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Original Paper

K6PC-5 Activates SphK1-Nrf2 Signaling to Protect Neuronal Cells from Oxygen Glucose Deprivation/Re-Oxygenation

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Key Words
Neuron • K6PC-5 • SphK1 • Nrf2 • Oxidative stress

Abstract
Background/Aims: New strategies are required to combat neuronal ischemia-reperfusion injuries. K6PC-5 is a novel sphingosine kinase 1 (SphK1) activator whose potential activity in neuronal cells has not yet been tested. ***Methods:*** Cell survival and necrosis were assessed with a Cell Counting Kit-8 assay and lactate dehydrogenase release assay, respectively. Mitochondrial depolarization was tested by a JC-1 dye assay. Expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) signaling components were examined by quantitative real-timePCR and western blotting. ***Results:*** K6PC-5 protected SH-SY5Y neuronal cells and primary murine hippocampal neurons from oxygen glucose deprivation/re-oxygenation (OGDR). K6PC-5 activated SphK1, and SphK1 knockdown by targeted short hairpin RNA (shRNA) almost completely abolished K6PC-5-induced neuronal cell protection. Further work showed that K6PC-5 inhibited OGDR-induced programmed necrosis in neuronal cells. Importantly, K6PC-5 activated Nrf2 signaling, which is downstream of SphK1. Silencing of Nrf2 by targeted shRNA almost completely nullified K6PC-5-mediated neuronal cell protection against OGDR. ***Conclusion:*** K6PC-5 activates SphK1-Nrf2 signaling to protect neuronal cells from OGDR. K6PC-5 might be a promising neuroprotective strategy for ischemia-reperfusion injuries.

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Introduction

During the pathogenesis of stroke, ischemia-reperfusion can severely damage neurons [1, 2]. Ischemia-reperfusion injury can be mimicked in cultured neuronal cells using oxygen glucose deprivation and re-oxygenation (OGDR) [3-6]. OGDR-induced neuronal cell death is mediated by oxidative stress [5, 7], with sustained oxygen glucose deprivation (OGD; often over 1 h) disrupting mitochondrial functions. After re-oxygenation, massive amounts of reactive oxygen species (ROS) are produced, causing oxidative stress, lipid peroxidation, DNA damage, and neuronal cell death [5, 7].

Evidence shows that the mitochondrial permeability transition pore (mPTP) mediates neuronal cell death after oxidative stress [8]. The mPTP is primarily composed of two structural components, the adenine nucleotide translocase-1 (ANT-1) and the voltage-dependent anion transporter (VDAC), as well as the regulatory component, cyclophilin D (Cyp-D) [9-11]. Cyp-D and ANT-1 are found in the inner mitochondrial membrane, whereas VDAC is in the outer mitochondrial membrane [9-11]. Oxidative stress can trigger the translocation of p53 to mitochondria, where it associates with Cyp-D to cause a conformational change in ANT-1, mPTP opening, and eventually cell necrosis (but not apoptosis) [9, 11].

Sphingolipid metabolites, including ceramide, sphingosine, and sphingosine-1-phosphate (S1P), are important signaling determinants of cell survival/death [12]. Sphingosine kinase 1 (SphK1) phosphorylates sphingosine to S1P, which depletes pro-death sphingosine and ceramide but increases the production of pro-survival S1P [13, 14]. Thus, SphK1 activation promotes cell survival [13, 14]. Recent studies have led to the development of a novel, specific, and highly potent SphK1 activator, named K6PC-5 (*N*-(1, 3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide) [15, 16]. Here, we show that activation of SphK1 by K6PC-5 protects neuronal cells from OGDR.

Materials and Methods

Reagents

K6PC-5 was provided by Dr. Fei [17]. Puromycin and cell culture reagents were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies were all provided by Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Shanghai, China).

SH-SY5Y cell culture

Human neuronal SH-SY5Y cells were provided by the iBS Cell Bank of Fudan University (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and the necessary antibiotics. Before OGDR treatment, SH-SY5Y cells were cultured for 5 days with 10 μM retinoic acid (RA) in DMEM plus 10% FBS, 2 mM glutamine, and necessary antibiotics, followed by another 5 days culture in serum-free DMEM with BDNF (brain-derived neurotrophic factor, 50 ng/mL) and glutamine (2 mM).

Primary culture of murine hippocampal neurons

Primary hippocampal neurons were derived from the CA1 hippocampus of C57 mouse E12-E14 embryos. CA1 neurons were plated in poly-lysine-coated 48-well plates at a density of 1×10^5 cells/well in neurobasal medium plus 2% B27, 500 μM L-glutamine, 20 ng/mL trichostatin A (TSA) and antibiotics (P/S). At day8, over 95% of cells were neurons. All animal studies were performed in accordance with the ethical treatment standards of the Institutional Animal Care and Use Committee of Jiangsu University.

Cell survival, apoptosis and death assay

The Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was performed to examine the viability of neuronal cells. The CCK optical density (OD) at 450 nm was recorded. Cell necrosis was tested via examination of the release of lactate dehydrogenase (LDH) to the conditioned medium using a two-step enzymatic reaction LDH assay kit (Takara, Tokyo, Japan). The LDH level in the medium was

normalized to total LDH (medium LDH plus cellular LDH). The TUNEL assay and the caspase-3 activity were described in detail elsewhere [18].

