.H.K. revised manuscript, All authors provided critical insight and review of the manuscript. All authors have read and agreed to published version of the manuscript.

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Data Availability Statement The datasets used and/or analyzed in the current study are available from the corresponding authors on reasonable request.

Ethics Approval All animal procedures were conducted in accordance with Hanyang University's guidelines for the care and use of laboratory animals, and approved by the Institutional Animal Care and Use Committee (IACUC) of Hanyang University (2019-0162A and 2021-0039A). All efforts were made to minimize the number of animals used and animal suffering.

Consent to Participate Not applicable.

Consent for Publication All authors consent to the publication of this manuscript.

Conflict of Interest The authors declare no competing interests.

References

1. Feigin VL, Forouzanfar MH, Krishnamurthi R, Mensah GA, Connor M, Bennett DA, Moran AE, Sacco RL, Anderson L, Truelsen T, O'Donnell M, Venketasubramanian N, Barker-Collo S, Lawes CM, Wang W, Shinohara Y, Witt E, Ezzati M, Naghavi M, Murray C, Global Burden of Diseases I, Risk Factors S, the GBDSEG (2014) Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. Lancet 383 (9913):245-254. doi:10.1016/s0140-6736(13)61953-4
2. Lakhan SE, Kirchgessner A, Hofer M (2009) Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J Transl Med 7:97. doi:10.1186/1479-5876-7-97
3. del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feuerstein GZ (2000) Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. Brain

Pathol 10 (1):95-112. doi:10.1111/j.1750-3639.2000.tb00247.x

4. McColl BW, Rothwell NJ, Allan SM (2007) Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. J Neurosci 27 (16):4403-4412. doi:10.1523/JNEUROSCI.5376-06.2007
5. Elkind MS, Cheng J, Rundek T, Boden-Albala B, Sacco RL (2004) Leukocyte count predicts outcome after ischemic stroke: the Northern Manhattan Stroke Study. J Stroke Cerebrovasc Dis 13 (5):220-227. doi:10.1016/j.jstrokecerebrovasdis.2004.07.004
6. Baird TA, Parsons MW, Barber PA, Butcher KS, Desmond PM, Tress BM, Colman PG, Jerums G, Chambers BR, Davis SM (2002) The influence of diabetes mellitus and hyperglycaemia on stroke incidence and outcome. J Clin Neurosci 9 (6):618-626. doi:10.1054/jocn.2002.1081
7. Huang J, Upadhyay UM, Tamargo RJ (2006) Inflammation in stroke and focal cerebral ischemia. Surg Neurol 66 (3):232-245. doi:10.1016/j.surneu.2005.12.028
8. Gao L, Dong Q, Song Z, Shen F, Shi J, Li Y (2017) NLRP3 inflammasome: a promising target in ischemic stroke. Inflamm Res 66 (1):17-24. doi:10.1007/s00011-016-0981-7
9. Fann DY, Lee SY, Manzanero S, Chunduri P, Sobey CG, Arumugam TV (2013) Pathogenesis of acute stroke and the role of inflammasomes. Ageing Res Rev 12 (4):941-966. doi:10.1016/j.arr.2013.09.004
10. Kelley N, Jeltema D, Duan Y, He Y (2019) The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. Int J Mol Sci 20 (13). doi:10.3390/ijms20133328
11. Ismael S, Zhao L, Nasoohi S, Ishrat T (2018) Inhibition of the NLRP3-inflammasome as a potential approach for neuroprotection after stroke. Sci Rep 8 (1):5971. doi:10.1038/s41598-018-24350-x
12. Haas S, Weidner N, Winkler J (2005) Adult stem cell therapy in stroke. Curr Opin Neurol 18 (1):59-64. doi:10.1097/00019052-200502000-00012
13. Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U, Frisen J (2000) Generalized potential of adult neural stem cells. Science 288 (5471):1660-1663. doi:10.1126/science.288.5471.1660
14. Qi D, Liu H, Niu J, Fan X, Wen X, Du Y, Mou J, Pei D, Liu Z, Zong Z, Wei X, Song Y (2012) Heat shock protein 72 inhibits c-Jun N-terminal kinase 3 signaling pathway via Akt1 during cerebral ischemia. Journal of the neurological sciences 317 (1-2):123-129. doi:10.1016/j.jns.2012.02.011
15. Kim YS, Yoo A, Son JW, Kim HY, Lee YJ, Hwang S, Lee KY, Lee YJ, Ayata C, Kim HH, Koh SH (2016) Early Activation of Phosphatidylinositol 3-Kinase after Ischemic Stroke Reduces Infarct Volume and Improves Long-Term Behavior. Molecular neurobiology:In press. doi:10.1007/s12035-014-9030-0
16. Koh SH, Lo EH (2015) The Role of the PI3K Pathway in the Regeneration of the Damaged Brain by Neural Stem Cells after Cerebral Infarction. J Clin Neurol 11 (4):297-304. doi:10.3988/jcn.2015.11.4.297
17. Nguyen N, Lee SB, Lee YS, Lee KH, Ahn JY (2009) Neuroprotection by NGF and BDNF against neurotoxin-exerted apoptotic death in neural stem cells are mediated through Trk receptors,

activating PI3-kinase and MAPK pathways. Neurochem Res 34 (5):942-951. doi:10.1007/s11064-008-9848-9

