Ketamine ameliorates hypoxia-induced endothelial injury in human umbilical vein endothelial cells

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OBJECTIVES: Hypoxia leads to endothelial cell inflammation, apoptosis, and damage, which plays an important role in the complications associated with ischemic cardiovascular disease. As an oxidoreductase, p66Shc plays an important role in the regulation of reactive oxygen species (ROS) production and apoptosis. Ketamine is widely used in clinics. This study was designed to assess the potential protective effect of ketamine against hypoxia-induced injury in human umbilical vein endothelial cells (HUVECs). Moreover, we explored the potential mechanism by which ketamine protected against hypoxia-induced endothelial injury.

METHODS: The protective effects of ketamine against hypoxia-induced injury was assessed using cell viability and adhesion assays, quantitative polymerase chain reaction, and western blotting.

RESULTS: Our data showed that hypoxia reduced HUVEC viability, increased the adhesion between HUVECs and monocytes, and upregulated the expression of endothelial adhesion molecules at the protein and mRNA levels. Moreover, hypoxia increased ROS accumulation and upregulated p66Shc expression. Furthermore, hypoxia downregulated sirt1 expression in HUVECs. Alternatively, ketamine was shown to reverse the hypoxia-mediated reduction of cell viability and increase in the adhesion between HUVECs and monocytes, ameliorate hypoxia-induced ROS accumulation, and suppress p66Shc expression. Moreover, EX527, a sirt1 inhibitor, reversed the protective effects of ketamine against the hypoxia-mediated reduction of cell viability and increase in adhesion between HUVECs and monocytes.

CONCLUSION: Ketamine reduces hypoxia-induced p66Shc expression and attenuates ROS accumulation via upregulating sirt1 in HUVECs, thus attenuating hypoxia-induced endothelial cell inflammation and apoptosis.

KEYWORDS: Hypoxia; Ketamine; p66Shc; Sirt1.
and apoptosis (15). Additionally, hypoxic stress leads to a reduction in sirt1 transcription levels, while ketamine treatment increases the sirt1 transcription levels in the vertebrate brain (16). The present study aims to investigate the protective role ketamine plays against hypoxia-induced injury in human umbilical vein endothelial cells (HUVECs) in vitro, and the mechanism thereof.

# MATERIALS AND METHODS

## Cell culture and reagents

First, HUVECs (Clonetics; Lonza, Basel, Switzerland) were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS ( Gibco, Australia), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C and maintained under 5% CO2 conditions. The hypoxia-treated cells were cultured in a modular incubator at 37°C in an atmosphere of 1% O2, 5% CO2, and 94% N2 (17).

EX527, a sirt1 inhibitor, was purchased from Selleck, China. Ketamine (Sigma-Aldrich, St. Louis, Mo, USA) was dissolved in DMEM. The clinically acceptable concentration of ketamine is approximately 2–80 μM (18,19). Moreover, the half-life time of ketamine is about 2h. Therefore, after hypoxia pretreatment, cells were incubated with different concentrations (1, 2, and 4 μg/mL) of ketamine for 2h. The minimal concentration of ketamine with notable inhibitory effects on the hypoxia-induced reduction of cell viability and increase of monocyte/endothelial cell adhesion was recorded.

### Cell viability assay

HUVECs suspension (3000 cells/100 μL) was added to each well in a 96-multiwell culture plate, followed by incubation at 37°C for 24h. After the corresponding treatments, 10 μL of Cell Counting Kit-8 reagent (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) was added into each well, followed by incubation for a further 2h. Cell viability was measured using the CCK-8 assay. The optical density of the sample was measured at 450 nm using a microplate reader (BioTek).

### Adhesion assay

The isolation of human peripheral monocytes was acquired with the use of Histopaque-1077 (Sigma). Briefly, 8 mL of heparinized blood from healthy volunteers was layered onto 8 mL of Histopaque-1077. The monocytes were obtained by centrifugation of the blood samples at 400g for 30 min. Thereafter, monocytes were washed twice with PBS, and then, HUVECs were added into each well, followed by incubation for a further 2h. The HUVECs and monocytes were incubated at 37°C for 24h. After the corresponding treatments, 10 μL of Cell Counting Kit-8 reagent (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) was added into each well, followed by incubation for a further 2h. Cell viability was measured using the CCK-8 assay. The optical density of the sample was measured at 450 nm using a microplate reader (BioTek).