OGDR

The OGDR procedure was as described previously [3]. Briefly, neuronal cells were first placed in an airtight chamber and equilibrated for 10 min with a continuous flux of gas (95% N_2/5% CO_2). The chamber was sealed and placed in an incubator for an additional 4 h of OGD. Cells were then re-oxygenated in the normal aerobic environment. Mock cells were placed in norm-oxygenated DMEM containing glucose.

Mitochondrial immunoprecipitation

The mitochondrial immunoprecipitation (mito-IP) protocol has been detailed previously [3, 19, 20]. Briefly, 10 × 10^6 SH-SY5Y cells per treatment were homogenized by lysis buffer A (250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, and 1mM dithiothreitol). After centrifugation, the cell pellets were resuspended in buffer B (1mL buffer A containing 10μL NP-40) and collected as the mitochondrial fraction. The pre-cleared mitochondrial lysates (600 μg per sample) were incubated overnight with anti-Cyp-D antibody [19, 21]. The mitochondrial immune complexes were then captured by protein A/G beads (Sigma-Aldrich). Cyp-D–p53 associations were then tested by western blotting.

Western blot

Neuronal cells were first incubated with RIPA lysis buffer (Biyuntian, Wuxi, China). Forty micrograms of lysate proteins per lane were separated on 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) gels, then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Suzhou, China)[22]. After blocking, the blots were incubated with the appropriate primary and secondary antibodies. Enhanced chemiluminescence (ECL) reagents (Pierce, Suzhou, China) were applied to visualize the targeted protein bands (based on the molecular weight). The total gray value of each band was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Mitochondrial depolarization assay

JC-1 dye was used to test for mitochondrial membrane potential changes [23]. After mitochondrial depolarization, JC-1 red aggregates form green monomers [24]. Briefly, neuronal cells were stained with JC-1 (10 μg/mL; Invitrogen, Shanghai, China) for 15 min at room temperature. JC-1 green intensity was examined immediately at 550 nm via a fluorescence spectrofluorometer.

Quantitative real-time PCR assay

Cellular RNA was extracted with TRIzol reagents (Promega, Shanghai, China). A SYBR Green PCR kit (Applied Biosystems, Suzhou, China) was used for reverse transcription, and the ABI Prism7600 Fast Real-Time PCR system was used for the quantitative real-time PCR (qPCR) assay. Melting curve analysis was performed to calculate the product melting temperature. Glyceraldehyde-3-phosphatedehydrogenase(*GAPDH*) was always tested as the reference gene. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative mRNA expression. The mRNA primers for *SphK1* were as described previously [25]. The mRNA primers for nuclear factor erythroid 2-related factor 2 (*Nrf2*), heme oxygenase 1 (*HO1*), NAD(P)H quinone oxidoreductase 1 (*NQO1*), γ-glutamylcysteine synthetase catalytic subunit (*GCLC*), and *GAPDH* were also described [26, 27].

Superoxide assay

The cellular superoxide level was tested with a superoxide assay kit (Beyotime Institute of Biotechnology, Wuxi, China) according to the manufacturer's protocol. Briefly, neuronal cells were cultured in 6-well plates at a density of 3×10^5 cells/well. After the applied treatment, the superoxide detection reagent (200 μL/well) was added at 37°C for 20 min. The absorbance was recorded at 450 nm in a 96-well plate reader.

SphK1 activity assay

After the applied treatment, 100 μg lysates per treatment was incubated with 25 μM D-erythro-sphingosine dissolved in 0.1% Triton X-100, 2 mM ATP, and [γ-^{32}P] ATP for 30 min at 37°C [28]. The reaction was terminated via the addition of 20 μL of HCl, plus 800 μL of chloroform/methanol/HCl (100:200:1, v/v). After vortexing, 250 μL of chloroform and 250 μL of KCl were added, and the phases were

separated by centrifugation. The organic layer was dried and resuspended in chloroform/methanol/HCl 37% (100:100:0.2, v/v). Lipids were resolved on silica TLC plates in 1-butanol/acetic acid/water (3:1:1, v/v). Labeled S1P spots were visualized by autoradiography and quantified by scraping and counting in a scintillation counter. SphK1 activity was evaluated as pmol/h/g protein.

Short hairpin RNA

Two lentiviral SphK1 short hairpin RNAs (shRNAs) were provided by Dr. Qin from Nanjing University of TCM [28]. Nrf2 lentiviral shRNA and the scrambled control shRNA were provided by Dr. Jiang from Nanjing Medical University [26]. shRNA lentivirus (10 μL/mL medium per well) was added to neuronal cells for 24 h. Stable cells were selected by puromycin (2.5 μg/mL; Sigma-Aldrich) for another 6 days. Expression of the targeted protein was determined by western blot.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Statistical analysis among different groups was performed via one-way analysis of variance with Scheffe's test using SPSS18.0 software (SPSS Inc., Chicago, IL). Experiments were repeated at least three times and consistent results were always obtained.

