

Hypoxic Preconditioning Protects SH-SY5Y Cell against Oxidative Stress through Activation of Autophagy

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Xiaomu Tan^{1,2,3}, Sherwin Azad⁴, and Xunming Ji²

Abstract

Oxidative stress plays a role in many neurological diseases. Hypoxic preconditioning (HPC) has been proposed as an intervention that protects neurons from damage by altering their response to oxidative stress. The aim of this study was to investigate the mechanisms by which HPC results in neuroprotection in cultured SH-SY5Y cells subjected to oxidative stress to provide a guide for future investigation and targeted interventions. SH-SY5Y cells were subjected to HPC protocols or control conditions. Oxidative stress was induced by H₂O₂. Cell viability was determined via adenosine triphosphate assay. Rapamycin and 3-methylxanthine (3-MA) were used to induce and inhibit autophagy, respectively. Monodansylcadaverine staining was used to observe the formation of autophagosomes. Levels of Microtubule-associated protein light chain 3 B (LC3B), Beclin 1, and p53 were measured by Western blot. Reactive oxygen species (ROS) were also determined. Cell viability in the HPC group following 24-h exposure to 600 μM H₂O₂ was 65.04 ± 12.91% versus 33.14 ± 5.55% in the control group. LC3B, Beclin 1, and autophagosomes were increased in the HPC group compared with controls. Rapamycin mimicked the protection and 3-MA decreased the protection. There was a moderate increase in ROS after HPC, but rapamycin can abolish the increase and 3-MA can enhance the increase. p53 accumulated in a manner consistent with cell death, and HPC-treated cells showed reduced accumulation of p53 as compared with controls. Treatment with rapamycin decreased p53 accumulation, and 3-MA inhibited the decrease in p53 induced by HPC. HPC protects against oxidative stress in SH-SY5Y cells. Mechanisms of protection may involve the activation of autophagy induced by ROS generated from HPC and the following decline in p53 level caused by activated autophagy in oxidative stress state. This is in line with recent findings in nonneuronal cell populations and may represent an important advance in understanding how HPC protects neurons from oxidative stress.

Keywords

oxidative stress, hypoxic preconditioning (HPC), autophagy, p53, reactive oxygen species

Introduction

Accumulation of reactive oxygen species (ROS) within cells is a well-known factor that contributes to the development of many neurological diseases such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and stroke among others^{1–3}. ROS such as superoxide, hydrogen peroxide (H₂O₂), and hydroxyl radicals are considered important stressors that induce neuronal damage and cell death through oxidation of macromolecules such as DNA, enzymatic proteins, and membrane lipids. Consequently, events such as disruption in energy production via loss of mitochondrial membrane potential and release of cytochrome c further activate proapoptotic signaling pathways such as caspase-mediated cell death. Therefore, inhibition of oxidative stress-induced damage is an important target for clinical treatment of these neurological diseases.

¹ Beijing Key Laboratory of Hypoxia Conditioning Translational Medicine, Xuanwu Hospital, Capital Medical University, Beijing, China

² Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Beijing, China

³ Department of Neurology, Beijing Luhe Hospital, Capital Medical University, Beijing, China

⁴ Department of Neurosurgery, Wayne State University School of Medicine, Detroit, MI, USA

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Corresponding Author:

Xunming Ji, Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, No. 45 Changchun Street, Xicheng District, Beijing 100069, China.

Email: jixm@ccmu.edu.cn



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Preconditioning is an important endogenous mechanism observed in a wide number of cell populations, whereby exposure to a nonlethal dose of a noxious stimuli induces protection against further exposure to the same stimuli⁴. This inducible response to a stressor, such as hypoxia, might offer a partial explanation for the protective effect of exercise in preventing the development of disease among other important implications⁵. As such, hypoxic preconditioning (HPC) has been the subject of intense investigation throughout the medical and scientific community to harness the therapeutic potential it may afford⁶. Numerous studies have indeed confirmed that a sublethal hypoxic pretreatment improves neuronal survival during exposure to a later state of oxidative stress both *in vitro* and *in vivo*^{7,8}.

Despite the proven benefits, clinical applications to date have been limited by a number of factors. Among these are the need for identification and pretreatment of patient populations at risk of a given disease, for example, stroke, as well as the possibility of treatment-related adverse effects in high-risk populations, which may have multiple comorbidities including hypertension, diabetes mellitus, and coronary heart disease. Indeed, the desire to “first, do no harm” results in a narrow therapeutic window, which varies not only with the level of existing disease but also according to patient-specific factors such as age, gender, and sex⁶. Understanding the mechanisms that underlie the damage caused by oxidative stress as well as the protection induced by HPC could allow for the development of targeted therapeutic interventions and drugs, which overcome these limitations.

