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Notoginsenoside R1 attenuates high-altitude pulmonary edema by targeting the NRF2-RIPK3/MLKL axis to suppress necroptosis and inflammation

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ABSTRACT: Nuclear factor erythroid 2-related factor 2 (NRF2) is crucial for cell survival during oxidative stress by regulating antioxidant genes, with its transcriptional activity influenced by nuclear localization. Analysis of high-throughput transcriptomic data from GSE260910 in the GEO database indicates that NRF2 and RIPK3 are implicated in the pathogenesis of high-altitude pulmonary edema (HAPE). In vivo research has shown that Notoginsenoside R1 (NGR) mitigates hypobaric hypoxia-induced HAPE. Furthermore, network pharmacology analysis identified necroptosis as a significantly enriched pathway associated with the protective effects of NGR against HAPE. However, it remains unclear whether the pharmacological effects of NGR against HAPE are mediated by promoting NRF2 nuclear translocation, thereby suppressing oxidative stress and alleviating necroptosis and inflammation. In this study, we established a hypobaric hypoxia-induced HAPE model using NR8383 alveolar macrophages and rats. Our experimental results reaffirmed the pharmacodynamic effects of NGR on HAPE, demonstrating that NGR attenuated pulmonary edema, blood acid-base imbalances, and inflammation caused by hypobaric hypoxia in rats. Furthermore, NGR was found to promote NRF2 nuclear translocation, reduce oxidative stress, and inhibit the activation of the RIPK3/MLKL pathway, thereby diminishing necroptosis and subsequent inflammation, offering protective effects against HAPE. Mechanistically, this study highlights the involvement of NRF2 and RIPK3-mediated necroptosis as crucial mechanisms in the development of HAPE, with NRF2 playing a regulatory role in the RIPK3/MLKL pathway. In conclusion, NGR mitigates oxidative stress, necroptosis, and inflammation through the promotion of NRF2 nuclear translocation, subsequently improving hypobaric hypoxia-induced HAPE in both NR8383 cells and rats. These findings underscore the potential of NGR as an innovative therapeutic agent for HAPE.

Keywords: High-altitude pulmonary edema; Notoginsenoside R1; Nuclear factor erythroid 2-related factor 2; Necroptosis; Inflammation

1. Introduction

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High-altitude pulmonary edema (HAPE) occurs in healthy persons who reach altitudes >2500–3000 m rapidly.^[1] It has been estimated that up to 62% of HAPE-susceptible individuals may develop HAPE after reaching an altitude of 4,559 m in some areas.^[2] Although the incidence of HAPE is low in healthy individuals, the mortality rate from untreated HAPE patients reaches up to 50%.^[3] The pathogenesis of HAPE has been implicated in multiple molecular pathways, such as inflammation, apoptosis, ferroptosis, and oxidative stress.^[4] Nuclear factor Erythroid 2-related factor 2 (NRF2) is the body's innate antioxidative defense mechanism.^[5, 6] The modulatory architecture governing NRF2 activity has emerged as an appealing therapeutic target for its intricate mechanisms.^[7] Several studies have shown that NRF2 activation is a promising prevention strategy against HAPE.^[5, 8, 9] Of note, NRF2 may attenuate HAPE by eliminating reactive oxygen species (ROS) formed in the course of acute high-altitude exposure. Thus, studies targeting the NRF2 transcription factor have important implications and are emerging as a prospective target for preventing HAPE.

Necroptosis, an inflammatory form of programmed cell death, is a cellular response to environmental stress that can cause the release of cellular contents, like damage-associated molecular patterns (DAMPs), cell debris and inflammatory cytokines, which stimulate immune cell populations.^[10–12] Necroptosis begins with the assembly of receptor-interacting protein kinase 1 (RIPK1)-RIPK3 complexes, which then induce activation of mixed lineage kinase domain-like (MLKL) to form the necrosome.^[11–13] Oligomerization of MLKL results in the transfer of whole necrosomes from the cytoplasm to the cell membrane and the formation of permeable pores, which leads to cell membrane rupture and necroptotic cell death.^[12, 14, 15] As a classical necroptosis pathway, there is mounting evidence that the RIPK1/RIPK3/MLKL may play a role in the pathogenesis of various diseases throughout the body, including cardiovascular and pulmonary diseases.^[10] Thus, necroptosis inhibition has been widely studied as a prospective therapeutic strategy.^[16] Our earlier study indicated that the phosphorylation level of MLKL protein was higher after hypobaric hypoxia-induced HAPE, suggesting that necroptosis might occur in HAPE rats.^[9] Nevertheless, the precise mechanisms for the association between HAPE and necroptosis is not clear and needs to be further investigated *in vivo* and *in vitro* experiments.

Several studies have provided evidence that heightened levels of ROS can initiate the process of necroptosis in macrophages.^[17–19] Moreover, recent research has revealed that the promotion of nuclear translocation of NRF2 can effectively reduce ROS production, thereby attenuating necroptosis.^[20, 21] However, the regulatory relationship between NRF2 and necroptosis in the context of HAPE pathogenesis has rarely been investigated. Thus, targeting NRF2 to inhibit necroptosis can be a highly promising strategy for preventing and treating hypobaric hypoxia-induced HAPE.

Notoginsenoside R1 (NGR) is a bioactive component extracted from *Panax notoginseng* (which belongs to the category of substances that are both food and medicine). It has antioxidant, anti-inflammatory, pulmonary protective, cardiac protective, and anti-apoptotic effects.^[22, 23] Despite reports in the literature that NGR prevents or attenuates hypoxia-induced disorders such as brain damage and cardiac injury^[24, 25], the

exact mechanism is still far from clear. Remarkably, our previous work demonstrated that NGR protected rats from hypobaric hypoxia-induced HAPE and cardiac damage^[4, 26], which were similar to those reported above. However, the mechanism of action of NGR to attenuate HAPE is limited to inhibition of apoptosis. The potential involvement of additional cell death mechanisms in the protective role of NGR against HAPE remains uncertain at this time. Thus, in this work, we verified that NGR mitigated oxidative stress, pulmonary edema, and inflammation induced by hypobaric hypoxia *in vivo* and *in vitro* by targeting the regulation of NRF2-RIPK3/MLKL axis, which provides a novel therapeutic candidate for HAPE.

2. Materials and methods

2.1 Reagents and antibodies

NGR (HPLC $\geq$ 98%, A0273) was bought from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). Selleck Chemicals (Houston, TX, US) provided dexamethasone, ML385, and GSK'872. The anti-NRF2 (AF7006), anti-p-RIPK1 (AF7088), anti-p-RIPK3 (AF7443), and anti-p-MLKL (AF7420) were obtained from Affinity (New Jersey, USA). Anti-RIPK1 (A7414), anti-RIPK3 (A5431), and anti-MLKL (A21894) were purchased from ABclonal Technology (Wuhan, China). Anti-HIF-1 α (ab179483), anti-VEGF (ab32152), anti-AQP1 (ab168387), anti-HMGB1 (ab191583), anti-Lamin B1 (ab16048) and anti- β -actin (ab227387) were provided by Abcam (Cambridge, UK). Anti-AQP5 (#59558) and anti-NF- κ B p65 (8242s) were provided by Cell Signaling Technology (Massachusetts, USA).

2.2 Cell culture and experimental design

NR8383 cells were cultured in F12K medium at 37°C in 5% CO₂ containing 20% heat-inactivated fetal bovine serum (FBS) (GIBCO, USA). NR8383 cells were plated at a density of 2×10^4 cells per well in 96-well plates. The subsequent experiments were carried out when cells grew into 80~90% confluence.

Experiment I. To determine optimum duration of exposure to hypobaric hypoxia, we conducted preliminary model experiments. Cells were divided into six groups based on exposure time: 0 h, 6 h, 12 h, 24 h, 36 h, and 48 h groups. The 0 h group was routinely cultured in the cell culture incubators (37 °C, 95% air, 5% CO₂ in gas phase). The 6 h, 12 h, 24 h, 36 h, and 48 h groups were subjected to hypobaric hypoxia for their respective time periods. Each group was exposed individually to ensure that the conditions were consistent.