18. Noh MY, Kim YS, Lee KY, Lee YJ, Kim SH, Yu HJ, Koh SH (2013) The early activation of PI3K strongly enhances the resistance of cortical neurons to hypoxic injury via the activation of downstream targets of the PI3K pathway and the normalization of the levels of PARP activity, ATP, and NAD(+). Mol Neurobiol 47 (2):757-769. doi:10.1007/s12035-012-8382-6
19. Bradberry JC, Raebel MA (1981) Continuous infusion of naloxone in the treatment of narcotic overdose. Drug Intell Clin Pharm 15 (12):945-950. doi:10.1177/106002808101501205
20. Olinger CP, Adams HP, Jr., Brott TG, Biller J, Barsan WG, Toffol GJ, Eberle RW, Marler JR (1990) High-dose intravenous naloxone for the treatment of acute ischemic stroke. Stroke 21 (5):721-725. doi:10.1161/01.str.21.5.721
21. Jabaily J, Davis JN (1984) Naloxone administration to patients with acute stroke. Stroke 15 (1):36-39. doi:10.1161/01.str.15.1.36
22. Liao SL, Chen WY, Raung SL, Chen CJ (2003) Neuroprotection of naloxone against ischemic injury in rats: role of mu receptor antagonism. Neurosci Lett 345 (3):169-172. doi:10.1016/s0304-3940(03)00540-8
23. Jan WC, Chen CH, Hsu K, Tsai PS, Huang CJ (2011) L-type calcium channels and mu-opioid receptors are involved in mediating the anti-inflammatory effects of naloxone. J Surg Res 167 (2):e263-272. doi:10.1016/j.jss.2010.03.039
24. Wang TY, Su NY, Shih PC, Tsai PS, Huang CJ (2014) Anti-inflammation effects of naloxone involve phosphoinositide 3-kinase delta and gamma. J Surg Res 192 (2):599-606. doi:10.1016/j.jss.2014.06.022
25. Lin HY, Chang YY, Kao MC, Huang CJ (2017) Naloxone inhibits nod-like receptor protein 3 inflammasome. J Surg Res 219:72-77. doi:10.1016/j.jss.2017.05.119
26. Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA, Moskowitz MA (2002) Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. Proc Natl Acad Sci U S A 99 (23):15188-15193. doi:10.1073/pnas.232473399
27. Chojnacki A, Weiss S (2008) Production of neurons, astrocytes and oligodendrocytes from mammalian CNS stem cells. Nat Protoc 3 (6):935-940. doi:10.1038/nprot.2008.55
28. Currle DS, Hu JS, Kolski-Andreaco A, Monuki ES (2007) Culture of mouse neural stem cell precursors. Journal of visualized experiments : JoVE (2):152. doi:10.3791/152
29. Park HH, Lee KY, Kim S, Lee JW, Choi NY, Lee EH, Lee YJ, Lee SH, Koh SH (2014) Novel vaccine peptide GV1001 effectively blocks beta-amyloid toxicity by mimicking the extra-telomeric functions of human telomerase reverse transcriptase. Neurobiology of aging 35 (6):1255-1274. doi:10.1016/j.neurobiolaging.2013.12.015
30. Studer L, Tabar V, McKay RD (1998) Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nature neuroscience 1 (4):290-295. doi:10.1038/1105

31. Choi YK, Maki T, Mandeville ET, Koh SH, Hayakawa K, Arai K, Kim YM, Whalen MJ, Xing C, Wang X, Kim KW, Lo EH (2016) Dual effects of carbon monoxide on pericytes and neurogenesis in traumatic brain injury. Nat Med 22 (11):1335-1341. doi:10.1038/nm.4188
32. Kim M, Moon S, Jeon HS, Kim S, Koh SH, Chang MS, Kim YM, Choi YK (2022) Dual Effects of Korean Red Ginseng on Astrocytes and Neural Stem Cells in Traumatic Brain Injury: The HO-1-Tom20 Axis as a Putative Target for Mitochondrial Function. Cells 11 (5). doi:10.3390/cells11050892
33. Kim M, Kim J, Moon S, Choi BY, Kim S, Jeon HS, Suh SW, Kim YM, Choi YK (2021) Korean Red Ginseng Improves Astrocytic Mitochondrial Function by Upregulating HO-1-Mediated AMPKalpha-PGC-1alpha-ERRalpha Circuit after Traumatic Brain Injury. Int J Mol Sci 22 (23). doi:10.3390/ijms222313081
34. Park HH, Han MH, Choi H, Lee YJ, Kim JM, Cheong JH, Ryu JI, Lee KY, Koh SH (2019) Mitochondria damaged by Oxygen Glucose Deprivation can be Restored through Activation of the PI3K/Akt Pathway and Inhibition of Calcium Influx by Amlodipine Camsylate. Sci Rep 9 (1):15717. doi:10.1038/s41598-019-52083-y
35. Kwon HS, Ha J, Kim JY, Park HH, Lee EH, Choi H, Lee KY, Lee YJ, Koh SH (2021) Telmisartan Inhibits the NLRP3 Inflammasome by Activating the PI3K Pathway in Neural Stem Cells Injured by Oxygen-Glucose Deprivation. Mol Neurobiol 58 (4):1806-1818. doi:10.1007/s12035-020-02253-1
36. Noh MY, Koh SH, Kim Y, Kim HY, Cho GW, Kim SH (2009) Neuroprotective effects of donepezil through inhibition of GSK-3 activity in amyloid-beta-induced neuronal cell death. Journal of neurochemistry 108 (5):1116-1125. doi:10.1111/j.1471-4159.2008.05837.x
37. Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC (2010) A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem cell research & therapy 1 (1):8. doi:10.1186/scrt8
38. Bimonte S, Barbieri A, Cascella M, Rea D, Palma G, Del Vecchio V, Forte CA, Del Prato F, Arra C, Cuomo A (2018) The effects of naloxone on human breast cancer progression: in vitro and in vivo studies on MDA.MB231 cells. Onco Targets Ther 11:185-191. doi:10.2147/OTT.S145780
39. Bimonte S, Barbieri A, Cascella M, Rea D, Palma G, Luciano A, Forte CA, Cuomo A, Arra C (2019) Naloxone Counteracts the Promoting Tumor Growth Effects Induced by Morphine in an Animal Model of Triple-negative Breast Cancer. In Vivo 33 (3):821-825. doi:10.21873/invivo.11545
40. Cheng W, Li Y, Hou X, Zhang N, Ma J, Ding F, Li F, Miao Z, Zhang Y, Qi Q, Li G, Shen Y, Liu J, Huang W, Wang Y (2014) HSP60 is involved in the neuroprotective effects of naloxone. Mol Med Rep 10 (4):2172-2176. doi:10.3892/mmr.2014.2411
41. Abulafia DP, de Rivero Vaccari JP, Lozano JD, Lotocki G, Keane RW, Dietrich WD (2009) Inhibition of the inflammasome complex reduces the inflammatory response after thromboembolic stroke in mice. J Cereb Blood Flow Metab 29 (3):534-544. doi:10.1038/jcbfm.2008.143
42. Liu L, Chan C (2014) The role of inflammasome in Alzheimer's disease. Ageing Res Rev 15:6-15. doi:10.1016/j.arr.2013.12.007