In recent years, there has been increasing appreciation for how oxidative stress contributes to aberrant protein accumulation and upregulation of cell death transcription factors in several neurodegenerative diseases⁹. Among others, the tumor protein p53 has been implicated in animal models of injury and disease as one such protein that may accumulate and cause cell death¹⁰. It is well known that p53 promotes apoptosis through transcriptional activation of specific target genes or by directly affecting mitochondrial pathways^{11,12}. As a transcription factor, p53 upregulates proapoptotic genes such as Bcl-2-Associated X (*Bax*), *Noxa*, and *PUMA*¹³. In addition, p53 can interact with Bax and Bcl-2 homologous antagonist/killer (Bak) to induce opening of the outer mitochondrial membrane¹⁴. The autophagy pathway provides a mechanism for degradation of proteins and serves to keep in check their accumulation¹⁵; and thus it can be seen why autophagy has been shown to promote cell survival under diverse stress conditions^{16,17}. Several studies have shown a link between increased autophagy and neuroprotection, for example, finding that HPC can activate autophagy to alleviate Methyl-4-phenylpyridinium (MPP)⁺/ethanol-induced neuronal death^{18,19}. However, questions remain about how HPC promotes oxidative stress resistance through its activation of autophagy. Specifically, the downstream effects of autophagy activation and the effects on individual proteins that may be degraded are

still unclear. Tumor protein p53, well known as a regulator of cell survival, was proposed as one protein that may be impacted by increased autophagy in neurons in yet unknown ways with important ramifications. Thus, the goal of the current study was to investigate the interaction, if one exists, between increased autophagy as a result of HPC and downstream effect or proteins such as p53.

Materials and Methods

Materials

Hypoxia Incubator Chamber (Stemcell Technologies, Vancouver, Canada 27310), Single Flow Meter (Stemcell Technologies, 27311), hypoxic gas (3% O₂, 5% CO₂, and 92% N₂, Beijing Yongsheng Gas Technology Limited Company, Beijing, China), H₂O₂ (Sigma-Aldrich, St. Louis, MO, USA H1009), rapamycin (Sigma-Aldrich, V900930), 3-methoxyanthine (3-MA; Sigma-Aldrich, M9281), anti-β-actin antibody (TransGen Biotech, Beijing, China HC201-01), anti-LC3B antibody (Cell Signaling Technology, Inc., Danvers, MA, USA 3868), anti-Becclin 1 antibody (Cell Signaling, 3495), anti-p53 antibody (Cell Signaling, 2524), IRDye[®] 800CW Goat anti-rabbit IgG (LI-COR, Lincoln, NE, USA 926-32211), IRDye[®] 800CW Goat anti-mouse IgG (LI-COR, 926-32210), CellTiter-Glo[®] Luminescent Cell Viability Assay Kit (Promega Corporation, Madison, WI, USA G7571), Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA 11995073), monodansylcadaverine (MDC; Sigma-Aldrich, D4008), and ROS Assay Kit (Beyotime, Shanghai, China S0033) were used.

Cell Culture and HPC

Human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection. The cells were maintained in DMEM, containing 15% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO₂ in a humidified incubator (Thermo Fisher Scientific, HEARCELL 150i). For HPC, cultured cell dishes were put into a sealed chamber. The chamber was then purged with a gas mixture of 3% O₂, 5% CO₂, and 92% N₂ at the speed of 20 L/min for 4 min. The cells were treated under this hypoxic condition for 4, 8, 16, or 24 h and subsequently recovered under normoxic conditions for 24 h. To induce oxidative stress, the cells were treated with H₂O₂ (600 μM) for 24 h.

Determination of Cell Viability

Cell viability was determined by adenosine triphosphate (ATP) assay, previously reported as a useful method²⁰. The CellTiter-Glo[®] Luminescent Cell Viability Assay Kit was used. Cells were seeded in the 96-well tissue culture plates, incubated for 24 h, and then exposed to 600-μM H₂O₂ for 24 h with or without HPC. After the H₂O₂ exposure, 100 μL of the CellTiter-Glo[®] mixture was added to each well, and culture plates were incubated at room temperature for an

additional 10 min. At that time, the ATP level was quantified by aluminometer. Viability was calculated using the background-corrected emission as follows:

$$\text{Viability (\%)} = \frac{\text{ATP of experiment well}}{\text{ATP of control well}} \times 100.$$

Western Immunoblotting

Cells were collected and washed with phosphate-buffered saline (PBS; pH 7.4) and lysed with radioimmunoprecipitation assay buffer, 1% Nonidet P-40 (NP-40), 150 mM NaCl, 50 mM Tris (pH 8.0), 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid sodium, 1 mM sodium orthovanadate, 0.1 mg/mL phenylmethylsulfonyl fluoride, and 3% aprotinin, on ice for 10 min; following which solubilized cells were centrifuged and the supernatant was collected. Protein concentration was determined for supernatant samples, and aliquots of the protein samples (20 to 40 μ g) were loaded into the lanes of an SDS-polyacrylamide gel. The protein samples were separated by electrophoresis, and the separated proteins were transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in tris-buffered saline tween-20 (TBST; 0.02 M Tris, 0.008% NaCl, pH 7.4, and 0.1% Tween-20) at room temperature for 1 h. Subsequently, the membranes were probed with primary antibodies directed against target proteins for 2 h at room temperature or overnight at 4 °C. After 3 quick washes in TBST, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase diluted at 1:10,000 in TBST for 1 h. Immune complexes were detected by enhanced chemiluminescence method (Odyssey GLx). The blots were stripped and reprobed with an anti- β -actin antibody. Images of immunoblots were documented using Image Studio Software 3.2 version (Odyssey CLx; LI-COR), and the intensity of specific proteins was quantified using the same software.