Experiment II. To ascertain the optimal dose of NGR, we performed the cytotoxicity and pharmacodynamic experiments. First, different concentrations of NGR (0, 5, 10, 20, 40, 80, 100 M) and treatment times (0 h, 24 h, 48 h, 72 h) were applied to cells. Next, cells were grouped into four: (1) control (Con) group, cells were cultured in F12K medium containing 10% FBS; (2) hypobaric hypoxia (HH) group, cells were cultured in F12K medium containing 10% FBS, then were exposed to hypobaric hypoxia for 24 h; (3) NGR+HH group, cells were treated with variable concentrations of NGR (10, 20, and 40 μ M) for 24 h, then were exposed to hypobaric hypoxia for 24 h.

Experiment III. To further investigate the underlying mechanisms, the cells were grouped into five: (1) HH group, cells were cultured in F12K medium containing 10% FBS, then were exposed to hypobaric hypoxia for 24 h; (2) NGR+HH group, cells were pre-treated with 20 μ M NGR for 24 h, then were exposed to hypobaric hypoxia for 24 h; (3) ML385+HH group, cells were pre-treated with 20 μ M ML385 for 1 h, then were exposed to hypobaric hypoxia for 24 h; (4) NGR+ML385+HH group, cells were pre-treated with 20 μ M NGR for 23 h, followed by 20 μ M ML385 for 1 h, followed by exposure to hypobaric hypoxia for 24 h; (5) GSK'872+HH group, cells were pre-treated with 3 μ M GSK'872 for 1 h, then were exposed to hypobaric hypoxia for 24 h; The dosage of NGR administered was determined according to preliminary experiments and prior research.^[22] We employed ML385, an NRF2 inhibitor known to arrest the transcriptional activity of NRF2^[27], to inhibit NRF2 in our study. The administration concentration for ML385 was established based on existing literature and primary experiments.^[28] Furthermore, RIPK3 is an upstream kinase that plays an essential role in necroptosis, so we suppressed necroptosis by using the RIPK3 inhibitor GSK'872. The concentrations of GSK'872 administered were determined based on references and preliminary experiments.^[29, 30]

2.3 Establishment of the high-altitude pulmonary edema cell model

A customized hypobaric hypoxic chamber (Tawang Intelligent Technology, Shanghai, China) was utilized to establish a high-altitude pulmonary edema cell model, following the methodology outlined in our previous research and other relevant literature.^[31] In brief, after pre-administration, NR8383 cells from each group except the Con group were placed in the customized chamber (altitude: 6000 m, pressure: 54.2 kPa, oxygen: 1%, CO₂: 5%, temperature: 37 °C) to simulate high-altitude conditions. Through Experiment I, 24 h was determined to be the optimal time point for hypobaric hypoxia exposure. Thus, after 24 h period, NR8383 cells were taken out of the hypobaric hypoxic chamber, and either the supernatant or cells were obtained for indicator assays.

2.4 Cell viability assay

Determination of cell viability using the CCK-8 assay kit (HY-K0301, MCE, China). Briefly, add 10 μ L of CCK-8 solution to each well and incubate at 37°C for 1 h. Afterwards, the value of absorbance was read at 450 nm on a microplate reader (Thermo Fisher, USA). Cell viability was calculated as per instructions.

2.5 Small interfering RNA (siRNA) assays

For NRF2 knockdown, NR8383 cells were seeded in 12-well plates at a density of 1×10^6 cells per well and transfected in antibiotic-free medium using Lipofectamine 3000 (L3000008, Thermo Fisher Scientific). NR8383 cells were transfected with siRNA targeting rat Nfe2l2 (AGACGGCCATGACTGATTAA) or negative control siRNA (TTCTCCGAACGTGTCACGT) for 12 h. The transfection medium was then replaced with complete DMEM containing 10% FBS and antibiotics. After an additional 48-72 h of culture, the transfected cells were harvested for subsequent experiments.

2.6 HAPE human transcriptome data analysis

To investigate key genes associated with the onset of HAPE and their correlation with disease phenotypes, this study conducted transcriptomic analysis on 12 blood samples (6 HAPE patients and 6 healthy controls) from the GSE260910 high-throughput transcriptomic dataset in the GEO database. Differential expression analysis was first performed using the “limma” package, with a significance threshold set at $p < 0.05$. Subsequently, weighted gene co-expression network analysis (WGCNA) was conducted using the “wgcna” package. In the WGCNA analysis, the study calculated the median absolute deviation (MAD) for each gene and excluded the bottom 50% of genes based on MAD values. The remaining gene expression data was used to construct a scale-free co-expression network, with the optimal soft threshold β value selected based on two criteria: the scale-free topological fit index (signed $R^2 > 0.85$) and average connectivity. The adjacency matrix was then transformed into a topological overlap matrix (TOM) to assess gene network connectivity. Gene modules were identified through average linkage hierarchical clustering (with a minimum module size of 30), and clusters with module distances less than 0.25 were merged to generate co-expression gene modules. Finally, gene modules significantly linked to HAPE onset were identified by conducting correlation analysis between module feature genes and disease phenotypes.

2.7 Data mining for network pharmacology

All known targets of NGR were obtained from the ETCM (<http://www.tcmip.cn/ETCM/>), CTD (<https://www.ctdbase.org/>), TCMSP (<https://tcmsp.91medicine.cn/#/home>), and PharmMapper (<https://www.lilab-ecust.cn/pharmmapper/>) databases. Genecards database (<https://www.genecards.org/>) and CTD database (<https://www.ctdbase.org/>) were used to collect therapeutic targets for HAPE. Subsequently, a Venn diagram was created based on the intersection of NGR and HAPE target genes using Venny 2.1 tool (<https://bioinfogp.cnb.csic.es/tools/venny/>). In addition, pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the Sangerbox platform (<http://www.sangerbox.com/>).

2.8 Molecular docking verification and molecular dynamics (MD) simulation

The chemical structure of Notoginsenoside R1 was obtained from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), saved in its SDF format, and converted to the mol2 format by OpenBabel-2.4.1. The PDB ID of the candidate NRF2 was derived from the UniProt database, and the corresponding protein three-dimensional structure was obtained from the RCSB PDB (<http://www.rcsb.org/>) database and saved in PDB format. Molecular docking was performed using Autodock Tools-1.5.7, and the docking score was used to assess the binding affinity of the target to the Notoginsenoside R1 molecule. The two-dimensional plan of the docking results was visualised by PyMOL software.

Molecular dynamics simulations of the complexes were performed using GROMACS 2023 for 100 ns. The CHARMM36 force field was applied to the protein, while ligand topology was generated with GAFF2 parameters. Systems were solvated in cubic boxes with TIP3P water molecules under periodic boundary

conditions. Electrostatic interactions were treated via Particle Mesh Ewald (PME), and van der Waals interactions utilized a cutoff of 1.0 nm. Prior to production runs, the systems underwent energy minimization followed by equilibration steps: 100,000 steps of isothermal-isovolumetric (NVT) and isothermal-isobaric (NPT) equilibration, each with a coupling constant of 0.1 ps and durations of 100 ps. The production MD simulations were conducted at a constant temperature of 300 K and pressure of 1 bar. All procedures ensured system stability and accurate representation of molecular interactions.

2.9 Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) measurements were performed on a Biacore 1K instrument (Cytiva, Sweden) at 25 °C. NRF2 protein was covalently immobilized onto a CM5 sensor chip via standard amine coupling. Serial dilutions of NGR were injected over the chip surface at a flow rate of 30 µL/min. The association and dissociation phases were monitored for 90 s and 120 s, respectively. The equilibrium dissociation constant (KD) was determined by fitting the data to a steady-state affinity model to assess the binding affinity between NRF2 and NGR.

2.10 Coimmunoprecipitation (co-IP)

NR8383 cells were harvested and subjected to lysis, followed by centrifugation to obtain the supernatant. The supernatant underwent pre-clearance through the addition of protein A + G magnetic beads, with incubation conducted for 1 h at 4°C. Post-incubation, the beads were separated by centrifugation, and the pre-cleared supernatant was preserved. RIPK1 and NRF2 antibodies were introduced to the pre-cleared supernatant, and the mixture was incubated overnight at 4°C with gentle agitation. A control IgG was included as a negative control. Subsequently, protein A + G magnetic beads were added to the supernatant and incubated for an additional hour at 4°C to facilitate the capture of antibody-protein complexes by the beads. The captured complexes were eluted from the beads using SDS-PAGE sample buffer and subjected to heating at 95°C for 5 min. Protein expression was subsequently analyzed via Western blotting.

2.11 Propidium iodide (PI) staining and lactate dehydrogenase (LDH) release

PI staining and LDH release were used to assess the integrity of the plasma membrane of NR8383 cells. NR8383 cells were embedded in 2% agarose, sectioned, circled with a histochemical pen, and stained with PI for nuclear visualization. Sections were sealed with an anti-fade fluorescence quencher and examined with a microscope.