### Western blot analysis

Whole-cell protein extracts were obtained by cell lysis buffer (Cell Signaling Technology, Danvers, MA). Same amounts of proteins (60 μg) from HUVECs subjected to different treatments were separated by 8 or 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes at a constant voltage (100 V) for 2h. After culturing in 5% fat-free milk solution, the membranes were washed with specific primary antibodies at 4°C overnight. The predominant antibodies used were anti-β-actin monoclonal antibodies, and anti-p66Shc, anti-sirt1, anti-E-selectin, anti-ICAM-1, and anti-active caspase-3 antibodies (Proteintech, Wuhan, P.R. China, 1:1000). Thereafter, primary antibodies were washed with tris-buffered saline containing Tween and the membranes were incubated with the corresponding secondary antibodies (Proteintech, Wuhan, P.R. China, 1:1000) for 1h at 26°C. Subsequently, the membranes were washed, and the specific protein bands were detected using an enhanced chemiluminescence (ECL) system (Milipore, Massachusetts). The respective densities of the protein bands were analyzed using Scan-gel-it software 7.1. In this study, β-actin was used as the loading control.

### Statistical analysis

Data were obtained from five experiments and expressed as means ± SDs. N represents the number of times the experiments were repeated using different cell cultures. Statistical significance between conditions was assessed by one-way ANOVA. p<0.05 was considered statistically significant.
RESULTS

Hypoxia reduced HUVEC viability and increased the adhesion between monocytes/HUVECs, which were reversed by ketamine

Compared with the control group, hypoxia reduced HUVEC viability and augmented the interactions between monocytes/HUVECs in a time-dependent manner (Figure 1A, C). Moreover, ketamine ameliorated hypoxia-mediated cell injury in a concentration-dependent manner. Compared with hypoxia treatment, 2 μg/mL ketamine was found to reverse hypoxia-mediated reduction in cell viability and increase in monocyte/HUVEC adhesion (Figure 1B, D). This treatment condition was employed in the further analyses to study the potential mechanism responsible for the protective effects of ketamine against hypoxia-mediated endothelial injury.

Compared with the control group, hypoxia enhanced the expression of ICAM-1, E-selectin, and caspase-3. Moreover, ketamine was found to inhibit the hypoxia-induced upregulation of ICAM-1, E-selectin, and caspase-3 expression (Figure 1E-G).

Hypoxia increased ROS accumulation and p66Shc expression and downregulated sirt1 expression, which were reversed by ketamine

Compared with the control group, hypoxia increased the accumulation of ROS in HUVECs, which was reversed by ketamine treatment (Figure 2A). Hypoxia increased the

Figure 1 - The effect of ketamine on HUVEC viability and monocyte/endothelial cell adhesion following the exposure of HUVECs to hypoxia. (A) Hypoxia reduced the viability of HUVECs in a time-dependent manner. (B) Ketamine enhanced the hypoxia-mediated reduction in cell viability in a concentration-dependent manner. (C) Hypoxia increased monocyte/endothelial cell adhesion in a time-dependent manner. (D) Ketamine inhibited the hypoxia-mediated monocyte/endothelial cell adhesion in a concentration-dependent manner. The optimal concentration of ketamine that began to ameliorate the hypoxia-mediated reduction of HUVEC viability and inhibit the hypoxia-mediated monocyte/endothelial cell adhesion was 2 μg/mL. (E) Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies against ICAM-1, E-selectin, and caspase-3. (F) The ratio of the protein expression of each specific protein (ICAM-1, E-selectin, and caspase 3) to the expression of β-actin (*p < 0.05 versus the control group, #p < 0.05 versus the hypoxia group, n=5).
The effects of ketamine on p66Shc and sirt1 expression in hypoxia-treated HUVECs. (A) Hypoxia induced ROS accumulation in HUVECs, which could be reversed by ketamine treatment. (B) Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies against p66Shc and sirt1. (C) The ratio of the protein expression of each specific protein (p66Shc and sirt1) to the expression of β-actin (* p < 0.05 versus the control group, # p < 0.05 versus the hypoxia group, n=5).

p66Shc and sirt1 expression and ROS accumulation were altered by hypoxia, ketamine, and EX527 in HUVECs. (A) Equal amounts of proteins from HUVECs following the corresponding treatments were separated by SDS-PAGE and immunoblotted with antibodies against p66Shc and sirt1. (B) The ratio of the protein expression of each specific protein (p66Shc and sirt1) to the expression of β-actin. (C) ROS accumulation in HUVECs following the corresponding treatments (* p < 0.05 versus the control group, # p < 0.05 versus the hypoxia group, & p < 0.05 versus the ketamine group, n=5).
expression of p66Shc and decreased the expression of sirt1, while these effects were counteracted by ketamine treatment (Figure 2B, C).