Determination of ROS

Cells were seeded in the 96-well tissue culture plates, incubated for 24 h, and then exposed to different treatments. ROS were determined by ROS Assay Kit with fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). As a nonpolar compound, DCFH-DA can cross the cell membrane, be deesterified intracellularly, and turn to 2',7'-DCFH, which is nonfluorescent and cannot cross the cell membrane. In the presence of intracellular ROS, DCFH is quickly oxidized to highly fluorescent 2',7'-dichlorofluorescein. This fluorescence can be used for quantifying intracellular ROS production by a fluorescence microplate reader with excitation and emission wavelengths at 488 nm and 525 nm, respectively.

MDC Staining

MDC is a fluorescent compound known to specifically label autophagic vacuoles. SH-SY5Y cells were stained with

50- μ M MDC and incubated at 37 °C for 30 min. After incubation, the cells were washed once with PBS and fixed with 4% paraformaldehyde. Following fixation, the samples were analyzed by confocal microscopy (Leica, Wetzlar, Germany, SP5) with excitation wave 335 nm and emission at 525 nm. The intensity of the sample was quantified by the Image-Pro Plus 6.0 software.

Statistical Analysis

Data are presented as mean \pm standard deviation (SD). Analysis of variance (ANOVA) was used to test differences in means among the treatment groups, and a $P < 0.05$ was considered statistically significant. For cases in which significant differences were detected, specific post hoc comparisons between treatment groups were examined by Student–Newman–Keuls tests. All the analyses were performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

HPC protects SH-SY5Y cells from oxidative stress induced by H₂O₂. SH-SY5Y cells were exposed to 4 levels of H₂O₂ concentration (200 μ M, 400 μ M, 600 μ M, and 800 μ M) for 3 different time periods (12, 24, and 48 h) to generate oxidative stress-induced cell death, following which cell viability was determined. The results showed that increased cell death occurred when higher concentration and longer treatments of H₂O₂ were applied (Fig. 1A). The 24-h exposure to 600- μ M H₂O₂ was selected as a model for oxidative stress for further experiments based on the fact that it reliably induced the death of approximately 65% of cells (Fig. 1A). To test the protective effects of HPC against oxidative stress, cells were pretreated with different hypoxic conditions, containing 1%, 3%, or 5% O₂, and 5% CO₂ balanced with N₂. We found that 3% O₂ for 8 h followed with normoxic recovery for 24 h showed the best protective effect with 65.04 \pm 12.91% cell viability in this group ($n = 4$), compared with 33.14 \pm 5.55% in the control group ($n = 4$, $P < 0.001$; Fig. 1B).

Hypoxia Activates Autophagy

Activation of autophagy by HPC has been reported to play a crucial protective role in vivo and in vitro^{18,21}; however, the exact mechanism of the protection against oxidative stress is unclear. During autophagy activation, cytoplasmic LC3I is processed and binds to the membrane of the autophagosome, where it is lipidated to Microtubule-associated protein light chain 3 II (LC3II); LC3 lipidation has proven to be a useful method to quantify activation of autophagy. Microtubule-associated protein light chain 3 (LC3) has 3 subtypes, but only LC3BII is correlated with autophagic activity. Another commonly used marker of autophagy is Beclin 1, which is a critical regulator involving autophagosome maturation and endocytic transportation. Western blotting for LC3B and