According to the instructions of the LDH Assay Kit (C0017, Biyuntian Biotechnology Co. Ltd., Shanghai, China), LDH release from NR8383 cells was measured. The LDH release percentage was computed using the formula: % LDH release = (sample LDH activity - background LDH)/ (total LDH activity - background LDH) × 100.^[32]

2.12 Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

An Isolation Kit (RE-03014, Foregene, China) for Total RNA was used to isolate RNA from NR8383 cells. For the qPCR, 2 µg of total RNA was added to PCR tubes. The qPCR was conducted using a CFX Connect (BIORAD, USA) fluorescence quantitative PCR instrument. The reaction mixture, totaling 20 µL, contained 2× qPCR MasterMix, 7.5 µM gene-specific primer mix, reverse transcription product, and ddH₂O. The amplification protocol was set at an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Primer sequences for *Tnf-α*, *IL-1β*, *Cxcl1*, and *Ccl20* are detailed in Table 1. Target gene expression levels were normalized to β-actin, a housekeeping gene.

Table 1. Primer sequences used in this study.

Name	Primer sequences (5'-3')	NCBI accession no.
<i>β-actin</i>	Forward: TGTCACCAACTGGGACGATA Reverse: GGGGTGTTGAAGGTCTCAAA	NM_031144
<i>Tnf-α</i>	Forward: CACCACGCTCTTCTGTCTACTGAAC Reverse: TGGGCTACGGGCTTGCTCACTC	NM_012675
<i>IL-1β</i>	Forward: ACAAATCCCTGTGGCCTTG Reverse: TGTGCCGTCTTTCATCACAC	NM_031512
<i>Cxcl1</i>	Forward: GCAGACAGTGGCAGGGATTACAC Reverse: AGTGTGGCTATGACTTCGGTTTGG	NM_030845
<i>Ccl20</i>	Forward: CAGTCAGAAGCAGCAAGCAACTTTG Reverse: TTCGTCGGCCATCTGTGTTGTG	NM_019233

2.13 Animal experiments

Animal experiments were conducted in compliance with the Animal Research Welfare and Ethics Committee of Chengdu University of Traditional Chinese Medicine (Grant Number: 2022-18). 49 male SD rats (180-200 g) were bought from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). Rats were housed in groups in a sterile room with a 12-hour light/dark cycle and unlimited food and water. In alignment with experimental requirements, rats were randomized into seven groups (n=7 per group): (1) Con group, rats were kept under normobaric normoxic conditions and received an intraperitoneal injection of saline with 2% dimethyl sulfoxide (DMSO) in a volume equivalent to that of NGR once daily for 3 days; (2) HH group, rats received an intraperitoneal injection of saline with 2% DMSO in a volume equivalent to that of NGR once daily for 3 days, then were exposed to hypobaric hypoxia for 48 h; (3) NGR+HH group, rats were pre-treated with NGR (100 mg/kg, i.p.) for 3 days, once a day, and then were exposed to hypobaric hypoxia for 48 h; (4) dexamethasone (Dex)+HH group, rats were pre-treated with dexamethasone (4 mg/kg, i.p.) for 3 days, once a day, and then were exposed to hypobaric hypoxia for 48 h; (5) GSK'872+HH group, rats were pre-treated with GSK'872 (4 mg/kg, i.p.) one hour before modeling, followed by exposure to hypobaric hypoxia for 48 h; (6) ML385+HH group, rats were pre-treated with ML385 (30 mg/kg, i.p.) one hour before modeling, followed by exposure to hypobaric hypoxia for 48 h; (7) NGR+ML385+HH group, rats were injected intraperitoneally with 100 mg/kg NGR once daily for 3 consecutive days. One hour after the final NGR administration, they received an intraperitoneal injection of 30 mg/kg ML385. One hour later, the rats were exposed to hypobaric hypoxia for 48 h. Given that dexamethasone prevents HAPE, it was employed as a positive drug, with its dosing regimen derived from existing studies.^[33] GSK'872, an inhibitor of RIPK3 kinase, was administered based on body surface area conversions and existing literature to determine the

appropriate dose.^[34] ML385 is an inhibitor of NRF2. The dissolution methods for both ML385 and NGR, as well as their respective dosing regimens, were consistent with the methodologies outlined in our preceding research.^[4, 9] HAPE rats were modeled as previously described ^[4]. In brief, rats were placed inside a ProOx-830 hypobaric hypoxia chamber (Tawang Intelligent Technology, Shanghai, China) under the following conditions: altitude 6,000 m, oxygen partial pressure 9.6 kPa, humidity 60%, temperature 20°C, and then rapidly elevated to an altitude of 6,000 m within 5 min. A humane euthanasia was performed 48 h after the rats were induced, and abdominal aortic blood, lung tissue and BALF were harvested for subsequent index analyses.

2.14 Arterial blood gas analysis

An EPOC blood gas analyzer (Siemens Healthcare, US), specifically designed for animal blood gas analysis, was utilized to assess variations in arterial blood gases following the induction of HAPE in rats. After the experimental period, blood samples were collected from the abdominal aorta. The samples were immediately stabilized in heparinized tubes to prevent coagulation and subsequently analyzed using the blood gas analyzer. Key parameters measured included: arterial oxygen saturation (SaO₂), partial pressure of oxygen (PaO₂), partial pressure of carbon dioxide (PaCO₂), pH values and bicarbonate (HCO₃).

2.15 Histological analysis

Right lung tissue was fixed in 4% paraformaldehyde for 24 h, subsequently embedded in paraffin, and sectioned at a thickness of 4 μm. Following this procedure, hematoxylin and eosin (H&E) staining and optical microscopy were used to examine the tissue sections. Blinded and independent scoring was conducted by two pathologists, following the McGuigan method.^[35]

2.16 Lung water content

The lungs were removed, and the surface liquid was dried off. The wet weight of the lungs was recorded. Subsequently, the lungs were baked at 60°C for 72 h until reaching a constant weight, after which their dry weight was measured. Lung water content was estimated by the formula (lung wet weight - lung dry weight)/lung wet weight × 100%.

2.17 Determination of total protein concentration in BALF

Rats underwent bronchoalveolar lavage as previously outlined ^[4], and BALF was collected accordingly. A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) was used to quantify BALF protein concentrations.

2.18 Extraction of nuclear and cytoplasmic protein

A Nuclear and Cytoplasmic Protein Extraction Kit (AR0106, Boster Biological Technology Co.Ltd, USA) was used to separate nuclear and cytoplasmic proteins. In brief, NR8383 cells or lung tissues were thoroughly

mixed with the cytoplasmic protein extraction reagent and incubated on ice for 15 min. Then, the mixture was centrifuged ($16,000 \times g$, 5 min, 4°C), and the resulting supernatant was promptly transferred to pre-cooled centrifuge tubes, which is the cytoplasmic protein. Subsequently, the remaining precipitate was treated with nuclear protein extraction reagent. After shaking and further incubation on ice for 40 min, the mixture was centrifuged ($16,000 \times g$, 5 min, 4°C). The supernatant was carefully aspirated as nuclear proteins. For further analysis, both cytoplasmic and nuclear proteins were stored at -80°C .

2.19 Western blotting assay

NR8383 cells and lung tissues were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer. Supernatants were collected after centrifugation ($12,000 \times g$, 10 min, 4°C) and protein concentration was assayed utilizing a BCA kit. Subsequently, equal amounts of proteins were loaded onto an 8%-12% gel electrophoresis (SDS-PAGE), and electroblotted to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with a quick sealing solution and incubated overnight at 4°C with the following primary antibodies at the indicated dilutions: HIF-1 α (1:1000), VEGF (1:1000), AQP1 (1:5000), AQP5 (1:1000), NRF2 (1:1000), p-RIPK1 (1:1000), RIPK1 (1:1000), p-RIPK3 (1:1000), RIPK3 (1:1000), p-MLKL (1:1000), MLKL (1:1000), HMGB1 (1: 1000), NF- κB p65 (1:1000), Lamin B1 (1:1000), and β -actin (1:5000). After incubation, the membranes were incubated with a horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (Abcam, ab205718, 1: 10000) for 2 h at room temperature. Following another round of washes, the blots were developed with supersensitive chemiluminescence substrate and imaged with BLT GelView 6000Plus imaging system.