43. Zhou K, Shi L, Wang Y, Chen S, Zhang J (2016) Recent Advances of the NLRP3 Inflammasome in Central Nervous System Disorders. J Immunol Res 2016:9238290. doi:10.1155/2016/9238290
44. Lenart N, Brough D, Denes A (2016) Inflammasomes link vascular disease with neuroinflammation and brain disorders. J Cereb Blood Flow Metab 36 (10):1668-1685. doi:10.1177/0271678X16662043
45. Liu SB, Mi WL, Wang YQ (2013) Research progress on the NLRP3 inflammasome and its role in the central nervous system. Neurosci Bull 29 (6):779-787. doi:10.1007/s12264-013-1328-9
46. Yang F, Wang Z, Wei X, Han H, Meng X, Zhang Y, Shi W, Li F, Xin T, Pang Q, Yi F (2014) NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke. J Cereb Blood Flow Metab 34 (4):660-667. doi:10.1038/jcbfm.2013.242
47. Ye X, Shen T, Hu J, Zhang L, Zhang Y, Bao L, Cui C, Jin G, Zan K, Zhang Z, Yang X, Shi H, Zu J, Yu M, Song C, Wang Y, Qi S, Cui G (2017) Purinergic 2X7 receptor/NLRP3 pathway triggers neuronal apoptosis after ischemic stroke in the mouse. Exp Neurol 292:46-55. doi:10.1016/j.expneurol.2017.03.002
48. Hong P, Li FX, Gu RN, Fang YY, Lai LY, Wang YW, Tao T, Xu SY, You ZJ, Zhang HF (2018) Inhibition of NLRP3 Inflammasome Ameliorates Cerebral Ischemia-Reperfusion Injury in Diabetic Mice. Neural Plast 2018:9163521. doi:10.1155/2018/9163521
49. Vaure C, Liu Y (2014) A comparative review of toll-like receptor 4 expression and functionality in different animal species. Front Immunol 5:316. doi:10.3389/fimmu.2014.00316
50. Sun SC (2011) Non-canonical NF-kappaB signaling pathway. Cell Res 21 (1):71-85. doi:10.1038/cr.2010.177
51. Sutterwala FS, Haasken S, Cassel SL (2014) Mechanism of NLRP3 inflammasome activation. Ann N Y Acad Sci 1319:82-95. doi:10.1111/nyas.12458
52. Tu XK, Yang WZ, Shi SS, Wang CH, Zhang GL, Ni TR, Chen CM, Wang R, Jia JW, Song QM (2010) Spatio-temporal distribution of inflammatory reaction and expression of TLR2/4 signaling pathway in rat brain following permanent focal cerebral ischemia. Neurochem Res 35 (8):1147-1155. doi:10.1007/s11064-010-0167-6
53. Guan T, Liu Q, Qian Y, Yang H, Kong J, Kou J, Yu B (2013) Ruscogenin reduces cerebral ischemic injury via NF-kappaB-mediated inflammatory pathway in the mouse model of experimental stroke. Eur J Pharmacol 714 (1-3):303-311. doi:10.1016/j.ejphar.2013.07.036
54. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I (2007) Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115 (12):1599-1608. doi:10.1161/CIRCULATIONAHA.106.603431
55. Stevens CW, Aravind S, Das S, Davis RL (2013) Pharmacological characterization of LPS and opioid interactions at the toll-like receptor 4. Br J Pharmacol 168 (6):1421-1429. doi:10.1111/bph.12028
56. Anttila JE, Albert K, Wires ES, Matlik K, Loram LC, Watkins LR, Rice KC, Wang Y, Harvey BK, Airavaara M (2018) Post-stroke Intranasal (+)-Naloxone Delivery Reduces Microglial Activation and Improves Behavioral Recovery from Ischemic Injury. eNeuro 5 (2). doi:10.1523/ENEURO.0395-17.2018