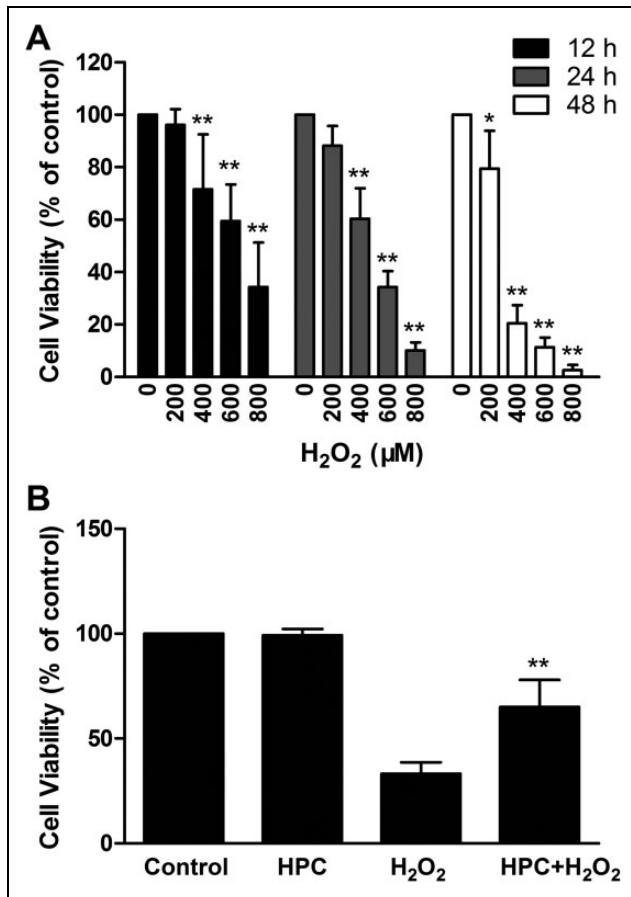


Fig. 1. Hypoxic preconditioning (HPC) has a protective effect on H₂O₂-induced cytotoxicity in SH-SY5Y cells. (A) Cell viability as a function of H₂O₂ treatment. SH-SY5Y cells were treated with 0 (control), 200-μM, 400-μM, 600-μM, or 800-μM H₂O₂ for 12, 24, or 48 h. (B) Effects of HPC on cell viability following treatment with 600-μM H₂O₂ for 24 h. HPC protocol consisted of pretreatment with 3% O₂ for 8 h followed with normoxic recovery for 24 h. Cell viability after normoxic recovery was near 100% in HPC-treated cells ($n = 4$). Following treatment with 600-μM H₂O₂ for 24 h, cell viability in HPC-treated cells was significantly higher than those that received no treatment. Data represent mean \pm SD, trial $n = 4$. ** $P < 0.01$ versus the control group (A). ** $P < 0.01$ versus the H₂O₂-treated group (B).

Beclin 1 in cells treated with 3% oxygen for 4 to 24 h showed HPC results in a time-dependent increase in LC3BII and Beclin 1 (Fig. 2A and B, $n = 4$, LC3BII/I-fold change vs. control was 1.24 ± 0.22 in 4-h group [$P = 0.226$], 1.79 ± 0.22 in 8-h group [$P = 0.002$], 2.24 ± 0.25 in 16-h group [$P < 0.001$], and 3.94 ± 0.45 in 24-h group [$P < 0.001$]; Beclin 1-fold change versus control was 1.31 ± 0.29 in 4-h group [$P = 0.142$], 2.23 ± 0.26 in 8-h group [$P < 0.001$], 2.84 ± 0.43 in 16-h group [$P < 0.001$], and 3.52 ± 0.25 in 24-h group [$P < 0.001$]). MDC staining for autophagic vacuoles revealed an increase in fluorescence intensity after 8-h exposure to HPC (Fig. 2C, $n = 4$, fluorescence intensity in HPC group [1.69 ± 0.23] vs. control group: $P = 0.004$).

Together, these results suggest HPC activates autophagy in SH-SY5Y cells.

Inactivation of Autophagy Abolished the Protective Effect of HPC

3-MA, an inhibitor of autophagosome formation, was added to HPC-treated cells to determine whether autophagy contributes to HPC-induced protection against H₂O₂ stress. Treatment with 3-MA (5 mM) during HPC abolished the protective effect of HPC (Fig. 3A, $n = 4$, cell viability of 3-MA + HPC + H₂O₂ group vs. HPC + H₂O₂ group: $40.05 \pm 8.03\%$ vs. $67.74 \pm 10.21\%$, $P < 0.001$; cell viability of 3-MA + HPC + H₂O₂ group vs. DMSO + H₂O₂ group: $40.05 \pm 8.03\%$ vs. $32.97 \pm 5.24\%$, $P = 0.154$). As a positive control, rapamycin (100 nM), an activator of autophagy, mimicked the protective effect of HPC against H₂O₂-induced cell death (Fig. 3A, $n = 4$, cell viability of rapamycin + H₂O₂ group vs. HPC + H₂O₂ group: $58.18 \pm 7.81\%$ vs. $67.74 \pm 10.21\%$, $P = 0.058$; cell viability of rapamycin + H₂O₂ group vs. DMSO + H₂O₂ group: $58.18 \pm 7.81\%$ vs. $32.97 \pm 5.24\%$, $P < 0.001$). At the protein level, rapamycin indeed activated autophagy (increased LC3B lipidation); and 3-MA inhibited autophagy (decreased LC3B lipidation; Fig. 3B, $n = 4$, LC3BII/I-fold change of rapamycin group vs. HPC group: 1.75 ± 0.13 vs. 1.73 ± 0.11 , $P = 0.882$; LC3BII/I-fold change of 3-MA + HPC group vs. HPC group: 0.75 ± 0.18 vs. 1.73 ± 0.11 , $P < 0.001$). MDC staining showed a similar result (Fig. 3C, $n = 4$, fluorescence intensity of rapamycin group vs. HPC group: 1.63 ± 0.26 vs. 1.62 ± 0.24 , $P = 0.944$; fluorescence intensity of 3-MA + HPC group vs. HPC group: 0.82 ± 0.12 vs. 1.62 ± 0.24 , $P < 0.001$). These results suggested that the activation of autophagy by HPC played a key functional role for the HPC against oxidative stress in SH-SY5Y cells. At the same time, ROS were also detected to determine whether the activation of autophagy is relative to it (Fig. 3D, $n = 4$). HPC can moderately increase the level of ROS (Fig. 3D, ROS-fold change of HPC group versus control group was 1.31 ± 0.15 [$P = 0.029$]). But rapamycin can eliminate the increase (Fig. 3D, ROS-fold change of rapamycin + HPC group vs. control group was 0.97 ± 0.15 [$P > 0.05$]), and 3-MA make the increase more obviously (Fig. 3D, ROS-fold change of 3-MA + HPC group vs. control group was 1.82 ± 0.23 [$P < 0.01$], and the difference between 3-MA + HPC group and HPC group was also statistically significant (1.82 ± 0.23 vs. 1.31 ± 0.15 , $P < 0.01$). The result of ROS detection suggested that ROS might play a role in the course of activation of autophagy by HPC in SH-SY5Y cells.

