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Glycine-nitronyl nitroxide conjugate protects human umbilical vein endothelial cells against hypoxia/reoxygenation injury via multiple mechanisms and ameliorates hind limb ischemia/reperfusion injury in rats

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ABSTRACT

Oxidative stress and inflammation play important roles in the pathogenesis of ischemia/reperfusion (I/R)-injury. The administration of antioxidants and anti-inflammatory agents has been applied to prevent I/R-injury for several decades. Of the numerous compounds administrated therapeutically in anti-oxidative stress, nitronyl nitroxide has gained increasing attention due to its continuous ability to scavenge active oxygen radicals. However, its effect is not ideal in clinical therapy. In previous study, we linked the anti-inflammatory amino acid glycine to nitronyl nitroxide and developed a novel glycine-nitronyl nitroxide (GNN) conjugate, which showed a synergetic protection against renal ischemia/reperfusion injury. However, the underlying mechanism remains unclear. In this study, a hypoxia/reoxygenation (H/R) injury model was established in human umbilical vein endothelial cells (HUVECs) and we found that the GNN conjugate significantly elevated the cell viability via reducing the apoptosis rate in H/R-treated HUVECs. Meanwhile, GNN conjugate attenuated H/R induced mitochondrial fragmentation, mitochondrial membrane potential reduction, Cytochrome *c* release and autophagy. To determine the extensive applicability of GNN conjugate in different I/R models and its effect in remote organs, an *in vivo* hind limb I/R model was established. As expected, GNN conjugate ameliorated damages of muscle and remote organs. These results demonstrate that GNN conjugate may be an effective agent against ischemia/reperfusion injury in clinical therapy.

1. Introduction

Multiple mechanisms are involved in I/R injury, including oxidative generation, Ca^{2+} overload, pH alteration and inflammatory cytokine release [1]. An emerging body of evidence indicates that the generation of reactive oxygen species (ROS) plays an important role in I/R injury. Normally, redundant ROS is effectively eliminated by the antioxidant defense system. Low and moderate amounts of ROS function as a beneficial mediator in many cellular signaling pathways, including clearance of invading pathogens, wound healing, and tissue repair. However, stressful environments, including infections, I/R injury, and various inflammatory processes significantly elevate the level of ROS, which results in disruption of normal cellular homeostasis. ROS generation of a single organ to ischemia/reperfusion may subsequently cause injury in distant non-ischemic organs, eventually leading to multiple organ failure [2]. In addition, inflammation is an essential step in the progression of I/R injury, which is characterized by production of cytokines, chemokines, and adhesion molecules that amplify tissue damage [3].

Despite continuous efforts through these years, minimizing or eliminating I/R injury remains challenging in clinical surgery and medical treatments of ischemia-related disease. Development of effective interventions and strategies to reduce I/R injury is of great importance. Stable nitroxides are protective agents against oxidative stress, which have been applied to attenuate oxidative damage in various experimental models of ischemia/reperfusion injury. In addition to scavenge active oxygen radicals, nitroxides also suppress the formation of reactive nitrogen species. Through

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continuous exchange, the three forms of nitroxides can act as self-replenishing antioxidants, implicating the potential of this unique class of antioxidants against ischemia/reperfusion injury [4]. On the other hand, glycine is an effective anti-inflammatory agent, which is beneficial against ischemia/reperfusion injury in various organs [5]. In previous study, in order to develop effective therapeutic agents against ischemia/reperfusion injury, we linked the antioxidant nitronyl nitroxide to the anti-inflammatory amino acid glycine and found this conjugate could provide a synergetic protection against renal reperfusion injury [6].

Microvascular disturbance is one of the main features of I/R injury. Endothelial dysfunction plays a critical role in I/R injury and the endothelial cell is one of attractive targets for treatment of I/R injury. The rapid evolution of knowledge of the mechanisms of endothelial I/R injury shed light on clinical therapy for alleviating I/R damage [7]. In this study, we established an *in vitro* H/R model in HUVECs to mimic I/R injury *in vivo*.

Mitochondria have emerged as a major source of I/R-induced ROS due to their ability to sense the cellular oxygen levels. In the process of I/R, ROS radicals, including NO•, O2•−, and NO3− are over-produced after reperfusion. In the mitochondria, excessive electron leakage from complexes I and III of the electron transport chain (ETC) is primarily responsible for O2•− generation. Moreover, over-production of ROS by ETC may in turn disturb adjacent complexes and then propagate mitochondrial dysfunction [8]. In the present study, we also detected the morphology and function of cellular mitochondria in H/R model of HUVECs under GNN treatment.

Autophagy is a double-edged sword in the process of I/R injury. The formation of autophagosomes can eliminate the destroyed organelles. However, excessive autophagy activation will digest the substances necessary for maintaining of normal cell life and induce cellular apoptosis [9]. In the present study, we also detected the regulatory function of GNN conjugate in H/R model of HUVECs.