**p66Shc and sirt1 expression and ROS accumulation were modified by hypoxia, ketamine, and EX527 in HUVECs**

Hypoxia treatment enhanced p66Shc expression, but downregulated sirt1 expression. However, ketamine was shown to reverse the hypoxia-mediated upregulation of p66Shc expression and reduction of sirt1 expression. Furthermore, EX527, a sirt1 inhibitor, was shown to counteract the effects of ketamine (Figure 3A, B). Compared with the control group, hypoxia enhanced ROS accumulation, which was reduced by ketamine. Moreover, EX527 could counteract the effects of ketamine against hypoxia-mediated ROS accumulation (Figure 3C).

**DISCUSSION**

Hypoxia is a state of insufficient oxygen supply in cells and tissues, and it remains the main reason for the high incidence rate of death and morbidity related to anesthesia (20).
Ischemia-induced endothelial injury that occurs after hypoxia results in the increased expression of cell adhesion molecules, such as ICAM-1 and E-selectin, which interact with circulating neutrophils, inducing them to migrate to the sites of endothelium, to further aggravate endothelial cell inflammation and damage (21). Moreover, ROS also play a vital role in hypoxia-mediated endothelial inflammation (22). An increase in ROS accumulation under hypoxic conditions can lead to endothelial cell apoptosis and necrosis (6). The 66-kDa subtype p66Shc of the growth factor adapter s6c is involved in ROS generation (23); p66Shc is phosphorylated on Ser36 and translocated to the mitochondria after activation by oxidative stress. In mitochondria, p66Shc combines with cytochromes to produce ROS as an oxidoreductase, which results in apoptosis (24). Sirt1 has been reported to suppress p66Shc transcription via epigenetic chromatin modification through decreased levels of acetylated histone H3, which binds to the promoter region of p66Shc (25). Furthermore, sirt1 was downregulated in the early phase of ischemia, which resulted in increased oxidative stress and apoptosis (26). The inhibition of p66Shc expression is useful to prevent endothelial cell aging and dysfunction caused by oxidative stress. Additionally, accumulation of ROS and expression of p66Shc were positively correlated with the endothelial apoptotic pathway related to oxidative stress (27). Similar results were obtained in the present study, as hypoxia inhibited sirt1 expression and upregulated p66Shc expression, inducing the expression of ICAM-1, -selectin, and active caspase-3, which increased the interactions between HUVECs/monocytes and endothelial cell apoptosis. Ketamine is an antagonist of the NMDA receptor. It is reported to be a neuroprotective agent that suppresses oxidative stress, cellular dysfunction, and apoptosis (28). Ketamine was found to elicit neuroprotective effects by markedly inhibiting oxidative stress in mice with traumatic brain injury (29). Moreover, a standard clinical dose of ketamine was noted to reduce the inflammatory response in the fetal cerebral cortex following transient hypoxia (30). Additionally, ketamine was found to ameliorate hypoxia-mediated inflammatory and apoptotic pathways in fetal ovine kidneys (31). However, few studies on the anti-inflammatory and anti-apoptotic effects of ketamine on vascular endothelial cells exist. Our findings indicated that ketamine could inhibit the hypoxia-mediated interactions between monocytes/endothelial cells and increase of endothelial cell apoptosis. Moreover, ketamine decreased hypoxia-induced ROS accumulation and p66Shc expression via the upregulation of sirt1 expression.

This study has a few limitations. First, we performed all experiments in vitro, and in vivo experiments are required to verify our hypothesis. Furthermore, we proposed that ketamine inhibits the hypoxia-induced increase in p66Shc expression via upregulating sirt1 expression in HUVECs. However, knockdown and overexpression of sirt1 should be further researched to provide more persuasive conclusions.

In summary, the present study indicated that ketamine reduced the hypoxia-induced upregulation of p66Shc expression and ROS accumulation via upregulating sirt1 expression in HUVECs, thus ameliorating the hypoxia-mediated reduction in HUVEC viability and reducing monocyte/endothelial cell adhesion.