HPC Downregulates p53

p53 is a key regulatory protein with numerous functions including the signaling of cell cycle arrest and DNA repair activation; high expression of p53 often induces cell death^{22,23}. Previous reports have indicated a link between

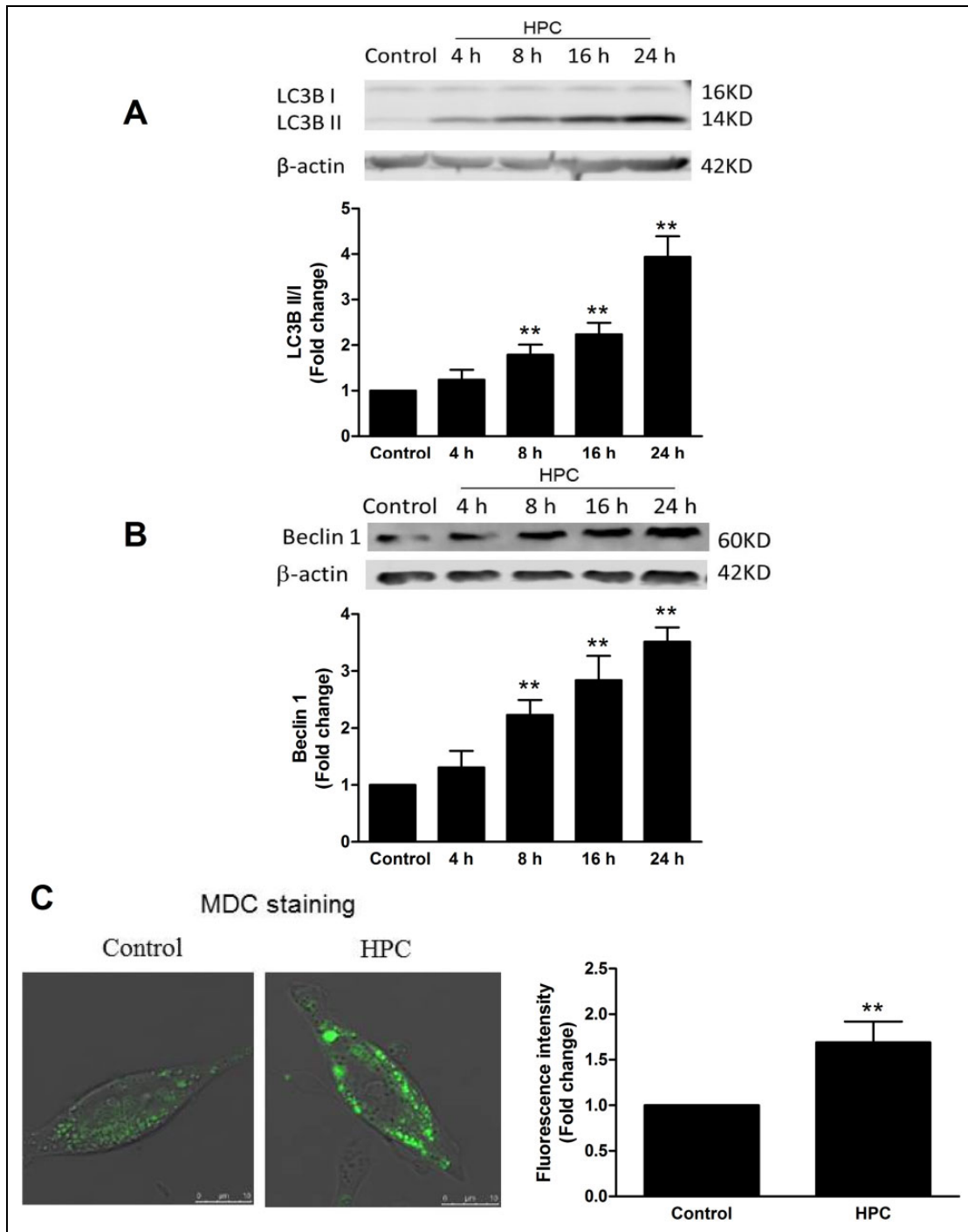


Fig. 2. Hypoxic preconditioning (HPC) activates autophagy in SH-SY5Y cells. Western immunoblotting for LC3B (A) and Beclin 1 (B) proteins, markers of autophagic function. SH-SY5Y cells were given HPC treatment with 3% O₂ for 0 (control), 4, 8, 16, or 24 h followed by normoxic recovery for 24 h, after which Western immunoblotting for LC3B and Beclin 1 proteins was performed. HPC significantly increased expression of LC3B II and Beclin 1 proteins, indicating an increase in autophagic function. (C) Monodansylcadaverine (MDC) staining for autophagic vacuoles. SH-SY5Y cells were either HPC treated with 3% O₂ for 8 h followed by 24 h normoxic recovery or maintained under control conditions. MDC staining was then performed to indicate the presence of autophagic vacuoles. Data represent mean \pm SD, trial $n = 4$. ** $P < 0.01$ versus the control group.

activation of the autophagy pathway and p53 degradation in certain cancer cells^{23,24}. Western blotting for p53 showed that H₂O₂ treatment indeed enhanced accumulation of

p53 (Fig. 4A, p53-fold change of DMSO + H₂O₂ group [2.51 \pm 0.18] versus control group: $P < 0.001$, $n = 4$); and HPC decreased p53 accumulation from H₂O₂ treatment