Results

K6PC-5 protects neuronal cells from OGDR

SH-SY5Y, the established human neuronal cells [29, 30], were treated with different concentrations of K6PC-5, from 2.5 to 100 μM. The CCK-8 assay results, shown in Fig. 1A, revealed that K6PC-5 was generally safe to SH-SY5Y cells, except at very high concentrations (100 μM). The latter induced a reduction in CCK-8 OD (Fig. 1A). LDH release to the medium is a characteristic marker of cell necrosis. Treatment of SH-SY5Y cells with 100 μM of K6PC-5 induced a significant LDH release (Fig. 1B). At other tested concentrations (2.5, 10, and 25 μM), K6PC-5 failed to induce cell death (Fig. 1B). Recent studies have used K6PC-5 at 10 μM in vitro [17, 31]. This concentration was thus applied for the remaining experiments.

SH-SY5Y cells were subjected to OGD for different periods, from 2 to 6 h. Afterward, the cells were maintained in the normal aerobic environment (re-oxygenation, OGDR) for another 24 h. The CCK-8 assay results confirmed that OGD for 4 and 6 h significantly inhibited SH-SY5Y cell viability (Fig. 1C). Co-treatment with K6PC-5 (10 μM) attenuated the reduction in viability induced by OGDR (Fig. 1C). OGDR-induced LDH release was also significantly inhibited by K6PC-5 (Fig. 1D). These results indicate that K6PC-5 protects SH-SY5Y cells from OGDR.

The effect of the novel SphK1 activator on primary murine hippocampal neurons was next tested. The results showed that K6PC-5 (10 μM) co-treatment of primary neurons dramatically alleviated the OGDR (4 h OGD deprivation, 24 h re-oxygenation)-induced viability reduction (CCK-8 OD decrease, Fig. 1E) and cell necrosis (LDH release, Fig. 1F). These results again suggest that K6PC-5 protects neuronal cells from OGDR.

K6PC-5-mediated neuroprotection against OGDR requires SphK1

SphK1 activity and expression in K6PC-5-treated neuronal cells were tested next. K6PC-5 dose-dependently increased SphK1 activity in SH-SY5Y cells (Fig. 2A) without affecting SphK1 protein expression (Fig. 2B). Conversely, OGDR induced SphK1 expression in SH-SY5Y cells (Fig. 2C and D). SphK1Mrna (Fig. 2C) and protein (Fig. 2D) levels were increased by OGDR treatment.

To test the link between SphK1 activation and K6PC-5-mediated neuroprotection, the shRNA method was applied to knockdown SphK1. Two different lentiviral shRNAs against non-overlapping sequences (Seq-a/-b) of SphK1 were provided by Dr. Qin [28]. Each of the SphK1 lentiviral shRNAs led to dramatic downregulation of SphK1 in OGDR-treated SH-SY5Y cells (Fig. 2E and F). SphK1mRNA (Fig. 2E) and protein (Fig. 2F) levels were largely