2.20 Immunofluorescence

NR8383 cells and lung tissue sections ($10\text{ }\mu\text{m}$) were submerged in PBS and washed thrice for 5 min each, followed by blocking with 3% bovine serum albumin (BSA) for 30 min. Primary antibodies (NRF2, RIPK1, RIPK3, p-RIPK3, p-MLKL) were applied dropwise onto the sections and incubated overnight at 4°C . On the second day, slides were washed thrice with PBS on a shaker for 5 min each. The secondary antibodies (Jackson ImmunoResearch, USA) were then applied dropwise and incubated at room temperature for 50 min. After another three PBS washes, slides were stained with DAPI and sealed using an anti-fade mounting medium. All immunofluorescence images were photographed by using a NIKON Eclipse Ci fluorescence microscope (Nikon, Japan) and analyzed with Software Image-J.

2.21 ROS assay

ROS level in NR8383 cells and lung tissues was determined by ROS Detection Assay Kit (YJ026288, Shanghai Enzyme Link Biotechnology). Briefly, horseradish peroxidase (HRP)-labeled detection antibody was added to each well except the blanks, then incubated at 37°C for 60 min. We added substrates A and B to each well and incubated them at 37°C for 15 min after washing five times. Stop solutions were added to each

well to terminate the reaction. Optical density (OD) values of the wells were recorded at 450 nm wavelength. A standard curve was generated to work out the concentration of each sample.

2.22 Superoxide dismutase (SOD) and glutathione (GSH) assay

As instructed by the manufacturer, superoxide dismutase (SOD, A001-3) and glutathione (GSH, A006-2-1) activities in NR8383 cells and lung tissues were determined by the appropriate kits (Nanjing Jianjian Bioengineering Institute).

2.23 Transmission electron microscopy (TEM) assay

NR8383 cells and lung tissues were fixed in 3% glutaraldehyde at 4°C. After dehydrating, immersing, and embedding, ultrathin sections were obtained and stained before being observed under a TEM (HT7700, Hitachi, Tokyo, Japan).

2.24 Statistical analysis

GraphPad Prism version 10.0 was used to analyze the data, which was represented as mean $\pm$ SEM. We conducted tests for homogeneity of variance to confirm the normal distribution of the data sets. Statistical significance was evaluated using one/two-way ANOVA and T-tests. $P < 0.05$ were considered statistically significant differences.

3. Results and discussion

3.1 NGR alleviated the inhibition of viability in NR8383 cells exposed to hypobaric hypoxia and exerted protective effects against HAPE

The chemical structure of NGR is shown in **Fig. 1A**. To determine the optimal modeling time point, we initially performed time-dependent experiments. As shown in **Fig. 1B**, we observed a time-dependent decrease in cell viability. Next, we assessed the cytotoxic effect of NGR on NR8383 cells, treating them with varying concentrations of NGR (5, 10, 20, 40, 80 and 100 μ M) for varying time periods (24 h, 48 h and 72 h). The CCK8 assay indicated no decrease and a slight increase in cell viability following treatment with 10, 20, and 40 μ M NGR at each time point. However, cell viability significantly decreased after treatment with 80 and 100 μ M NGR for 72 h (**Fig. 1C**).

Next, to estimate the protective effect of NGR on cell viability under hypobaric hypoxia conditions, NR8383 cells were pre-treated with NGR at 10, 20, or 40 μ M for 24 h followed by exposure to hypobaric hypoxia for an additional 24 h. The results showed that NGR at 10, 20, and 40 μ M remarkably abrogated the decrease in cell viability caused by hypobaric hypoxia, with 20 μ M being the most effective concentration (**Fig. 1D**). Consequently, a 24 h pre-treatment with 20 μ M NRG was chosen for subsequent *in vitro* experiments to maximize the pharmacodynamic effect and minimize cytotoxicity.

Next, we first demonstrated the pharmacodynamic effect of NGR against HAPE using western blot analysis *in vitro*. As a transcription factor, hypoxia-inducible factor 1 alpha (HIF-1 α) plays a pivotal role in

the cellular response to hypoxia, which is quickly degraded under normal oxygen levels but stabilizes and activates under hypoxic conditions.^[36, 37] Vascular endothelial growth factor (VEGF) is a potent angiogenesis promoter and is regulated as a response gene by HIF-1 α . Under hypoxic conditions, the rapid accumulation of HIF-1 α leads to increased VEGF expression, contributing to HAPE.^[38, 39] Our results indicated that exposure to hypobaric hypoxia increased the expression of both HIF-1 α and VEGF (**Fig. 1E-G**). Aquaporin 1 (AQP1) and AQP5 facilitate water transport through cells, maintaining osmotic balance.^[40] Our findings revealed that the HH group exhibited lower expression levels of AQP1 and AQP5 compared to the Con group (**Fig. 1E, H and I**). These data suggested a successful establishment of an *in vitro* HAPE model. However, premedication with NGR decreased HIF-1 α and VEGF expression while increasing AQP1 and AQP5 expression (**Fig. 1E-I**).

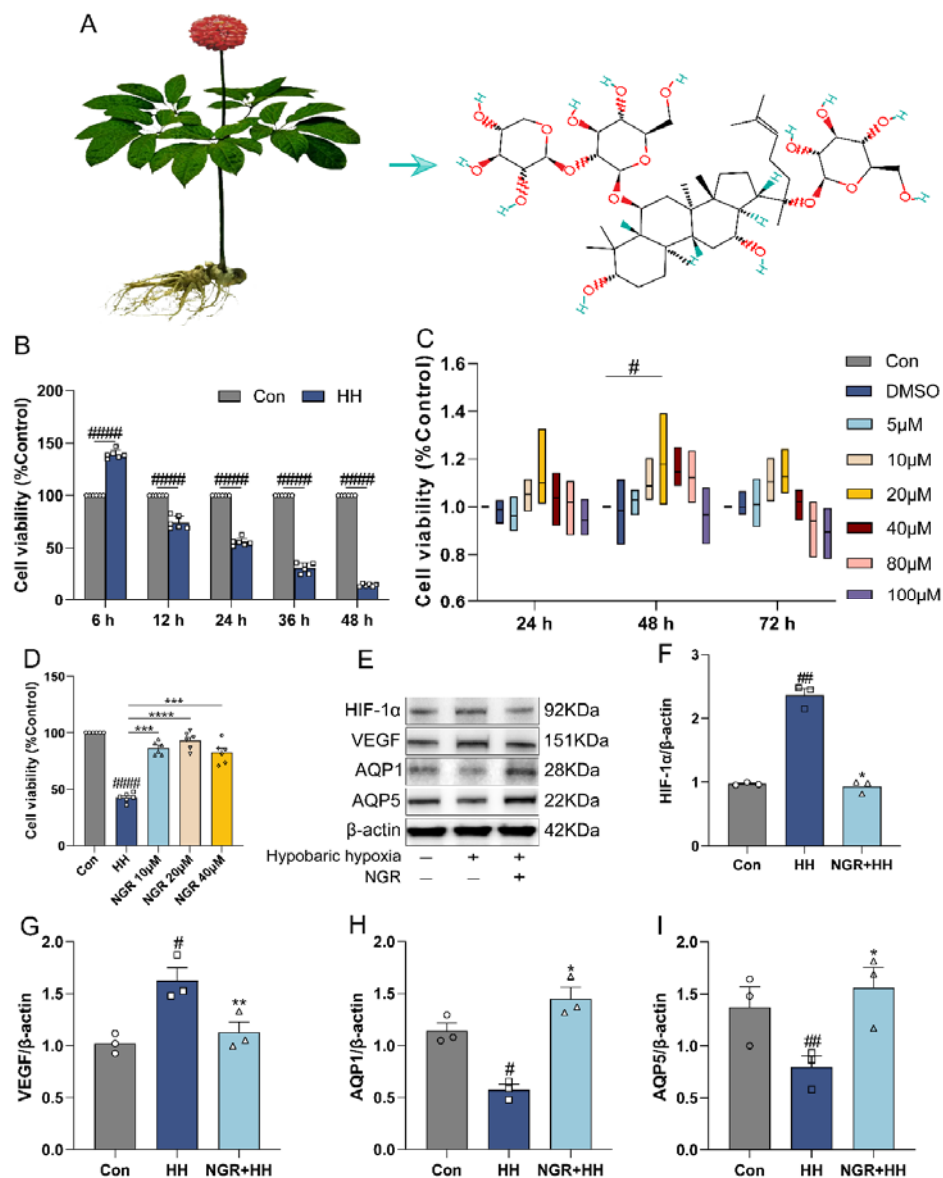


Fig. 1 Pharmacodynamic effects of NGR on HAPE in NR8383 cells. (A) The chemical structure of NGR. (B) Viability of NR8383 cells at various time points following exposure to hypobaric hypoxia. (C) Viability of NR8383 cells treated with different concentrations of NGR at different time points. (D) Viability of NR8383 cells treated with 10 μ M, 20 μ M, and 40 μ M NGR for 24 h followed by exposure to hypobaric hypoxia for an additional 24 h. (E-I) Protein expression levels of HIF-1 α , VEGF, AQP1, and AQP5 measured by Western blot. Data represent mean $\pm$ SEM (n=3). * P < 0.05, ## P < 0.01, #### P < 0.0001 compared with Con group; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared with HH group.