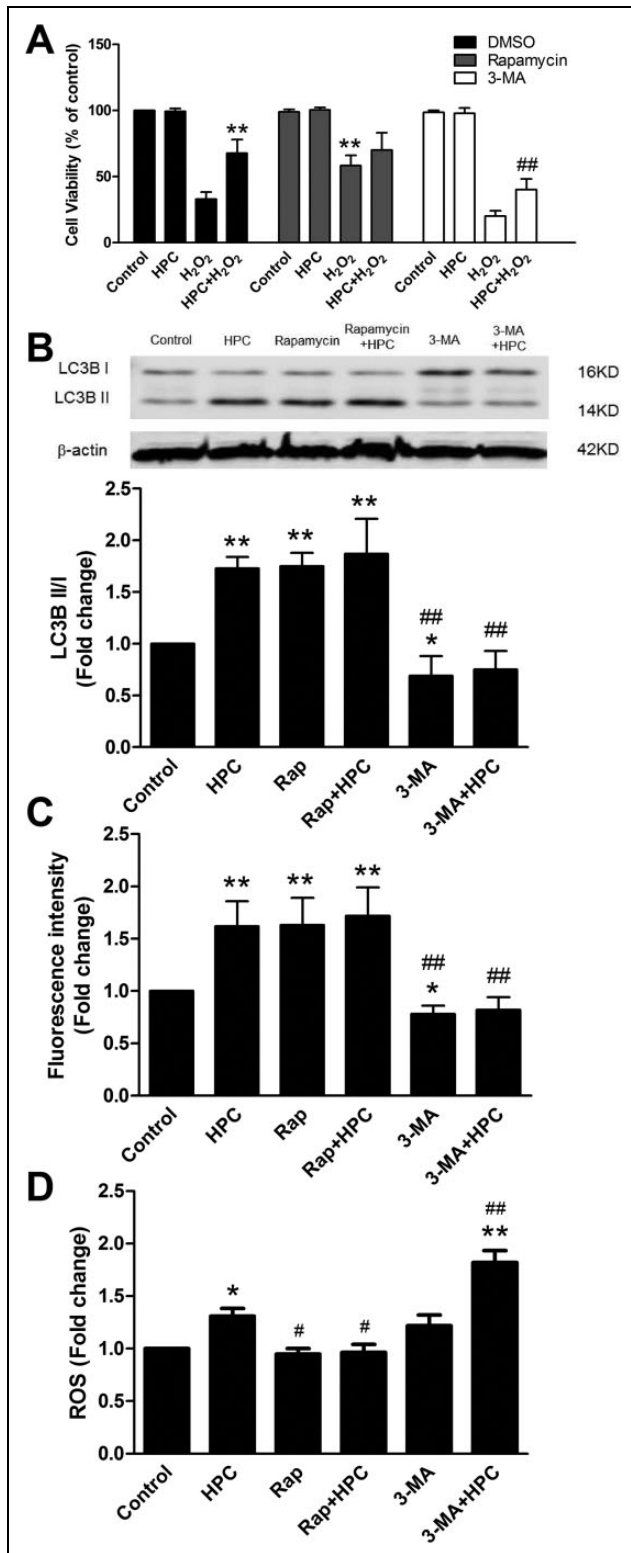


Fig. 3. Activation of autophagy may prevent oxidative damage in SH-SY5Y cells. Effects of DMSO (control), rapamycin (100 nM), and 3-methylxanthine (MA; 5 mM) on the viability of SH-SY5Y cells exposed to 600- μ M H₂O₂ for 24 h with or without hypoxic preconditioning (HPC) at 3% O₂ for 8 h (A). Effect of rapamycin (100 nM) and 3-MA (5 mM) on autophagic function as revealed by Western immunoblotting for LC3B protein (B) and

(Fig. 4A, p53-fold change of HPC + H₂O₂ group vs. DMSO + H₂O₂ group: 1.50 ± 0.19 versus 2.51 ± 0.18 , $n = 4$, $P < 0.001$). Further, we found that rapamycin treatment decreased p53 level (Fig. 4A, p53-fold change of rapamycin + H₂O₂ group versus DMSO + H₂O₂ group: 1.78 ± 0.19 vs. 2.51 ± 0.18 , $n = 4$, $P < 0.001$); and 3-MA treatment abolished the HPC effect on p53 (Fig. 4A, p53-fold change of 3-MA + HPC + H₂O₂ group vs. HPC + H₂O₂ group: 2.20 ± 0.15 vs. 1.50 ± 0.19 , $n = 4$, $P < 0.001$). These results suggest that p53 accumulation induced by H₂O₂ may be degraded by autophagy pathway activated by HPC.

Discussion

HPC results in significant protection against H₂O₂-induced death in SH-SY5Y cells with optimal protection occurring with an 8-h exposure to 3% O₂ followed by normoxic recovery for 24 h. Markers of autophagic function, such as LC3BII and Beclin 1, increased in the treatment group as compared with controls, with a corresponding decrease in the accumulation of p53, suggesting protection is mediated to some degree through activation of autophagy and degradation of p53. This is further supported by the finding that activation of autophagy with rapamycin mimics the protective effect of HPC and that inhibition of autophagy with 3-MA diminishes the protection of HPC.