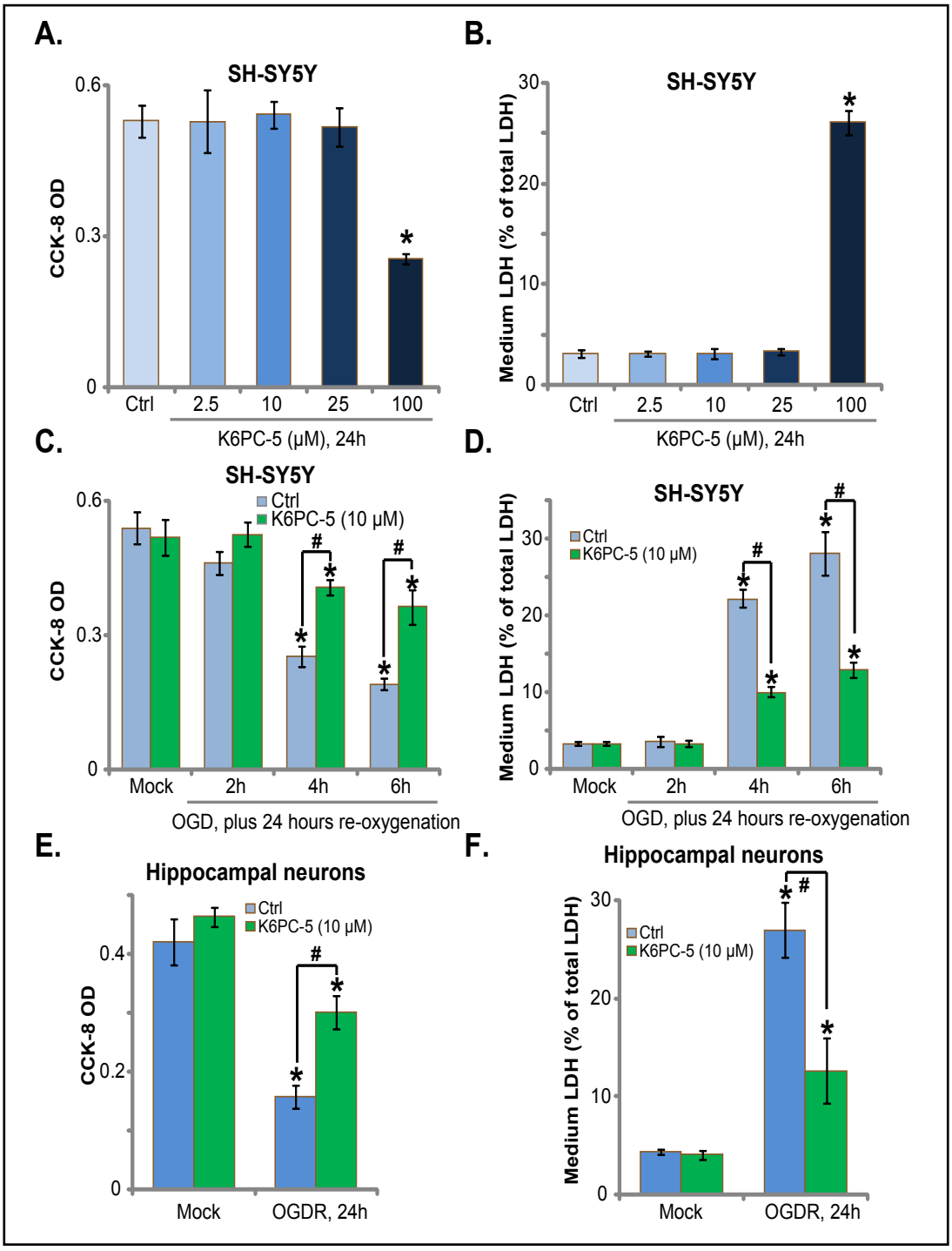

Fig. 1. K6PC-5 protects neuronal cells from OGDR. SH-SY5Y cells (A-D) or primary murine hippocampal neurons (E and F) were treated with the indicated concentrations of K6PC-5, with or without OGD; cells were further cultured in a normal aerobic environment (re-oxygenation, OGDR) for an additional 24 h. Cell viability was tested by a CCK-8 assay (A, C, and E), whereas cell necrosis was analyzed by an LDH release assay (B, D, and F). Ctrl indicates the untreated control group (same for all figures). Mock indicates the mock treatment group (no OGDR, regular medium) (same for all figures). Bars indicate the mean ± SD (n=5). *p<0.05 vs. Ctrl. #p<0.05 vs. OGDR only. Each experiment was repeated four times and similar results were obtained.