3.2 Prediction of NGR treatment targets for HAPE

Our previous research suggested that NRF2 may play a role in the pathogenesis of HAPE. To further validate this finding, we first analyzed the GSE260910 high-throughput transcriptomic dataset from the GEO database, which revealed 6,554 significantly differentially expressed genes (DEGs, $P < 0.05$). Among these, 3,227 genes were upregulated, while 3,327 were downregulated, including NFE2L2 (protein name NRF2). The top-ranked DEGs are presented in **Fig. 2A, B**. WGCNA was performed with a soft threshold of $\beta = 28$, determined based on Signed R^2 and average connectivity. This analysis resulted in the identification of eight co-expression gene modules (**Fig. 2C, D**). Notably, the turquoise module demonstrated the strongest correlation with the HAPE phenotype ($r = 0.92$, $P < 0.0001$), and included key genes such as NFE2L2 and RIPK3 (**Fig. 2E, F**).

To further investigate the potential therapeutic targets of NGR for the treatment of HAPE, we performed a network pharmacology analysis. A comprehensive search of the GeneCards and Comparative Toxicogenomics Database (CTD) identified 210 targets associated with HAPE (**Fig. 2G**). Additionally, using the ETCM, CTD, TCMSP, and PharmMapper databases, we predicted 346 potential target genes for NGR (**Fig. 2H**). Subsequent KEGG pathway enrichment analysis of the overlapping targets, conducted using the Venny 2.1 tool, revealed that these genes are predominantly involved in key signaling pathways such as HIF-1, tumor necrosis factor (TNF), necroptosis, and VEGF (**Fig. 2I**). These findings suggest that NGR may alleviate HAPE by modulating hypoxia- and necroptosis-related signaling networks.

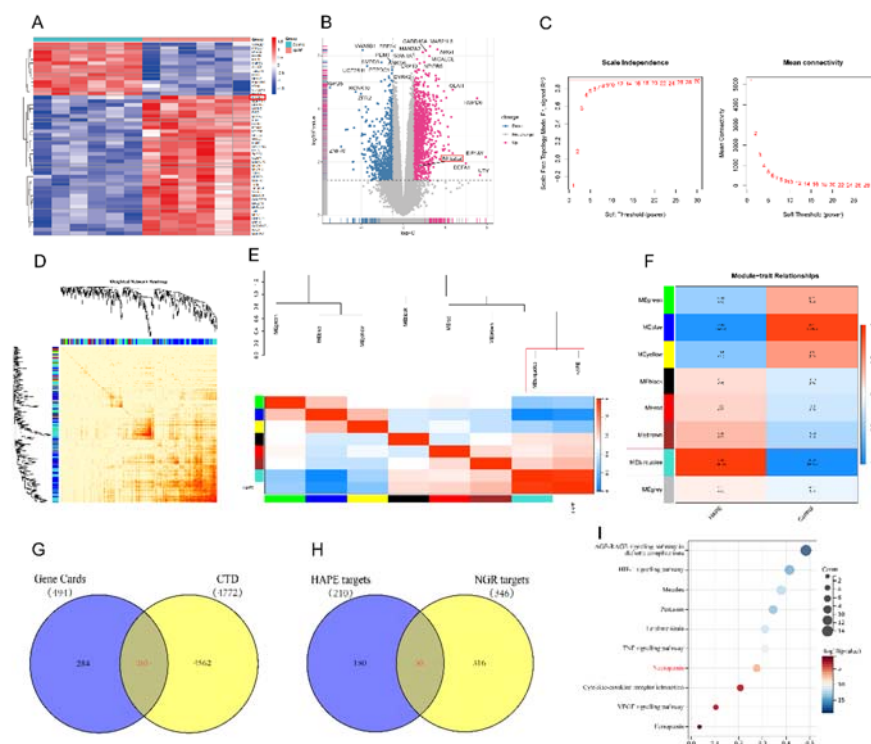


Fig. 2 High-Throughput Transcriptomics and Network Pharmacology Analysis. (A) Heat map showing differential analysis, with NFE2L2 highlighted in a red frame ($P < 0.05$). (B) Volcano plot representing differential analysis, with NFE2L2 marked in a red box. (C) Screening for soft thresholds. (D) WGCNA. (E, F) Correlation analysis between gene modules and disease phenotypes. (G) Venn diagram illustrating the overlapping target genes related to HAPE, identified through a combined analysis of GeneCards and the CTD. (H) Cross-tabulation analysis of targets associated with HAPE and those linked to NGR. (I) Pathway enrichment analysis of the overlapping genes identified in panel (H).

3.3 The therapeutic effect of NGR on HAPE was related to promoting nuclear translocation of NRF2 in NR8383 cells inhibited by hypobaric hypoxia

To clarify whether NGR alleviates hypobaric hypoxia-induced HAPE by regulating NRF2, we first employed molecular docking simulations to investigate the interaction between the NRF2 protein and the compound NGR. As shown in **Fig. 3A**, our results indicated that the binding energy of the NRF2-NGR complex was -14.12 kcal/mol. Further molecular dynamics simulations provided confirmation of a stable binding interaction between NGR and NRF2 (**Fig. S1A-E**). Furthermore, we evaluated the binding affinity between NGR and purified NRF2 protein using SPR. The analysis revealed a binding affinity of 49.4 μ M, indicating a robust interaction between NGR and NRF2 (**Fig. 3B**). Additionally, the Western blotting analysis demonstrated an elevation in NRF2 expression within the cytoplasm and a corresponding reduction in NRF2 expression within the nucleus following 24 h of hypobaric hypoxia exposure (**Fig. 3C and Fig. S1F**). Conversely, NGR pre-treatment resulted in an augmentation of NRF2 content within the nucleus when compared to the HH group (**Fig. 3C and Fig. S1F**). Consistently, immunofluorescence analysis showed the similar results (**Fig. 3D**).

Moreover, we assessed oxidative stress by examining the activities of ROS, SOD, and GSH after hypobaric hypoxia exposure in NR8383 cells. ROS serve as crucial indicators of oxidative stress, with excessive accumulation potentially leading to severe oxidative damage. Antioxidants such as SOD and GSH are essential for mitigating the detrimental effects of ROS.^[41] As shown in **Fig. 3E, F**, our results revealed a significant increase in ROS levels and a decrease in SOD and GSH activities in NR8383 cells following exposure to hypobaric hypoxia. Contrarily, pretreatment with NGR decreased the level of ROS and increased the antioxidant activities of SOD and GSH (**Fig. 3E, F**). Thus, we speculated that severe oxidative stress induced by hypobaric hypoxia hinders the nuclear translocation of NRF2, contributing to the dysfunction of the NRF2 antioxidant defense system. Conversely, pre-administration of NGR activated the expression of antioxidant genes by facilitating the nuclear translocation of NRF2.

To validate this conjecture, the NRF2 inhibitor, ML385, was used to inhibit the transcriptional activation function of NRF2. The CCK8 assay results indicated that ML385 markedly inhibited NRF2, with an IC₅₀ value of 20 μ M (**Fig. S1G**). The Western blotting results showed that a concentration of 20 μ M ML385 significantly inhibited the expression of NRF2 in NR8383 cells after 24 h (**Fig. S1H**). Therefore, we selected 20 μ M ML385 to pretreat NR8383 cells for *in vitro* experiments. Our findings indicated that ML385 not only further suppressed the nuclear translocation of NRF2 (**Fig. 3G, H and Fig. S1I**), but also exacerbated oxidative stress when compared to the HH group (**Fig. 3I**). Besides, ML385 offset the NRF2 nuclear translocation facilitated by NGR pre-administration and the subsequent reduction of oxidative stress (**Fig. 3G-I and Fig. S1I**).