Several signaling pathways may contribute to the considerable activation of autophagy observed following HPC such as the Adenosine Monophosphate Activated Protein Kinase (AMPK) pathway²⁵, upregulation of Hypoxia-inducible factors 1 (HIF1)²⁶, and Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3)/BNIP3L in mitochondria²⁷. However, the downstream effect of autophagy activation on neural protection, which specific proteins are targeted, is still unclear. Clearly, autophagy can degrade aggregated and/or misfolded proteins and damaged organelles²⁸⁻³⁰; but the exact proteins or organelles which are implicated in playing key detrimental roles in oxidative stress lesion are less clear. One study showed that neurons from heterozygous p53

Fig. 3. (continued) monodansylcadaverine staining for autophagic vacuoles of SH-SY5Y cells with or without HPC at 3% O₂ for 8 h (C). Effect of DMSO (control), rapamycin (100 nM), and 3-MA (5 mM) on the level of reactive oxygen species (ROS) of SH-SY5Y cells with or without HPC at 3% O₂ for 8 h (D). Rapamycin, an inducer of autophagy, mimics the protective effect of HPC, while 3-MA, an inhibitor of autophagy, decreases the protective effect of HPC. HPC can moderately increase the level of ROS, rapamycin can eliminate the increase, but 3-MA makes the increase intensify. Data represent mean \pm SD, trial $n = 4$. ** $P < 0.01$ versus the DMSO + H₂O₂ group, ### $P < 0.01$ versus the HPC + H₂O₂ group (A). * $P < 0.05$ versus the control group, ** $P < 0.01$ versus the control group, ### $P < 0.01$ versus the HPC group (B) and (C). * $P < 0.05$ versus the control group, ** $P < 0.01$ versus the control group, # $P < 0.05$ versus the HPC group, and ### $P < 0.01$ versus the HPC group (D). Rap indicates rapamycin.

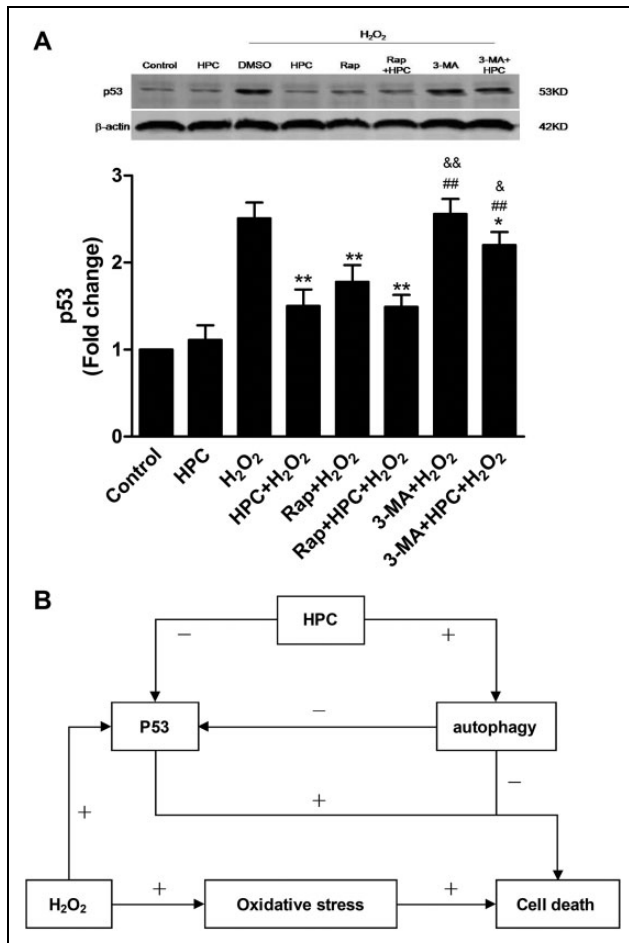


Fig. 4. Accumulation of p53 is downregulated by hypoxic preconditioning (HPC) and autophagy. Western immunoblotting for p53 and p53 expression (fold change) in HPC-treated SH-SY5Y cells and controls given different pretreatments (DMSO, control), rapamycin (100 nM), and 3-MA (5 mM) before being exposed to 600- μ M H₂O₂ for 24 h. H₂O₂ treatment resulted in an accumulation of p53 (DMSO), which was lessened by HPC. Pretreatment with rapamycin resulted in a similar decrease in the accumulation of p53, mimicking the protective effect of HPC, whereas pretreatment with 3-MA decreased the ability of HPC to attenuate the increase in p53 (A). Proposed model for the mechanism of protection induced by HPC. HPC activates autophagy, which decreases accumulation of p53 that may otherwise cause cell death (B). Data represent mean \pm SD, trial $n = 4$. * $P < 0.05$ versus the DMSO + H₂O₂ group. ** $P < 0.01$ versus the DMSO + H₂O₂. ### $P < 0.01$ versus the HPC + H₂O₂. & $P < 0.05$ versus the rap + H₂O₂. && $P < 0.01$ versus the rap + H₂O₂. Rap indicates rapamycin.

knockout mice display resistance to oxidative stress³¹; and yet another reported that accumulation of p53 induced by glutamate and calcium overload plays a causal role on neuronal cell death³². However, whether neuronal cell death caused by oxidative stress is also mediated through p53 accumulation is unknown. Our data indicate that p53 protein is accumulated in the SH-SY5Y cells upon H₂O₂ treatment, and activation of autophagy by rapamycin can decrease p53 protein levels. Consistently, inhibition of autophagy by