57. Wang X, Zhang Y, Peng Y, Hutchinson MR, Rice KC, Yin H, Watkins LR (2016) Pharmacological characterization of the opioid inactive isomers (+)-naltrexone and (+)-naloxone as antagonists of toll-like receptor 4. Br J Pharmacol 173 (5):856-869. doi:10.1111/bph.13394
58. Williams DL, Ozment-Skelton T, Li C (2006) Modulation of the phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. Shock 25 (5):432-439. doi:10.1097/01.shk.0000209542.76305.55
59. Laird MH, Rhee SH, Perkins DJ, Medvedev AE, Piao W, Fenton MJ, Vogel SN (2009) TLR4/MyD88/PI3K interactions regulate TLR4 signaling. J Leukoc Biol 85 (6):966-977. doi:10.1189/jlb.1208763
60. Fukao T, Koyasu S (2003) PI3K and negative regulation of TLR signaling. Trends Immunol 24 (7):358-363. doi:10.1016/s1471-4906(03)00139-x
61. Ha T, Hu Y, Liu L, Lu C, McMullen JR, Kelley J, Kao RL, Williams DL, Gao X, Li C (2010) TLR2 ligands induce cardioprotection against ischaemia/reperfusion injury through a PI3K/Akt-dependent mechanism. Cardiovasc Res 87 (4):694-703. doi:10.1093/cvr/cvq116
62. Ha T, Hua F, Liu X, Ma J, McMullen JR, Shioi T, Izumo S, Kelley J, Gao X, Browder W, Williams DL, Kao RL, Li C (2008) Lipopolysaccharide-induced myocardial protection against ischaemia/reperfusion injury is mediated through a PI3K/Akt-dependent mechanism. Cardiovasc Res 78 (3):546-553. doi:10.1093/cvr/cvn037
63. Yang L, Cai X, Liu J, Jia Z, Jiao J, Zhang J, Li C, Li J, Tang XD (2013) CpG-ODN attenuates pathological cardiac hypertrophy and heart failure by activation of PI3Kalpha-Akt signaling. PLoS One 8 (4):e62373. doi:10.1371/journal.pone.0062373
64. Li C, Ha T, Kelley J, Gao X, Qiu Y, Kao RL, Browder W, Williams DL (2004) Modulating Toll-like receptor mediated signaling by (1–>3)-beta-D-glucan rapidly induces cardioprotection. Cardiovasc Res 61 (3):538-547. doi:10.1016/j.cardiores.2003.09.007
65. Luo X, Zeng H, Fang C, Zhang BH (2021) N-acetylserotonin Derivative Exerts a Neuroprotective Effect by Inhibiting the NLRP3 Inflammasome and Activating the PI3K/Akt/Nrf2 Pathway in the Model of Hypoxic-Ischemic Brain Damage. Neurochem Res 46 (2):337-348. doi:10.1007/s11064-020-03169-x
66. Wang K, Zhang Y, Cao Y, Shi Z, Lin Y, Chen Y, Zhao H, Liu X (2020) Glycyrrhetinic acid alleviates acute lung injury by PI3K/AKT suppressing macrophagic Nlrp3 inflammasome activation. Biochem Biophys Res Commun 532 (4):555-562. doi:10.1016/j.bbrc.2020.08.044
67. Zhao W, Shi CS, Harrison K, Hwang IY, Nabar NR, Wang M, Kehrl JH (2020) AKT Regulates NLRP3 Inflammasome Activation by Phosphorylating NLRP3 Serine 5. J Immunol 205 (8):2255-2264. doi:10.4049/jimmunol.2000649
68. De Filippis L, Binda E (2012) Concise review: self-renewal in the central nervous system: neural stem cells from embryo to adult. Stem Cells Transl Med 1 (4):298-308. doi:10.5966/sctm.2011-0045