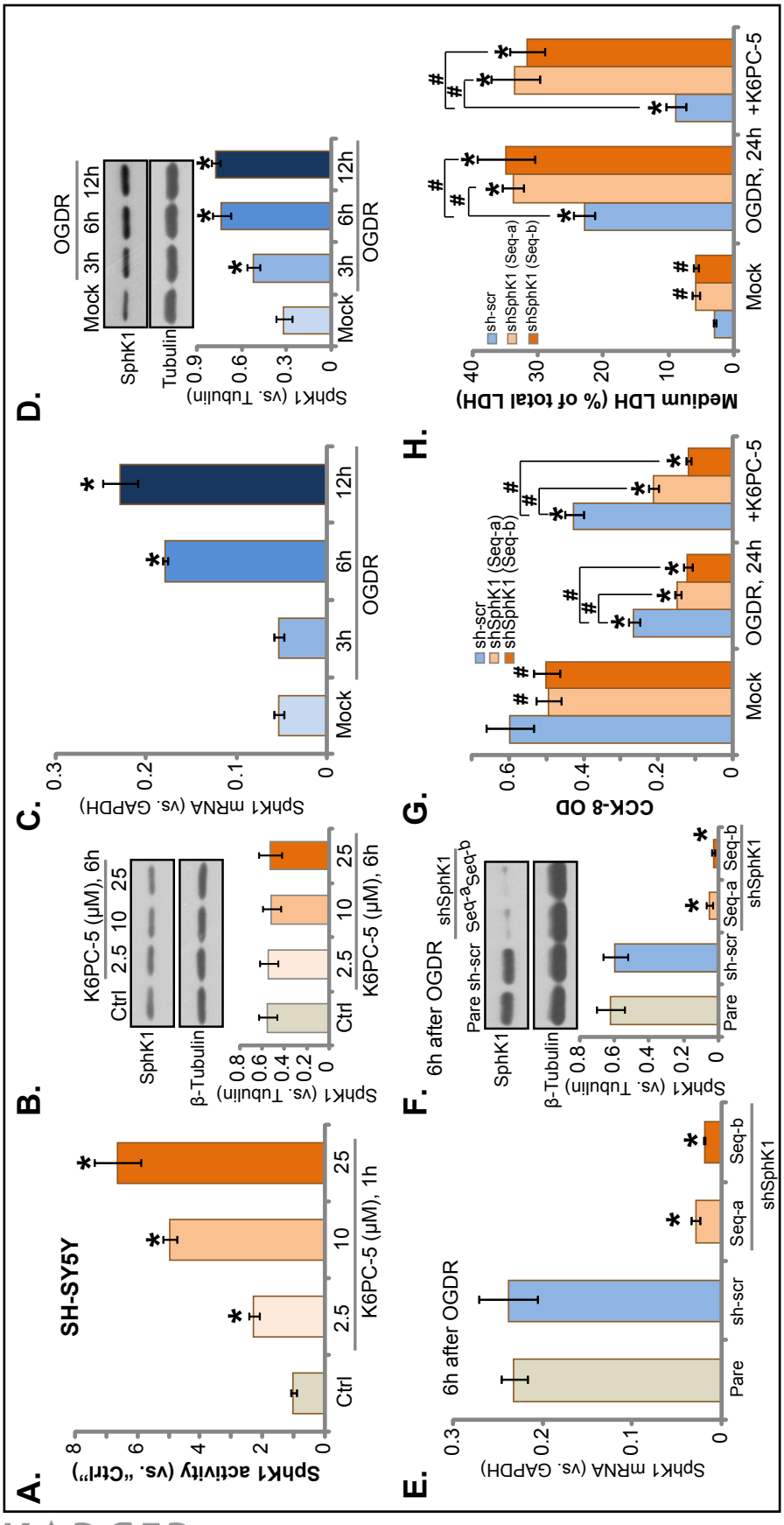

Fig. 2. K6PC-5-mediated neuroprotection against OGDR requires SphK1. SH-SY5Y cells were treated with the indicated concentrations of K6PC-5 for the indicated times and then the relative SphK1 activity (A) and protein expression (B) were tested. SH-S5Y5 cells were subjected to OGDR (OGD for 4 h) for the indicated times and SphK1mRNA (C) and protein (D) expression were measured. Stable SH-SY5Y cells, expressing scrambled control shRNA (sh-scr) or SphK1 shRNA (Seq-a/-b), as well as the parental SH-SY5Y cells (Pare), were subjected to OGDR with or without K6PC-5 (10 μM) for the indicated times and SphK1mRNA (E) and protein (F) expression were determined. Cell survival (CCK-8 assay, G) and death (LDH release assay, H) were additionally tested. Five sets of western blotting data were quantified (B, D, and F, lower panel). Bars indicate the mean ± SD (n=5). *p<0.05 vs. Ctrl/Mock. #p<0.05 vs. sh-scr cells (G and H). Each experiment was repeated three times and similar results were obtained.