3-MA resulted in increased p53 levels in HPC-treated cells. Recently, Xu et al. demonstrated that HPC downregulates p53 through activation of the PI3K/protein kinase B (Akt)-DNA methyltransferase 1 (DNMT1)-p53 pathway in cardiac progenitor cells and protects them from oxygen-serum deprivation damage³³. Therefore, activation of autophagy by HPC to downregulate p53 protein may be a general principle for organ protection. Previous reports have indicated that when cells are subjected to hypoxia, the ROS can be increased and ROS can activate autophagy. We also know autophagy can decrease ROS from other's research. The results of our experiment was similar to theirs, showing that ROS might play a role in the course of activation of autophagy by HPC in SH-SY5Y cells.

Although the data presented in our current study suggest that HPC-induced protection in an in vitro neuronal cell model is likely modulated in a significant manner via activation of autophagy with a subsequent decrease in the accumulation of p53, there are important limitations that should be considered. While exposure to H₂O₂ serves as a useful model for studying oxidative stress in vitro, it should be noted that it may differ from the oxidative stress generated by in vivo hypoxia, for example, by causing direct cell membrane oxidative attack in addition to intracellular oxidative stress, and thus the protection of HPC seen in our model may differ from the effect seen in vivo. Additionally, we only tested the neuron-like (neuroblast) SH-SY5Y cell line that differs from primary neurons in important ways, including the ability to proliferate as well as undergo further cell divisions. Despite this, SH-SY5Y cells are widely used in neurological research as they have many properties of neurons, such as the morphological characteristics of adrenergic neurons and the expression of markers of dopaminergic neurons. In the future, we hope to study additional cell lines and primary neurons with the hope that our results will be reproducible in those lineages. Another consideration is that hypoxia could have had an influence on cell proliferation. However, our data showed that cell viability was not influenced in an appreciable manner by 8-h hypoxia (Fig. 3B), and therefore, we do not expect that a change in proliferation was a significant confounder on the result of this experiment.

Even so, there is a strong likelihood that other contributing factors exist, as our data showed that activation of autophagy alone does not fully account for the effect of HPC. This was evidenced by the fact that rapamycin, an activator of autophagy, also increased cell viability and decreased p53 accumulation; however, the observed effect was weaker than that of HPC. Similarly, 3-MA, an inhibitor of autophagy, did not fully eliminate protection nor fully restore p53 levels when combined with HPC, again pointing to the possibility of additional protective mechanisms. Many such mechanisms have been proposed, for instance, that HPC might protect cells through activation of specific antiapoptotic pathways²⁵, reducing energy consumption³⁴, increasing activity of ROS scavengers^{35,36}, activating autophagy^{18,19}, promoting transportation of glucose^{37,38}, improving function

of ionic channels and mitochondria^{39,40}, upregulating Erythropoietin (EPO) and p-p38^{36,41}, enhancing the expression of Heat shock protein 70 (HSP-70) and Heme oxygenase 1 (HO-1)^{42,43}, and promoting the expression of HIF-1 α ⁴⁴. These HPC effects may play distinct protective roles to different stresses. Further work is needed to determine what interplay, if any, exists between the mechanism of increased autophagy we observed and the mechanisms proposed by other authors, and to determine whether they account for the incompleteness of the association of HPC protection with autophagy or whether there is a yet unknown mechanism that contributes as well. With regard to the incomplete association of p53 and activated autophagy that was observed, an additional HPC mechanism that merits consideration as a contributing factor would be the PI3K/Akt-DNMT1-p53 pathway, as previously discussed³³.

Development of neuronal protective agents has long been a key area of pharmaceutical research. Many drugs including several traditional Chinese medicine have shown promise as neuroprotective agents through activation of autophagy^{45,46}. Similarly, rapamycin has been widely used clinically as an immunosuppressant. However, rapamycin also has many undesirable side effects, such as thrombocytopenia, anemia, lymphocele, and bone necrosis, which limit its therapeutic potential in the treatment of neurological disease. These effects seem unrelated with its actions on autophagy and instead depend on other effects of rapamycin such as inhibition of mitochondrial respiration⁴⁷. HPC is a low-cost, safe, and noninvasive therapeutic alternative to rapamycin proven to be beneficial in patients, without observable side effects^{48–50}. However, because of limitations in application of HPC, pursuing drug design or targeted therapies based on mechanistic observations remains an important topic.

In conclusion, our study suggests that HPC is protective against neuronal damage induced by H₂O₂ oxidative stress in vitro; and activation of autophagy by HPC plays a functional role in this protection. An important downstream effect of autophagy may be that it decreases the accumulation of p53, which can otherwise induce neuronal cell death. A connection between HPC-induced neuroprotection, autophagy, and p53 opens the door to potential treatments for a number of neurological diseases and warrants further study to determine how this mechanism might be exploited to overcome the limitations associated with HPC.

Ethical Approval

Ethical Approval is not applicable.

Statement of Human and Animal Rights

Statement of Human and Animal Rights is not applicable.

Statement of Informed Consent

Statement of Informed Consent is not applicable.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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