Figures

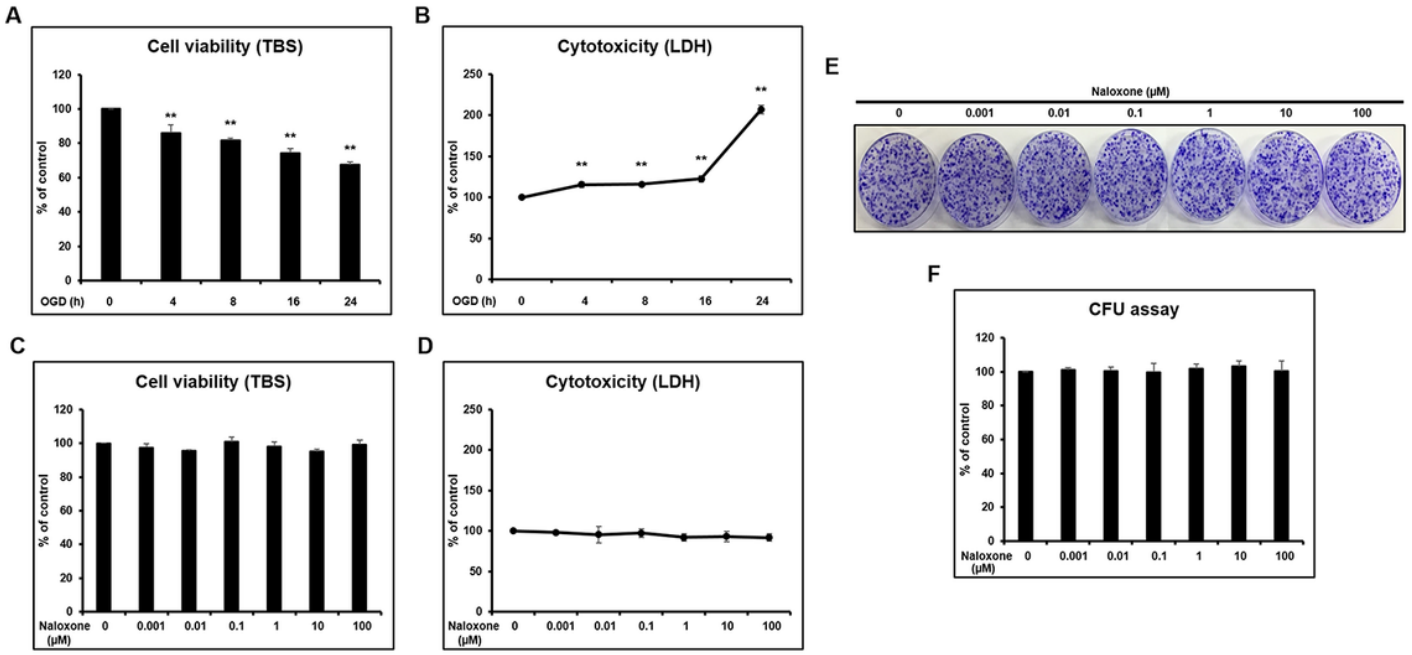

Figure 1

Effects of OGD and naloxone on NSCs viability, cytotoxicity, and proliferation. (**A, B**) OGD markedly decreased viability and increased cytotoxicity in NSCs in a time-dependent manner. Naloxone at concentrations up to 100 μM did not affect cell viability (**C**), cytotoxicity (**D**), or proliferation (**E**, **F**) of NSCs. All data are represented as mean (% of control) ± standard deviation of a minimum of three independent experiments and were analyzed using Tukey's test after a one-way analysis of variance (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ (vs. the control group). OGD, oxygen-glucose deprivation, NSCs, neural stem cells.

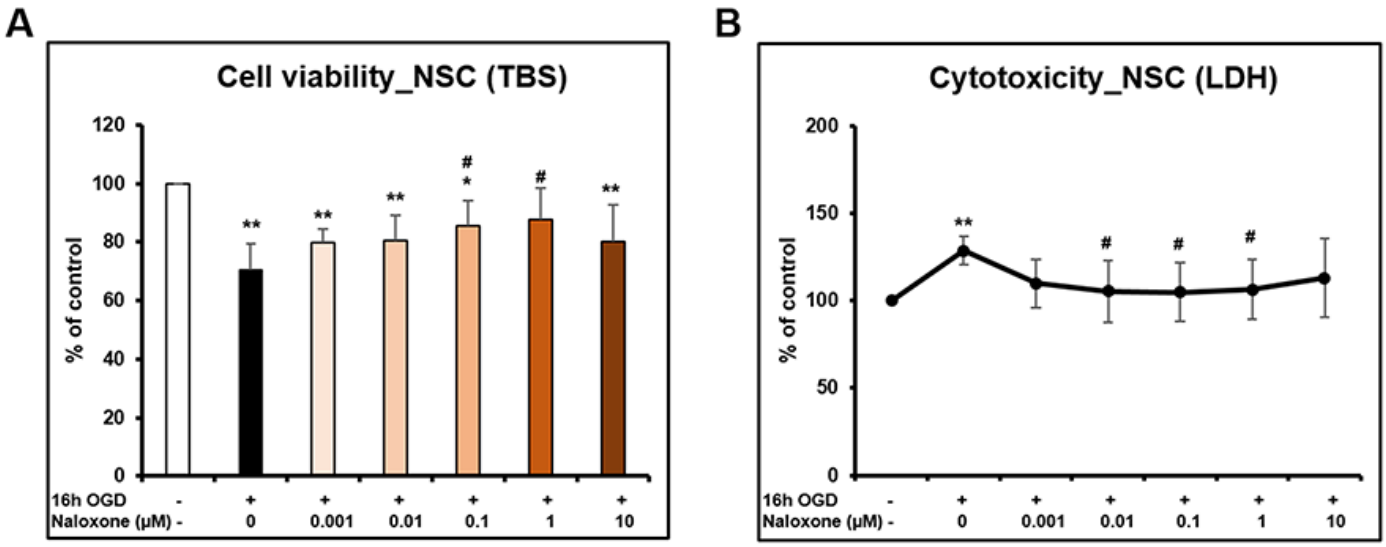

Figure 2

Effects of naloxone on viability and cytotoxicity of OGD-damaged NSCs. (**A**) NSC viability was significantly reduced after 16 h of OGD but significantly increased after naloxone treatment. (**B**) Cytotoxicity also increased after OGD but decreased after naloxone treatment. All data are represented as mean (% of control) ± standard deviation of a minimum of three independent experiments and were analyzed using Tukey's test after a one-way analysis of variance ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ (vs. the control group), and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (vs. the group subjected to OGD alone). NSCs, neural stem cells, OGD, oxygen-glucose deprivation.