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AUTHOR CONTRIBUTIONS
Meng Z and Hu Y designed the study, supervised the experiments, analyzed the data and wrote the manuscript. Zhou X and Lai J performed the experiments. Yang S and Su Y collected the data.

REFERENCES
1. Abe H, Semb a H, Takeda N. The Roles of Hypoxia Signaling in the Pathogenesis of Cardiometabolic Diseases. J Atheroscler Thromb. 2017; 24(9):884-94. https://doi.org/10.5531/jat.1701909
2. Ali MH, Schildt SA, Hynes KL, Marcus BC, Gewertz BL. Prolonged hypoxia alters endothelial barrier function. Surgery. 1998;124(3):491-7. https://doi.org/10.1016/S0039-6010(98)00947-7
3. Flamm A, Toffoli S, Raes M, Michiels C. Hypoxia regulates inflammatory gene expression in endothelial cells. Exp Cell Res. 2009;315(5):733-47. https://doi.org/10.1016/j.yexcr.2008.11.020
4. Fasanaro P, D’Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem. 2008;283(23):15878-83. https://doi.org/10.1074/jbc.M800731200
5. Hu H, Wang F, Wu Z, Gu H, Dong N, Jiang X, et al. FOXO3a-dependent up-regulation of Mxi1-0 promotes hypoxia-induced apoptosis in endothelial cells. Cell Signal. 2018;51:233-42. https://doi.org/10.1016/j.cellsig.2018.08.009
6. Kondoh M, Ohga N, Akiyama K, Hida Y, Maishi N, Towfik AM, et al. Hypoxia-induced reactive oxygen species cause chromosomal abnormalities in endothelial cells in the tumor microenvironment. PLoS One. 2013;8(11)e80349. https://doi.org/10.1371/journal.pone.0080349
7. Yao S, Yin J, Hou X, Liu J. Growth factor milt38/SP-1 promotes hypoxia-induced vascular endothelial cell injury via HIF-1a. Exp Cell Res. 2018;370(1):31-38. https://doi.org/10.1016/j.yexcr.2018.06.001
8. Galimov ER. The Role of p66Shc in Oxidative Stress and Apoptosis. Acta Physiol. 2010;2(4):44-51. https://doi.org/10.22037/20758253-2010-2-4-44-51
9. Cosentino F, Francia P, Camici GG, Pellicci PG, Luescher TF, Volpe M. Final common molecular pathways of aging and cardiovascular disease: role of the p66Shc protein. Arterioscler Thromb Vase Biol. 2008;28(4):622-8. https://doi.org/10.1161/ATVBAHA.107.156059
10. Giblin W, Skinner ME, Lombard DB. Sirhins: guardians of mammalian healthspan. Trends Genet. 2014;30(5):271-8. https://doi.org/10.1016/j.tig.2014.04.007
11. Xu X, Hu Y, Zhai X, Lin M, Chen Z, Tian X, et al. Salvianolic Acid A preconditioning confers protection against concanavalin A-induced liver injury through SIRT1-mediated repression of p66Shc in mice. Toxicol Appl Pharmacol. 2013;273(1):68-76. https://doi.org/10.1016/j.taap.2013.08.021
12. Zhou S, Chen HZ, Wan YZ, Zhang QJ, Wei YS, Huang S, et al. Repression of p66Shc expression by SIRT1 contributes to the prevention of hypoglycemia-induced endothelial dysfunction. Circ Res. 2011;109(6):639-48. https://doi.org/10.1161/CIRCRESAHA.111.243992
13. Ota H, Akishita M, Eto M, Iijima K, Kaneki M, Ouchi Y. Sirt1 modulates premature senescence-like phenotype in human endothelial cells. J Mol Cell Cardiol. 2007;43(5):571-9. https://doi.org/10.1016/j.yjmcc.2007.08.008
14. Krystal JH, Karper LP, Seibyl JP, Freeman GK, Delaney R, Brenner JD, et al. Subnuclear effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. Arch Gen Psychiatry. 1994;51(3):199-214. https://doi.org/10.1001/archpsyc.1994.03950030035004
15. Liang J, Wu S, Xie W, He H. Ketamine ameliorates oxidative stress-induced apoptosis in experimental traumatic brain injury via the Nrf2 pathway. Drug Des Devel Ther. 2018;12:845-53. https://doi.org/10.2147/DDDDT.S160046
16. Zakhar SM, Ayubacha D, Anseri F, Kamran K, Karim M, Leheste JR, et al. A behavioral and molecular analysis of ketamine in zebrabfish. Synapse. 2011;65(2):160-7. https://doi.org/10.1002/syn.20830
17. Liu H, Shi C, Deng Y. MALAT1 affects hypoxia-induced vascular endothelial cell injury and autophagy by regulating miR-19b-3p/HIF-1α. Mol Cell Biochem. 2020;466(1-2):25-34. https://doi.org/10.1007/s11010-020-03684-z
18. Domino EE, Zsigaand EF, Domino LE, Domino KE, Kothary SP, Domino SE. Plasma levels of ketamine and two of its metabolites in surgical patients using a gas chromatographic mass fragmentographic assay. Anesth Analg. 1982;64(2):87-92. https://doi.org/10.1213/00000539-19820200-00004
19. Meltoniemi MA, Hagelberg NM, Ollikka KT, Saari TI. Ketamine: A Review of Clinical Pharmacokinetics and Pharmacodynamics in Anesthesia and Pain Therapy. Clin Pharmacokinet. 2016;55(9):1059-77. https://doi.org/10.1007/s40262-016-0383-6
20. Ward DS, Kanan SR, Pandit JJ. Hypoxia: developments in basic science, physiology and clinical studies. Anaesthesia. 2011;66 Suppl 2:19-26. https://doi.org/10.1111/j.1365-2044.2011.06930.x