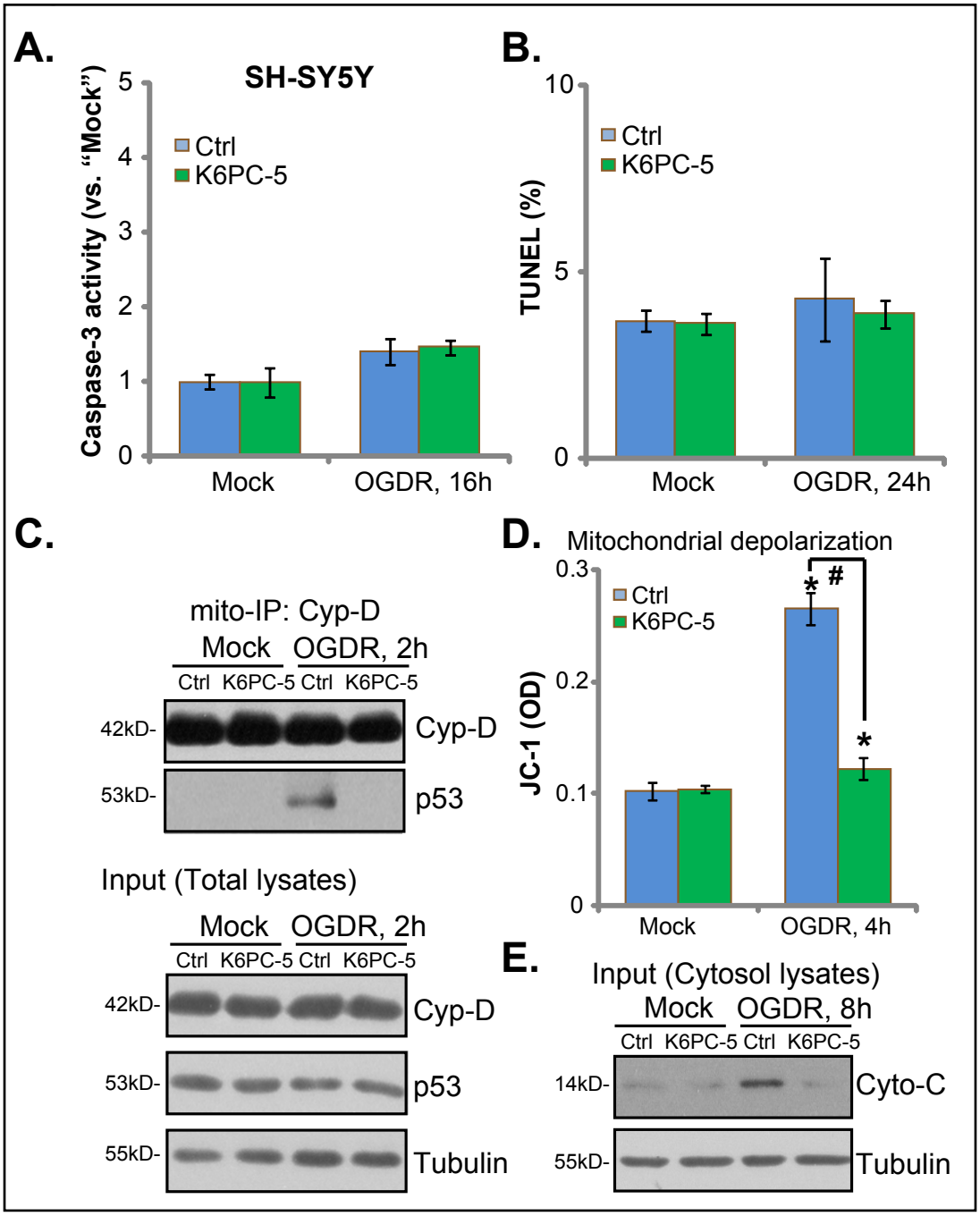

Fig. 3. K6PC-5 inhibits OGDR-induced programmed necrosis in neuronal cells. SH-SY5Y cells were subjected to OGDR with or without K6PC-5 (10 μM) for the indicated times and he caspase-3 activity (A), nuclear TUNEL percentage (B), mitochondrial p53–Cyp-D association (C, "mito-IP"), mitochondrial depolarization (JC-1 dye assay, D), and expression of the listed proteins (C, "Input", and E) were tested by the appropriate assays. Bars indicate the mean ± SD(n=5). *p<0.05 vs. Mock. #p<0.05. Each experiment was repeated four times and similar results were obtained.

inhibited. Importantly, OGDR-induced cytotoxicity (CCK-8 OD reduction and LDH release) was increased in SH-SY5Y cells treated with SphK1 shRNA (Fig. 2G and H). Furthermore, K6PC-5-mediated neuroprotection against OGDR was almost completely abolished in SphK1-silenced cells (Fig. 2G and H). Based on these results, we propose that SphK1 is important to combat OGDR. K6PC-5 activated SphK1 to protect SH-SYRY cells from OGDR. The scrambled control shRNA (sh-scr), as expected, did not affect SphK1 expression (Fig. 2E and F) nor OGDR-induced cytotoxicity (Fig. 2G and H).

K6PC-5 inhibits OGDR-induced programmed necrosis in neuronal cells

Studies have shown that OGDR mainly induces cell programmed necrosis (not apoptosis [5, 19, 32]). We showed that OGDR failed to increase caspase-3 activity (Fig. 3A) or nuclear terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining percentage (Fig. 3B) in SH-SY5Y cells. OGDR induces p53 translocation to mitochondria, where it associates with Cyp-D[19, 32]. The mitochondrial p53–Cyp-D association then triggers mitochondrial depolarization, mPTP opening, cytochrome C (Cyto-C) release, and eventually cell necrosis [19, 32]. Here, OGDR treatment of SH-SY5Y cells induced the mitochondrial p53–Cyp-D association (Fig. 3C), mitochondrial depolarization (JC-1 OD increase, Fig. 3D), and Cyto-C release (Fig. 3E). Importantly, such effects of OGDR were significantly attenuated by K6PC-5 (Fig. 3C-E). p53 and Cyp-D expression levels in total cell lysates were unaffected (Fig. 3C, "Input"). These results suggest that K6PC-5-induced neuronal cell protection against OGDR might occur through inhibition of the programmed necrosis pathway.

K6PC-5 activates Nrf2 signaling in neuronal cells

Nrf2 disassociates from its inhibitor protein Keap1, causing Nrf2 stabilization and nuclear translocation. Nrf2 then binds to antioxidant response element (ARE) in the nucleus, mediating the transcription and expression of several key antioxidant genes, including NQO1, HO1 and GCLC [33-36]. Here, we showed that the mRNA expression levels of the Nrf2-dependent genes NQO1 (Fig. 4A), HO1 (Fig. 4B), and GCLC (Fig. 4C) were significantly elevated after K6PC-5 treatment of SH-SY5Y cells. However, the Nrf2 mRNA level was unchanged (Fig. 4D). The protein expression levels of NQO1, HO1, and GCLC were also increased by K6PC-5 in SH-SY5Y cells (Fig. 4E). Additionally, the Nrf2 protein level was increased by K6PC-5 treatment (Fig. 4E), indicating Nrf2 protein stabilization. The latter is an essential step forNrf2 nuclear translocation and activation [33-36].

Next, we tested whether SphK1 is required for K6PC-5-induced Nrf2 activation. The qPCR assay results in Fig. 4F and G show that K6PC-5-induced expression of NQO1 mRNA and HO1 mRNA were largely inhibited by SphK1 shRNA (Seq-a). K6PC-5-induced upregulations of NQO1, HO1, and GCLC proteins were also inhibited (Fig. 4H). These results suggest that SphK1 is required for K6PC-5-induced Nrf2 activation in SH-SY5Y cells. In the primary murine hippocampal neurons, K6PC-5 induced upregulation of Nrf2, NQO1, HO1, and GCLC proteins (Fig. 4I). These results again confirmed that K6PC-5activates Nrf2 signaling in neuronal cells.