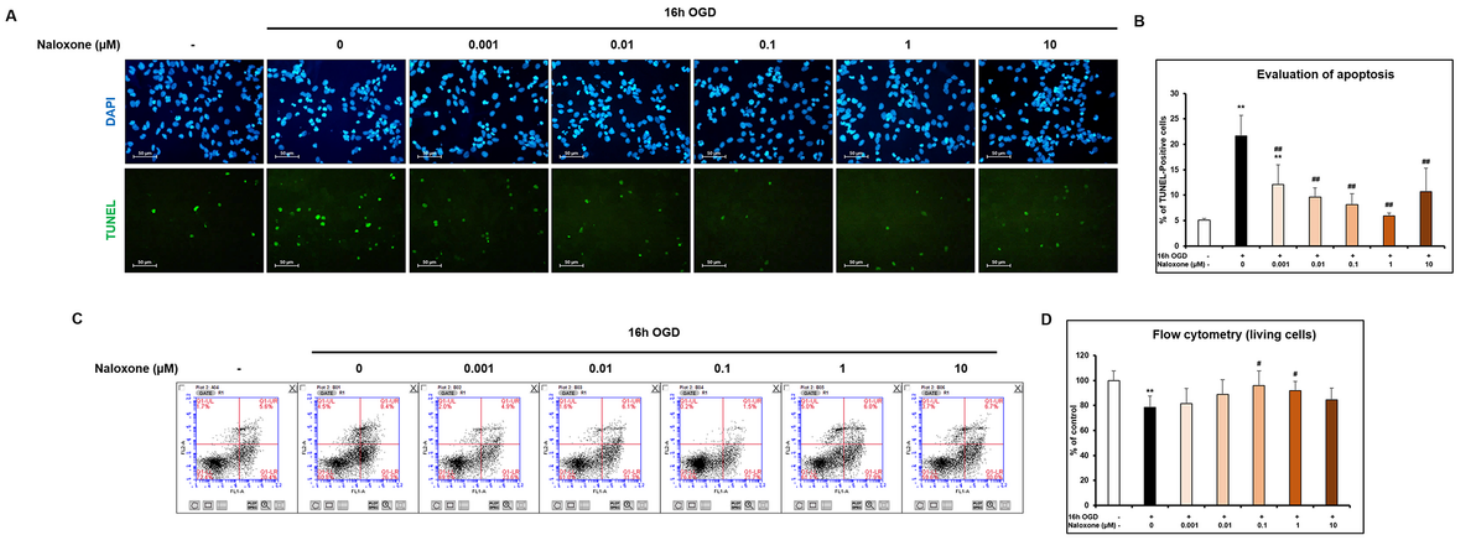

Figure 3

Effects of naloxone on apoptosis, necrosis, and cell death of NSCs damaged by OGD. Apoptosis of NSCs was evaluated using TUNEL staining. (**A**, **B**) OGD upregulated apoptosis but it was significantly reduced by naloxone treatment. The data show TUNEL-positive cells. (**C**, **D**) Flow cytometry analysis using Annexin V and PI showed that living cells decreased after OGD but increased with naloxone treatment. All data are represented as mean (% of control) ± standard deviation of a minimum of three independent experiments and were analyzed using Tukey's test after a one-way analysis of variance ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ (vs. the control group), and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (vs. the group subjected to OGD alone). NSCs, neural stem cells, OGD, oxygen-glucose deprivation.