21. Yoshida N, Granger DN, Anderson DC, Rothlein R, Lane C, Kvietsy PR. Anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. Am J Physiol. 1992;262(6 Pt 2):H1891-8. https://doi.org/10.1152/ajpheart.1992.262.6.H1891

22. Zhu M, Ding J, Jiang H, Kong L, Sun Z, Chen J, et al. Propofol ameliorates endothelial inflammation induced by hypoxia/reoxygenation in human umbilical vein endothelial cells: Role of phosphatase A2. Vascul Pharmacol. 2015;73:149-57. https://doi.org/10.1016/j.vph.2015.06.002

23. Spescha RD, Glanzmann M, Simic B, Witassek F, Keller S, Akhmedov A, et al. Adaptor protein p66(Shc) mediates hypertension-associated, cyclic stretch-dependent, endothelial damage. Hypertension. 2014;64(2):347-53. https://doi.org/10.1161/HYPERTENSIONAHA.113.02129

24. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moreni M, Contursi C, et al. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell. 2005;122(2):221-33. https://doi.org/10.1016/j.cell.2005.05.011

25. Chen HZ, Wan YZ, Liu DP. Cross-talk between SIRT1 and p66Shc in vascular diseases. Trends Cardiovasc Med. 2013;23(7):237-41. https://doi.org/10.1016/j.tcm.2013.01.001

26. Wang G, Yao J, Li Z, Zu G, Feng D, Shan W, et al. miR-34a-5p Inhibition Alleviates Intestinal Ischemia/Reperfusion-Induced Reactive Oxygen Species Accumulation and Apoptosis via Activation of SIRT1 Signaling. Antioxid Redox Signal. 2016;24(17):961-73. https://doi.org/10.1089/ars.2015.6492

27. Skulachev VP. Cytochrome c in the apoptotic and antioxidant cascades. FEBS Lett. 1998;423(3):275-80. https://doi.org/10.1016/S0014-5793(98)00061-1

28. Marcoux FW, Goodrich JE, Dominick MA. Ketamine prevents ischemic neuronal injury. Brain Res. 1988;452(1-2):329-35. https://doi.org/10.1016/0006-8993(88)90037-6

29. Liang J, Wu S, Xie W, He H. Ketamine ameliorates oxidative stress-induced apoptosis in experimental traumatic brain injury via the Nrf2 pathway. Drug Des Devel Ther. 2018;12:845-53. https://doi.org/10.2147/DDDT.S160046

30. Chang EI, Zárate MA, Rabaglino MB, Richards EM, Arndt TJ, Keller-Wood M, et al. Ketamine decreases inflammatory and immune pathways after transient hypoxia in late gestation fetal cerebral cortex. Physiol Rep. 2016;4(6):e12741. https://doi.org/10.14814/phy2.12741

31. Chang EI, Zárate MA, Rabaglino MB, Richards EM, Keller-Wood M, Wood CE. Ketamine suppresses hypoxia-induced inflammatory responses in the late-gestation ovine fetal kidney cortex. J Physiol. 2016;594(5):1295-310. https://doi.org/10.1113/JPH271066

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