Nrf2 knockdown inhibitsK6PC-5-mediated neuroprotection against OGDR

Further studies showed that exposure of SH-SY5Y cells to OGDR also induced minor Nrf2 activation, Nrf2-dependent mRNAs (NQO1, HO1, and GCLC) were slightly increased in OGDR-treated cells (Fig. 5A-C, $p< 0.05$ vs. Mock cells). The Nrf2 mRNA level was unchanged (Fig. 5D). shRNA-mediated knockdown of Nrf2 in SH-SY5Y cells (Fig. 5D) blocked OGDR-induced NQO1, HO1, and GCLC mRNA expression (Fig. 5A-C). Compared with SH-SY5Y cells treated with the control shRNA, the OGDR-induced increase in the superoxide level (Fig. 5E) was significantly potentiated in Nrf2-silenced SH-SY5Y cells. Importantly, Nrf2-silenced SH-SY5Y cells were more vulnerable to OGDR, presenting with lower viability (Fig. 5F) and increased cell necrosis (LDH release, Fig. 5G). These results imply that OGDR induced minor but nonetheless significant Nrf2 activation to counteract neuronal cell necrosis. In contrast, Nrf2 knockdown intensified neuronal cell death by OGDR.

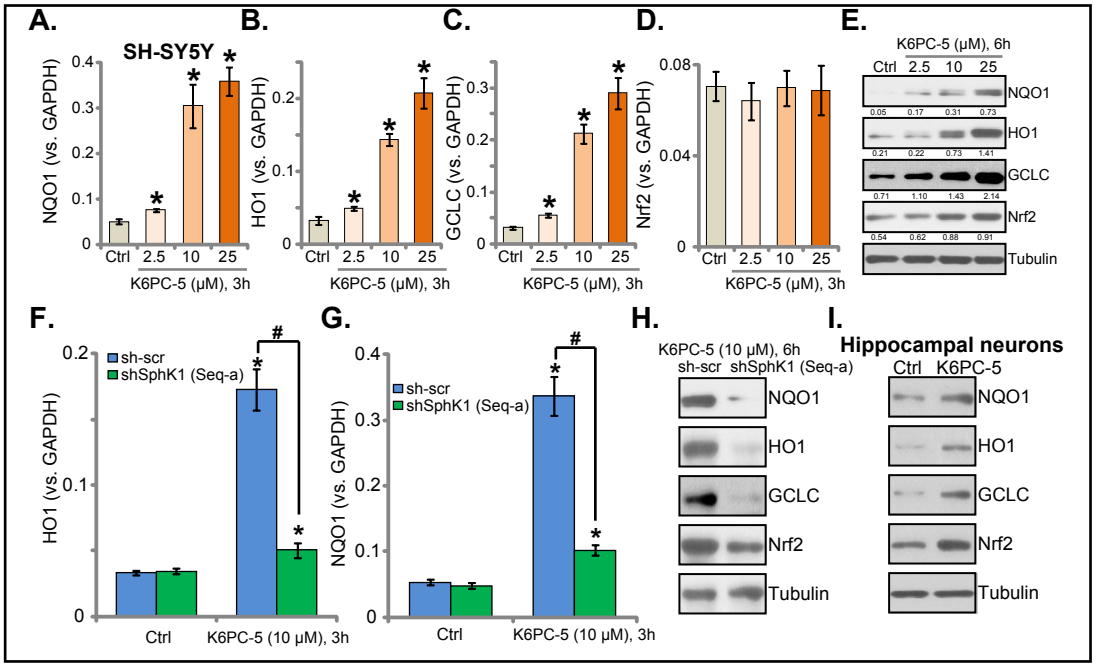

Fig. 4. K6PC-5 activates Nrf2 signaling in neuronal cells. SH-SY5Y cells, with or without lentiviral scrambled control shRNA (sh-scr) or the SphK1 shRNA (Seq-a, sh-SphK1), were treated with the indicated concentrations of K6PC-5 for the indicated times and the mRNA and protein expression levels of the examined Nrf2-related genes were tested by qPCR assay (A-D, F, and G) and western blot (E and H), respectively. The primary murine hippocampal neurons were treated with K6PC-5 (10 μM) for 6 h, with the expression determined of the listed proteins (I). Quantification of western blot results (E). Bars indicate the mean ± SD (n=5). *p<0.05 vs. Ctrl. #p<0.05 (F and G). Each experiment was repeated four times and similar results were obtained.