To further clarify that NRF2 is a critical target mediating the protective effects of NGR against HAPE, we performed siRNA-mediated knockdown of NRF2 in NR8383 cells. The results were consistent with those

obtained using ML385, further supporting the essential role of NRF2 in the anti-HAPE effects of NGR (Fig. 3J-L and Fig. S1J).

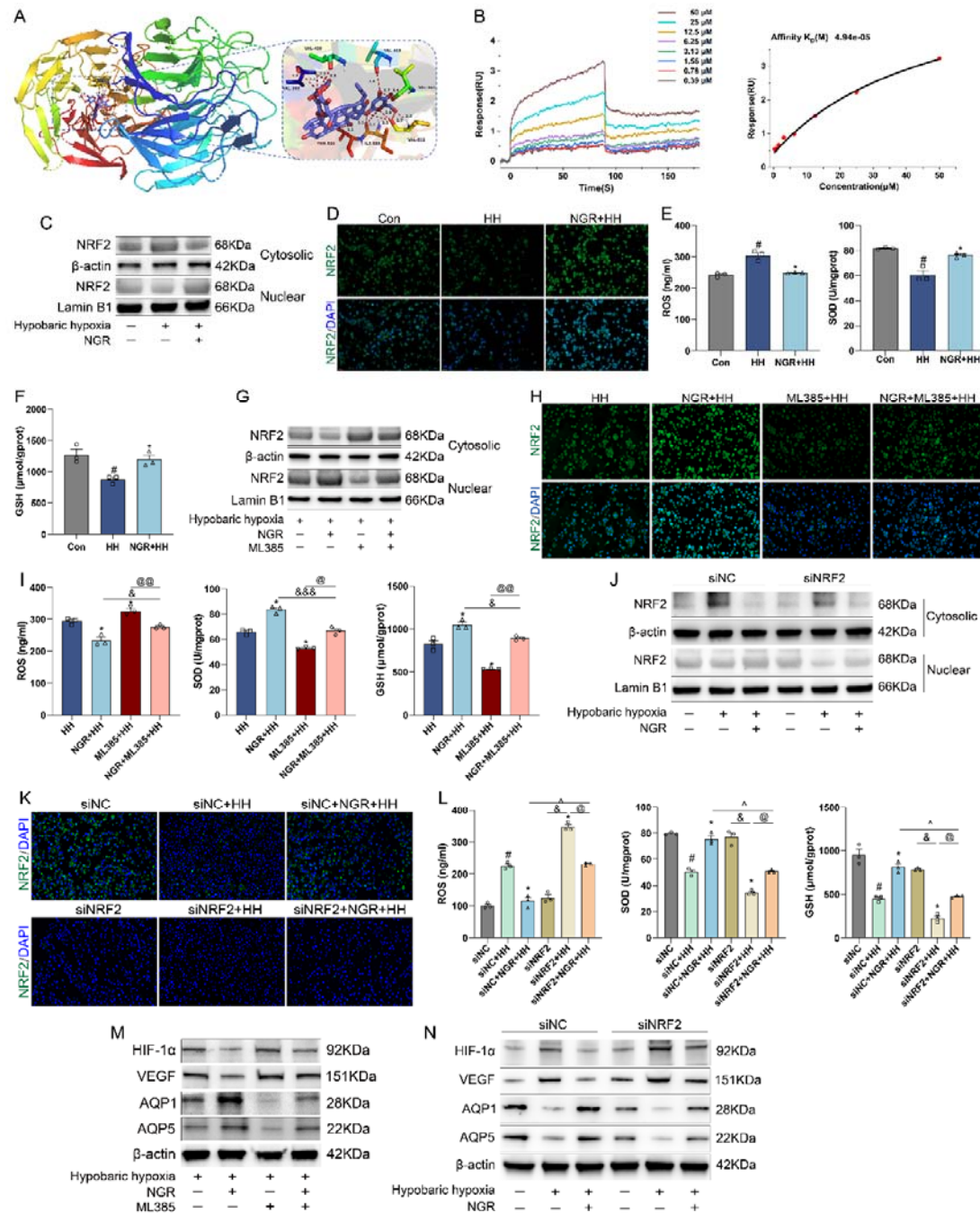


Fig. 3 NGR facilitated nuclear translocation of NRF2, inhibited oxidative stress, and attenuated HAPE in NR8383 cells. (A) NGR and NRF2 molecular docking. (B) SPR analysis of the binding affinity between NRF2 protein and NGR. (C) Western blot analysis shows NRF2 distribution in nuclear and cytoplasmic fractions. (D) Immunofluorescence depicting NRF2 distribution in NR8383 cells. (E, F) Analysis of ROS, SOD and GSH activities. (G) Western blot showing NRF2 distribution in the nucleus and cytoplasm post-ML385 treatment. (H) Immunofluorescence depicting NRF2 localization in NR8383 cells after ML385 treatment. (I) Evaluations of ROS, SOD, and GSH activities in NR8383 cells treated with ML385. (J) Western blot showing NRF2 distribution in the nucleus and cytoplasm post-NRF2 knockdown. (K) Immunofluorescence depicting NRF2 localization in NR8383 cells after NRF2 knockdown. (L) Evaluations of ROS, SOD, and GSH activities in NR8383 cells after NRF2 knockdown. (M) Western blot analysis detailing the expression of HIF-1 α , VEGF, AQP1, and AQP5 following ML385 treatment. (N) Western blot analysis detailing the expression of HIF-1 α , VEGF, AQP1, and AQP5 post-NRF2 knockdown. Data represent means $\pm$ SEM (n=3). # P < 0.05, ## P < 0.01, #### P < 0.0001 compared with Con group; * P < 0.05, ** P < 0.01 compared with HH group; & P < 0.05, && P < 0.01, &&& P < 0.001 compared with NGR+HH group; @ P < 0.05, @@ P < 0.01 compared with ML385+HH group; In NRF2 knockdown cells, # P < 0.05, ## P < 0.01 compared with siNC group; * P < 0.05, ** P < 0.01 compared with siNC+HH group; & P < 0.05 compared with siNRF2 group; @ P < 0.05, @@ P < 0.01 compared with siNRF2+HH group; ns presents no statistical significance. See also Fig. S1.

To further confirm that NRF2 is a critical regulator in NGR's mitigation of hypobaric hypoxia-induced HAPE, we conducted Western blot analysis following pre-treatment with ML385 or NRF2 knockdown. As shown in **Fig. 3M-N** and **Fig. S1K-N**, both ML385 and siNRF2 attenuated the therapeutic effects of NGR, as evidenced by increased HIF-1 α and VEGF levels, along with reduced AQP1 and AQP5 expression in NR8383 cells. These findings highlight the pivotal role of NRF2 in NGR's protective effects against HAPE, positioning NRF2 as a potential novel therapeutic target for HAPE.

3.4 Hypobaric hypoxia-induced necroptosis and subsequent inflammation contribute to HAPE development

The WGCNA analysis indicated that the onset of HAPE is associated with RIPK3 (**Fig. 2E, F**). Interestingly, network pharmacology analysis suggested that NGR may alleviate HAPE by modulating necroptosis (**Fig. 2I**). Therefore, to verify that the anti-HAPE efficacy of NGR is associated with the inhibition of necroptosis, we conducted Western blot analysis to assess the expression levels of necroptosis-related proteins. As shown in **Fig. 4A** and **Fig. S2A**, the phosphorylation of RIPK1, RIPK3, and MLKL was increased after exposure to hypobaric hypoxia. Moreover, immunofluorescence analysis revealed RIPK1 co-localization with RIPK3 in NR8383 cells (**Fig. 4B**). This co-localization was examined in detail using a plot profile (**Fig. S2B-E**). Notably, Pearson's co-localization coefficients for the whole image analysis revealed that the values were markedly larger in the HH group compared to the Con group (**Fig. S2F**). To gain a deeper understanding of the interaction between RIPK1 and RIPK3, we conducted a co-IP assay (**Fig. 4C**). These findings suggested that hypobaric hypoxia caused the formation of necrosome complexes.^[42] Simultaneously, the immunofluorescence intensity of p-MLKL was significantly increased in the HH group (**Fig. 4D** and **Fig. S2G**).