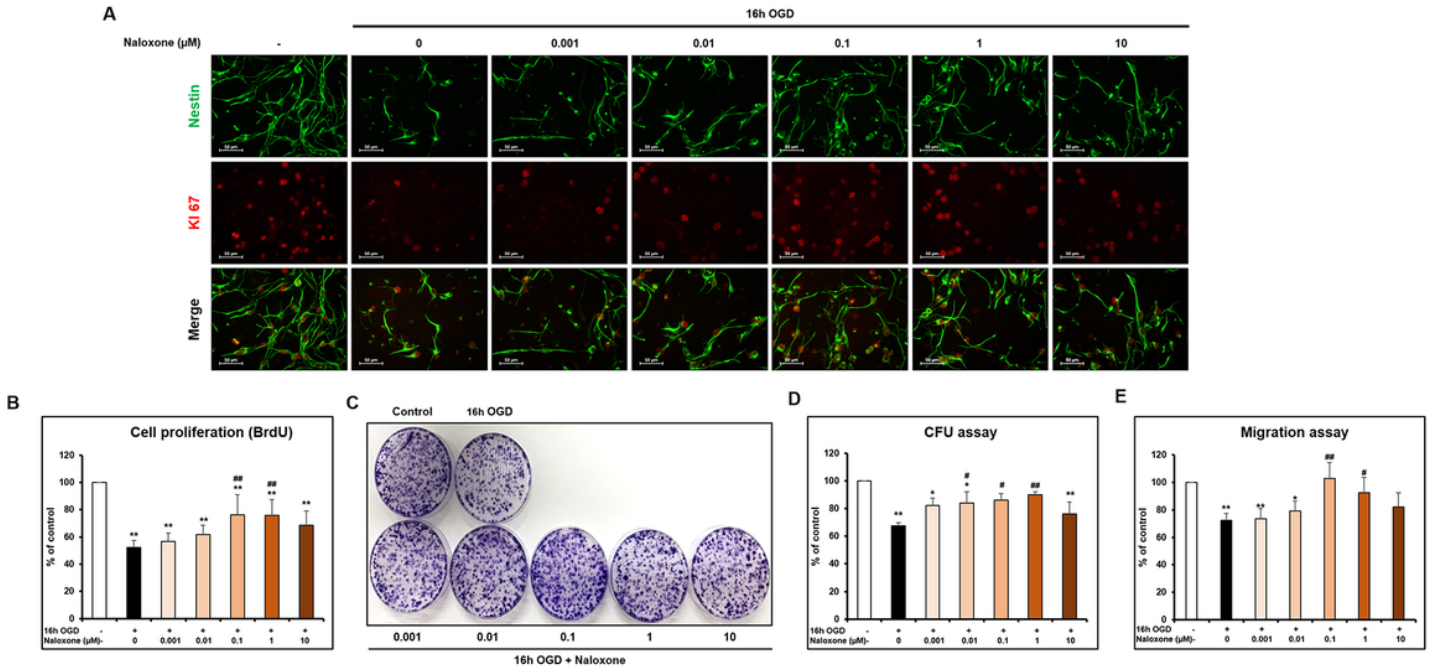

Figure 4

Effects of naloxone on proliferation and migration capacity of OGD-damaged NSCs. (**A**) Proliferation of NSCs injured by OGD was measured by immunostaining for KI 67 and nestin, which are markers for proliferation and neural stem cells, respectively. NSCs proliferation was also confirmed by BrdU (**B**) and colony forming unit (CFU) (**C**, **D**) assays. It was shown that naloxone improved the proliferation of OGD-exposed NSCs. (**E**) The migratory capacity of NSCs was reduced after exposure 16 h of OGD, however, naloxone treatment efficiently restored migratory capacity of NSCs. All data are represented as mean (% of control) ± standard deviation of a minimum of three independent experiments and were analyzed using Tukey's test after a one-way analysis of variance ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ (vs. the control group), and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (vs. the group subjected to OGD alone). NSCs, neural stem cells, OGD, oxygen-glucose deprivation.

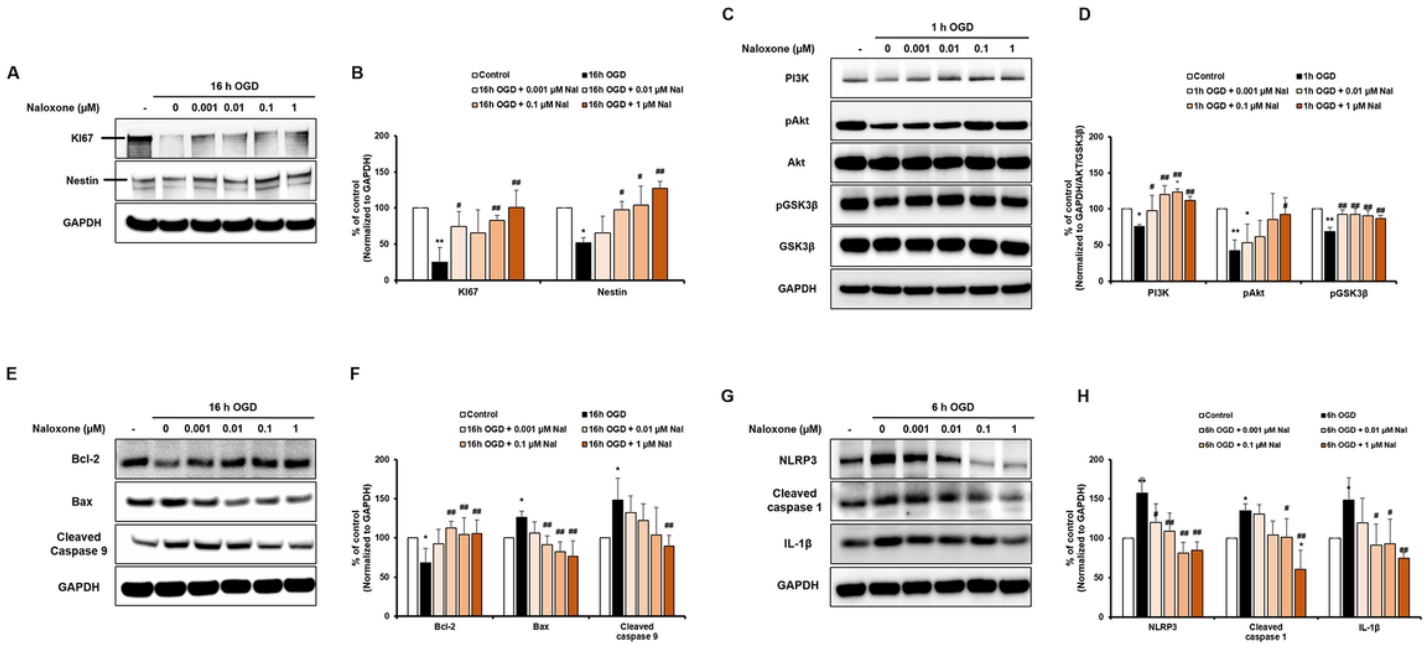

Figure 5

Effects of naloxone on the expression levels of intracellular signaling proteins impaired by OGD in NSCs. Expression levels of intracellular signaling proteins associated with cell proliferation and survival in NSCs subjected to OGD and treated with naloxone were evaluated via western blotting. Naloxone markedly restored expression levels of proteins related to survival and proliferation of OGD-damaged NSCs (**A–F**) and significantly restored the expression of inflammasome factors, such as NLRP3, cleaved caspase-1, and IL-1β, in NSCs impaired by 6 h OGD treatment (**G**, **H**). All data are represented as mean (% of control) ± standard deviation of a minimum of three independent experiments and were analyzed using Tukey's test after a one-way analysis of variance (**A**, **B:** KI67, $n = 3$, nestin, $n = 3$, **C**, **D:** PI3K, $n = 5$, pAKT, $n = 4$, AKT, $n = 4$, pGSK3β, $n = 4$, GSK3β, $n = 4$, **E**, **F:** Bcl-2, $n = 6$, Bax, $n = 5$, cleaved caspase 9, $n = 5$, **G**, **H:** NLRP3, $n = 5$, cleaved caspase 1, $n = 5$, IL-1β, $n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ (vs. the control group), and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (vs. the group subjected to OGD alone). OGD, oxygen-glucose deprivation, NSCs, neural stem cells.