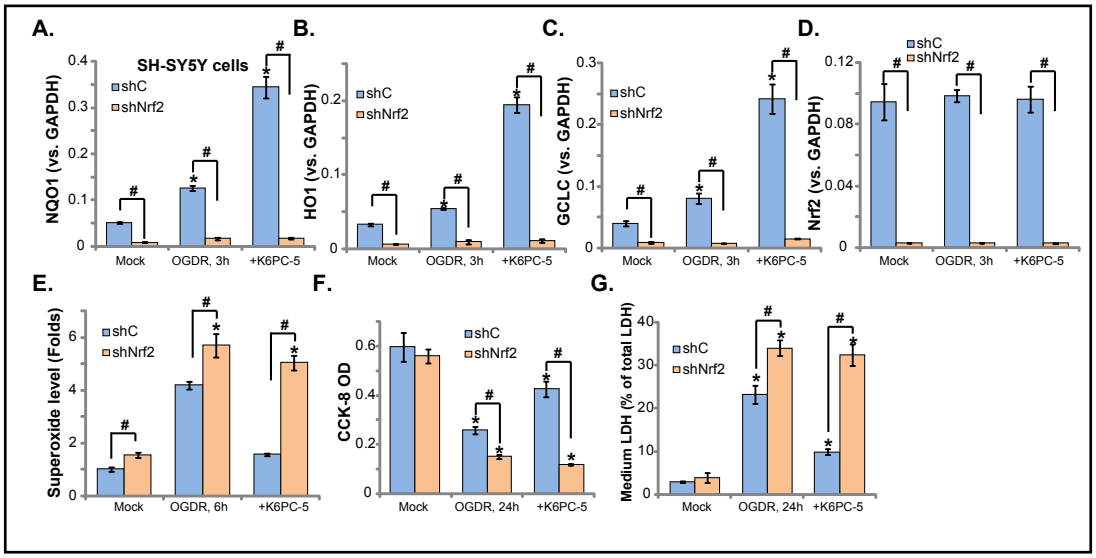

Fig. 5. Nrf2 knockdown inhibits K6PC-5-mediated neuroprotection against OGDR. SH-SY5Y cells, with lentiviral scrambled control shRNA (shC) or Nrf2 shRNA (shNrf2), were treated with K6PC-5 (10 μM) and/or OGDR for the indicated time: the expression levels of the listed mRNAs were examined by qPCR assay (A-D). The superoxide level (E) was also tested, as well as cell viability (CCK-8 assay, F) and necrosis (LDH release assay, G). Bars indicate the mean ± SD(n=5). *p<0.05 vs. Mock treatment of shC cells. #p<0.05. Each experiment was repeated three times and similar results were obtained.

Notably, K6PC-5 had no effect on the OGDR-induced superoxide increase (Fig. 5E) in Nrf2-silenced SH-SY5Y cells. More importantly, K6PC-5-mediated neuroprotection against OGDR was almost completely nullified by Nrf2 shRNA in SH-SY5Y cells (Fig. 5Fand G). These results suggest that Nrf2 activation is required for K6PC-5-induced anti-OGDR neuroprotective activity.

Discussion

In this study, we showed that K6PC-5, a SphK1 activator, efficiently protected SH-SY5Y cells and primary hippocampal neurons from OGDR. K6PC-5 activated SphK1, which is required for its activity in neuronal cells, with SphK1 knockdown by targeted shRNA almost completely abolishing K6PC-5-mediated neuroprotection. Considering the upregulation of SphK1 in OGDR-treated cells, our results suggest that SphK1 could be an important neuroprotection target for ischemic injury.

mPTP opening triggers mitochondrial swelling, outer membrane leakage, and Cyto-C release, which have been linked to cell apoptosis alone [37, 38]. However, this theory has recently been challenged. First, apoptosis is an active and ATP-consuming process, but mPTP disruption leads to ATP depletion [11]. Second, Cyp-D knockout mice show normal development, indicating that extensive apoptosis during development is not affected in Cyp-D knockout mice [8]. Third, cell apoptosis by pro-apoptotic Bcl-2 proteins is unaffected in Cyp-D-depleted cells [8, 39, 40]. Further studies have shown that *in vitro* apoptosis is not inhibited in cells with Cyp-D depletion. However, oxidative stress- and calcium overload-induced *in vitro* cell necrosis is clearly suppressed [8, 11, 39-41]. Thus, the current view is that the mitochondrion is the key hub of both apoptosis and necrosis through a mechanism involving the mPTP [9-11, 38].

A number of studies have confirmed that programmed necrosis, an active necrosis pathway, mediates cell death by OGDR and oxidative stress [3, 5, 19, 32]. OGDR induces ROS production and oxidative stress, which lead to p53 translocation to the mitochondria to form a complex with Cyp-D. The complex then triggers mitochondrial depolarization, mPTP opening, Cyto-C release, and eventually cell necrosis [3, 5, 19, 32]. In the present study, we show that treatment with K6PC-5 blocked the programmed necrosis pathway by OGDR in neuronal cells. This could explain the superior neuroprotective activity of this SphK1 activator.

Nrf2 is arguably one of the most important endogenous antioxidant signaling factors [33-36]. Activated Nrf2 splits from its inhibitor protein Keap1, causing its translocation to the cell nucleus. Freed Nrf2 binds to ARE, triggering the transcription and expression of multiple genes, including the endogenous antioxidant genes, phase II detoxification enzymes, and other cellular defensive genes [33-36]. Our results showed that activation of SphK1 by K6PC-5 also activated Nrf2 signaling in neuronal cells. K6PC-5 treatment of neuronal cells induced Nrf2 protein stabilization as well as transcription of Nrf2-regulated genes (*NQO1*, *HO1*, and *GCLC*). Importantly, activation of Nrf2 is required for K6PC-5-mediated neuroprotection against OGDR. Nrf2 shRNA not only exacerbated OGDR-induced injury in neuronal cells, but also abolished K6PC-5-mediated neuronal cell protection. Furthermore, SphK1 shRNA in SH-SY5Y cells almost entirely blocked Nrf2 activation by K6PC-5, suggesting that K6PC-5-activated SphK1 acts upstream of Nrf2 in neuronal cells.

Conclusion

Taken together, our results suggest that K6PC-5 activates SphK1-Nrf2 signaling to protect neuronal cells from OGDR.

Acknowledgements

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Disclosure Statement

The authors declare no conflicts of interest.

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