Necroptosis is characterized by the loss of plasma membrane integrity and the release of the cytosolic LDH. When membrane integrity is lost, propidium iodide (PI), a membrane impermeable fluorescent DNA intercalating dye, can label intracellular nucleic acids.^[43] Thus, we utilized PI staining and LDH release assays to assess hypobaric hypoxia-induced necroptosis. Following 24 h of hypobaric hypoxia exposure, we found a markedly enhanced PI-positive staining (**Fig. 4E** and **Fig. S2H**) and release of LDH (**Fig. 4F**). Moreover, we utilized TEM to observe ultrastructural alterations in NR8383 cells after hypobaric hypoxia exposure. As expected, the cells exhibited typical morphological features of necroptosis, such as organelle swelling, particularly in mitochondria, and plasma membrane rupture (**Fig. 4G**). This resulted in the leakage of cytoplasmic contents, which provoked robust inflammatory responses.^[18, 44]

In light of these considerations, we quantified the expression of high mobility group box 1 (HMGB1) protein in NR8383 cells, as well as the nuclear and cytoplasmic distribution of nuclear factor kappa B (NF- κ B) p65 protein within these cells. HMGB1, a pivotal inflammatory mediator, activates NF- κ B, triggering the release of cytokines like tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) and thereby enhancing the inflammatory response cascade.^[45, 46] Our findings demonstrated a significant elevation in HMGB1 and nuclear NF- κ B p65 expression, along with a concurrent decrease in cytoplasmic NF- κ B p65 upon exposure to hypobaric hypoxia (**Fig. 4H** and **Fig. S2I**).

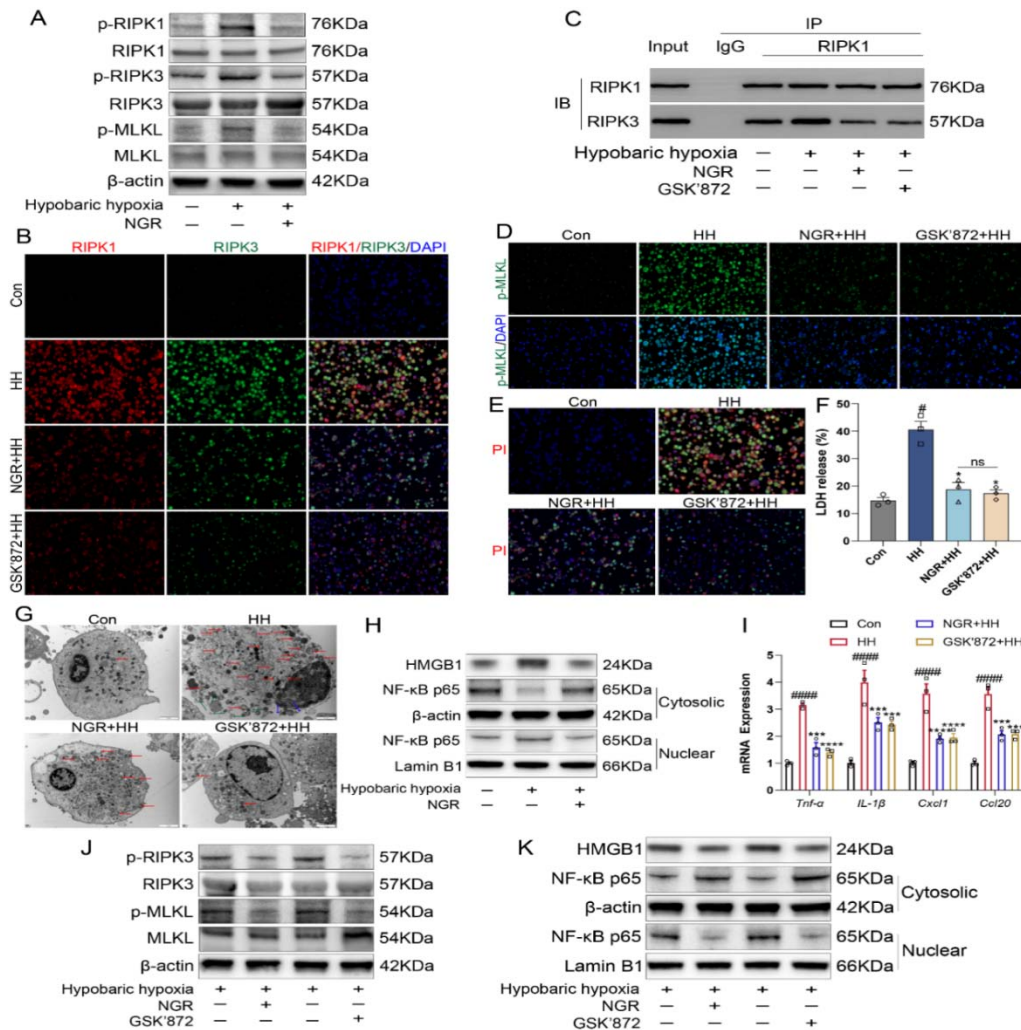


Fig. 4 NGR alleviated hypobaric hypoxia-induced necroptosis and subsequent inflammatory response in NR8383 cells. (A) RIPK1, RIPK3, and MLKL proteins analyzed by western blot. (B) Immunofluorescence staining shows co-localization of RIPK1 and RIPK3 in NR8383 cells. Scale bars: 20 μm. (C) Coimmunoprecipitation to detect the interaction between RIPK1 and RIPK3. (D) Immunofluorescence analysis of p-MLKL. Scale bars: 20 μm. (E) Cell death was assessed by PI staining. Scale bars: 20 μm. (F) Determination of LDH release levels. (G) TEM reveals ultrastructural changes in NR8383 cells. Scale bars: 2 μm. Red arrows, swollen and vacuolated mitochondria; blue arrows, ruptured plasma membrane; green arrows, leakage of cytosolic content. (H) Western blot analysis of HMGB1 and NF-κB p65 distribution in the nuclear and cytoplasmic fractions. (I) Quantitative RT-PCR analysis of *Tnf-α*, *IL-1β*, *Cxcl1*, and *Ccl20* mRNA levels. (J) Western blot analysis of p-RIPK3 and p-MLKL following treatment with GSK'872. (K) Western blot analysis of HMGB1 and NF-κB p65 distribution in the nuclear and cytoplasmic fractions following GSK'872 treatment. Data were means ± SEM (n=3). #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, ####*P* < 0.0001 compared with Con group; **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 compared with HH group; ns presents no statistical significance. See also Fig. S2.

Furthermore, we analyzed the mRNA levels of pro-inflammatory cytokines and chemokines, such as *TNF-α*, *IL-1β*, chemokine (C-X-C motif) ligand 1 (*CXCL1*), and C-C motif chemokine ligand 20 (*CCL20*), in NR8383 cells. As shown in **Fig. 4I**, the mRNA levels of *Tnf-α*, *IL-1β*, *Cxcl1*, and *Ccl20* were elevated following hypobaric hypoxia exposure.

However, to our surprise, the pre-administration of NGR reversed these phenomena to varying degrees (**Fig. 4A-I** and **Fig. S2A-I**).

To further validate whether hypobaric hypoxia-induced HAPE is attributable to RIPK3-mediated necroptosis, we used the RIPK3 kinase activity inhibitor GSK'872. As shown in **Fig. S2J**, 3 μM GSK'872 had no significant toxic effects on NR8383 cells after 24 h and even promoted cell growth. Consequently, we

selected this concentration for our subsequent *in vitro* experiments. The results showed that inhibition of RIPK3 activity strongly reduced cell death induced by hypobaric hypoxia. Specifically, NR8383 cells exhibited a number of significant changes, including decreased phosphorylation of RIPK3 and MLKL (**Fig. 4J and Fig. S2K**), reduced co-localization of RIPK1 and RIPK3 (**Fig. 4B and Fig. S2B-F**), decreased interaction between RIPK1 and RIPK3 (**Fig. 4C**), lower intensity of p-MLKL immunofluorescence (**Fig. 4D and Fig. S2G**), attenuated PI positivity (**Fig. 4E and Fig. S2H**), decreased LDH release (**Fig. 4F**), less organelle swelling (**Fig. 4G**), and lower mRNA levels of *Tnf- α* , *IL-1 β* , *Cxcl1*, and *Ccl20* (**Fig. 4I**). Additionally, there was a reduction in the protein expression of HMGB1 and nuclear NF- κ B p65, while the protein expression of cytoplasmic NF- κ B p65 exhibited a relative increase (**Fig. 4K and Fig. S2L**).