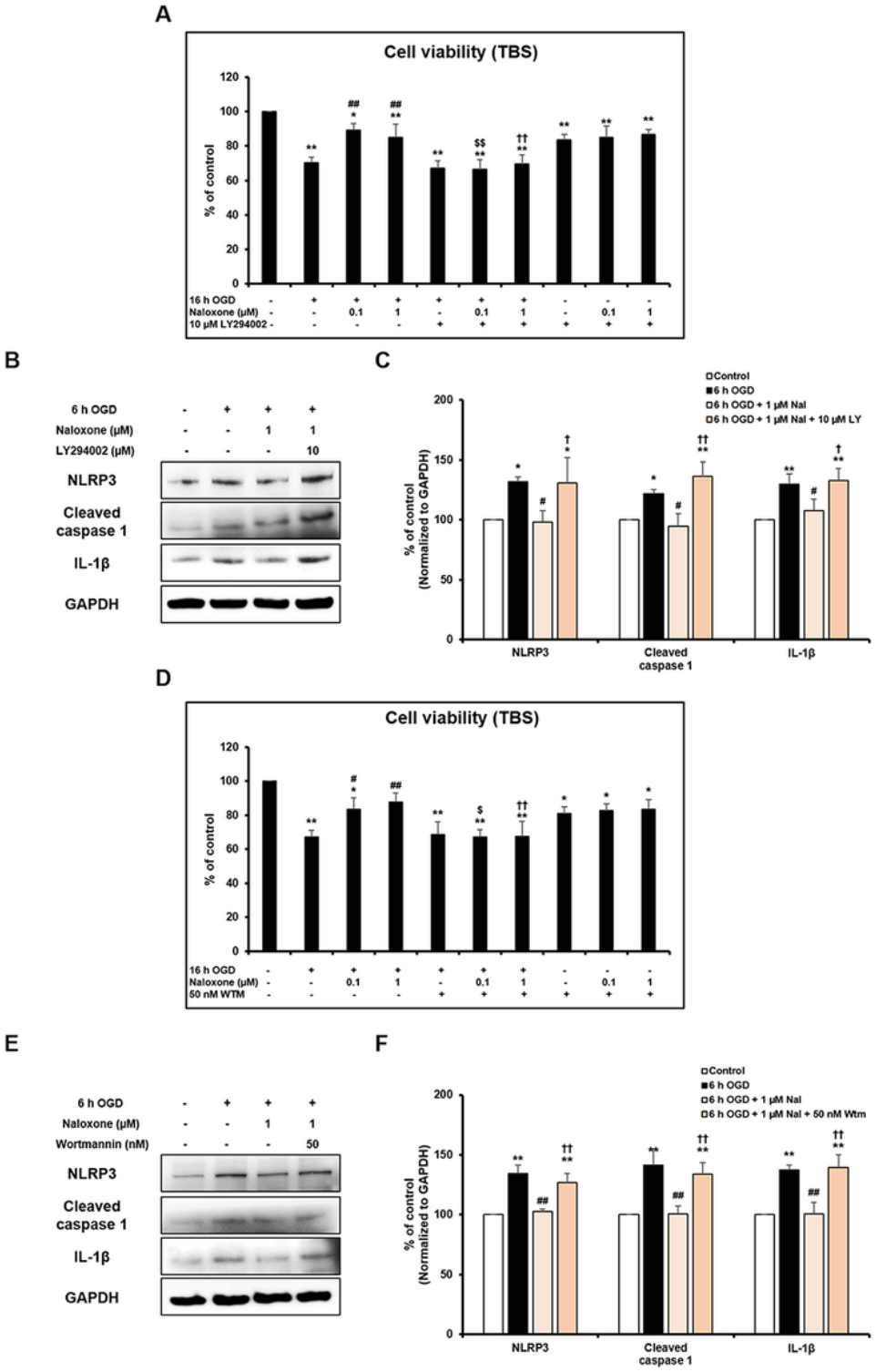

Figure 6

Role of the PI3K pathway in naloxone-induced recovery of OGD-damaged NSCs. Pre-treatment of NSCs with a PI3K inhibitor (LY294002 or wortmannin) significantly attenuated the protective effects of naloxone on NSC viability (**A**, **D**) and its inhibitory effects on inflammasome-associated proteins (**B**, **C**, **E**, **F**) in OGD-injured NSCs. Data are represented as the mean (% of control) ± standard deviation of a minimum of three independent experiments and were analyzed using Tukey's test after a one-way

analysis of variance (**A,** n = 5, **C,** NLRP3, n = 3, cleaved caspase 1, n = 3, IL-1β, n = 3, **D,** n = 3, **F,** NLRP3, n = 3, cleaved caspase 1, n = 4, IL-1β, n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ (vs. the control group), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (vs. the group subjected to OGD alone), $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ (vs. the group subjected to OGD and 0.1 μM naloxone), and $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ (vs. the group subjected to OGD and 1 μM naloxone). PI3K, phosphatidylinositol 3-kinase, OGD, oxygen-glucose deprivation, NSCs, neural stem cells.

Supplementary Files

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- SupplementaryFigure1300dpi.tif
- SupplementaryFigure2300dpi.tif
- SupplementaryFigure3300dpi.tif
- SupplementaryFigure4300dpi.tif