Acute I/R injury affects not only the local tissues but also remote organs due to pro-inflammatory and tissue injury. In this study, an in *vivo* rat model of hind limb I/R induced injury was established and the protective role of the GNN conjugate in local and remote organs was evaluated in local and remote organs.

2. Materials and methods

2.1. Culture and maintenance of HUVECs

HUVECs were purchased from Life Technologies and cultured in ECM medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco), 20 μg/ml vascular endothelial growth factor (VEGF; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Beyotime, Shanghai, China). The cells were cultured in an incubator with 5% CO_2 and saturated humidity (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. After reaching 90%–95% confluence, the cells were passaged by 0.05% trypsin (Gibco) and applied for the following experiments or storage.

2.2. Establishment of the in vitro H/R model of HUVECs

Hypoxia/reoxygenation model was established when the cells were in the logarithmic growth phase. The culture medium was replaced by hypoxic medium without FBS and glucose and the culture dish was put into a sterilized hypoxic box. 5% CO_2 and 95% N_2 was flushed into the hypoxic box until the concentration of oxygen in the hypoxic box reached less than 1%. Then the box was sealed and cultured in 37 °C incubator for 8 h. Then the cells were cultured in normal medium for 4 h with 4.5 g/ml glucose at 37 °C for reoxygenation.

2.3. Grouping and treatment

Samples were divided into 4 groups, the control group (mock, wild type HUVECs), the H/R model group (T8R4), the 2 μg/ml GNN conjugate + H/R treatment group (T8R4 + D 2) and 5 μg/ml GNN conjugate + H/R treatment group (T8R4 + D 5). For the last two groups, cells were treated with 2 μg/ml or 5 μg/ml of GNN conjugate before reoxygenation. All the experiments were repeated three times for each group.

2.4. SRB experiments

The treated cells in each group were seeded in 24-well plates at 1×10^5 cells/well with normal HUVEC culture medium. After adhering to plates, the cells were washed and incubated with fresh medium. Culture medium was renewed every other day. At day 0, 2, 4, and 6, cells in each well were fixed with 10% trichloroacetic acid (TCA), stained with 0.4% sulforhodamine (SRB) (Sigma, St. Louis, MO), washed three times with 1% acetic acid, and subjected to the measurement of optical density in a spectrometer at 490 nm.

2.5. MTT experiments to detect cell viability

The cultured HUVECs were treated as described above. After that, 100 μL HUVEC suspension (2×10^4 cells) was seeded into a 96-well plate; each group had six wells. 20 μL MTT at 5 mg/ml final concentration was added to each well. Then, the plates were incubated at 37 °C for an additional 4 h to allow the MTT to form formazan crystals by reacting with the viable cells. The MTT medium mixture was removed and the formazan crystals were dissolved in 150 μl DMSO at 37 °C for 10 min. The absorbance was measured by a microplate reader at 570 nm.

2.6. Detection of cellular apoptosis

Apoptosis was measured by staining cells with Annexin V-FITC and PI and detected by flow cytometry according to the manufacturer's protocols (BD Pharmingen, San Diego, CA). Briefly, 2×10^6 treated cells were washed in cold PBS and resuspended in 100 μl 1× staining buffer. The washed cells were stained with Annexin V and PI (5 μl each) (BD Pharmingen) for 30 min at room temperature in the dark and then washed twice with 500 μl 1 × staining buffer and subjected to flow cytometry, and the data were analyzed by the FlowJo software.

2.7. Reverse transcription and quantitative PCR to detect mRNA expression

Total RNA was extracted with the Trizol Reagent and reversely transcribed as previously described [10]. The SYBR green (DBI, Bioscience, Ludwigshafen, Germany) method and IQ5 Real-time PCR detection system (BioRad, Hercules, CA) were used for real-time PCR. Primer sequences were as follows: *p53*-forward: GCGAGCACTGCCCAACAACA, *p53*-reverse: GGATCTGAAGGGTGAAATAT- TCT; *BAD*-forward: CAGGCCTATGCAAAAAGAGGAT, *BAD*-reverse: CGCAC-CGGAAGGGAATCT; *Bax*-forward: TTTGCTTCAGGGTTTCATCC, *Bax*-reverse: ATCCTCTGCAGCTCCATGTT; *BCL2*-forward: GAGGATTGTGGCCTTCTTTG, *BCL2*-reverse: ACAGTTCCACAAAGGCATCC; *DRP1*-forward: GATGCCATAGTTGAAGTGGTGAC, *DRP1*-revrese: CCACAAGCATCAGCAAAGTCTGG; *MFN1*-forward: GGTGAATGAGCGGCTTTCCAAG, *MFN1*-reverse: TCCTCCACCAAGAAATGCAGGC; *MFN2*-forward: ATTGCAGAGGCGGTTCGACTCA, *MFN2*-reverse: TTCAGTCGGTCTTGCCGCTCTT; *OPA1*-forward: GTGGTTG-

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