3.5 NGR alleviated HAPE by suppressing necroptosis in NR8383 cells

Next, to further study the action of necroptosis in HAPE pathogenesis, we conducted Western blot analysis for HIF-1 α , VEGF, AQP1, and AQP5 following pre-administration of GSK'872. Our results indicated that GSK'872 produced effects similar to NGR, reducing the expression of HIF-1 α and VEGF while increasing that of AQP1 and AQP5 (**Fig. 5A-E**).

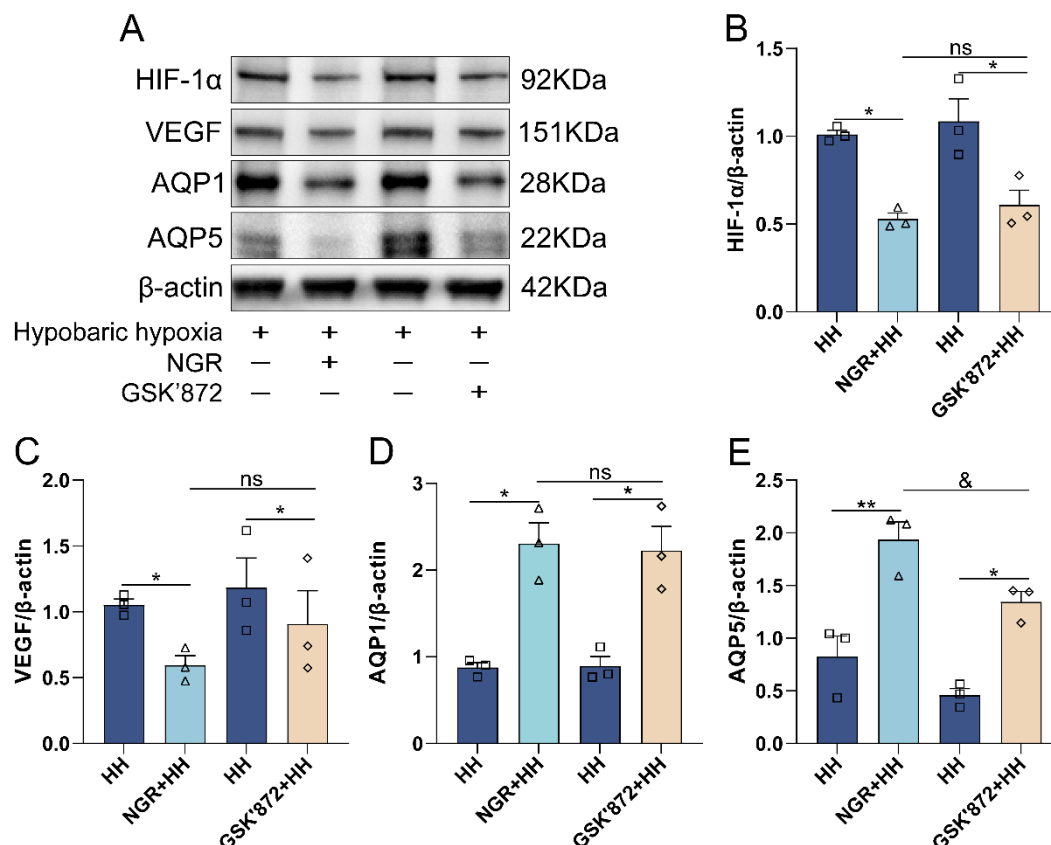
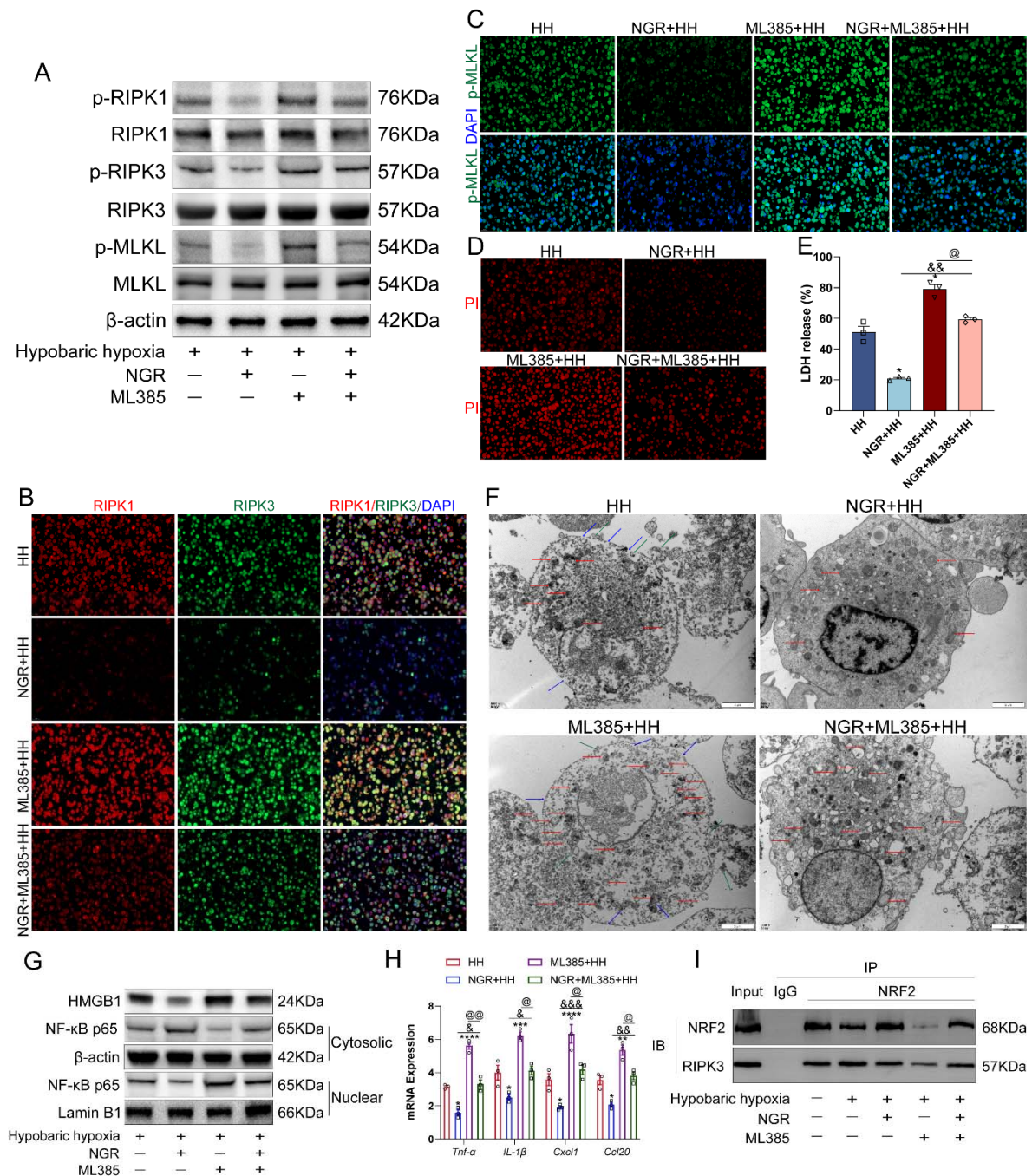


Fig. 5 NGR alleviated HAPE by suppressing necroptosis in NR8383 cells. (A-E) Western blotting was performed to detect the expression of HIF-1 α , VEGF, AQP1 and AQP5. Data represent means $\pm$ SEM (n=3). * P < 0.05, ** P < 0.01 compared with HH group; & P < 0.05 compared with NGR+HH group; ns presents no statistical significance.

3.6 NGR inhibited necroptosis in NR8383 cells under hypobaric hypoxia by regulating NRF2

Having demonstrated the important roles of the antioxidant-responsive transcription factor NRF2 and necroptosis in the pathogenesis of HAPE, we dissected the regulatory role of NRF2 in the attenuation of hypobaric hypoxia-induced necroptosis by NGR. As mentioned earlier, the NR8383 cells were pretreated with 20 μ M ML385. As expected, in western blot analysis, ML385 further increased RIPK1, RIPK3, and MLKL phosphorylation levels (**Fig. 6A and Fig. S3A**). It also enhanced the co-localization of RIPK1 and RIPK3 (**Fig. 6B and Fig. S3B-F**), and boosted the immunofluorescence intensity of p-MLKL under hypobaric hypoxia, counteracting the effects of NGR in reducing necroptosis (**Fig. 6C and Fig. S3G**). The above experimental results were further confirmed by PI staining (**Fig. 6D and Fig. S3H**), LDH release (**Fig. 6E**), and TEM analysis (**Fig. 6F**).



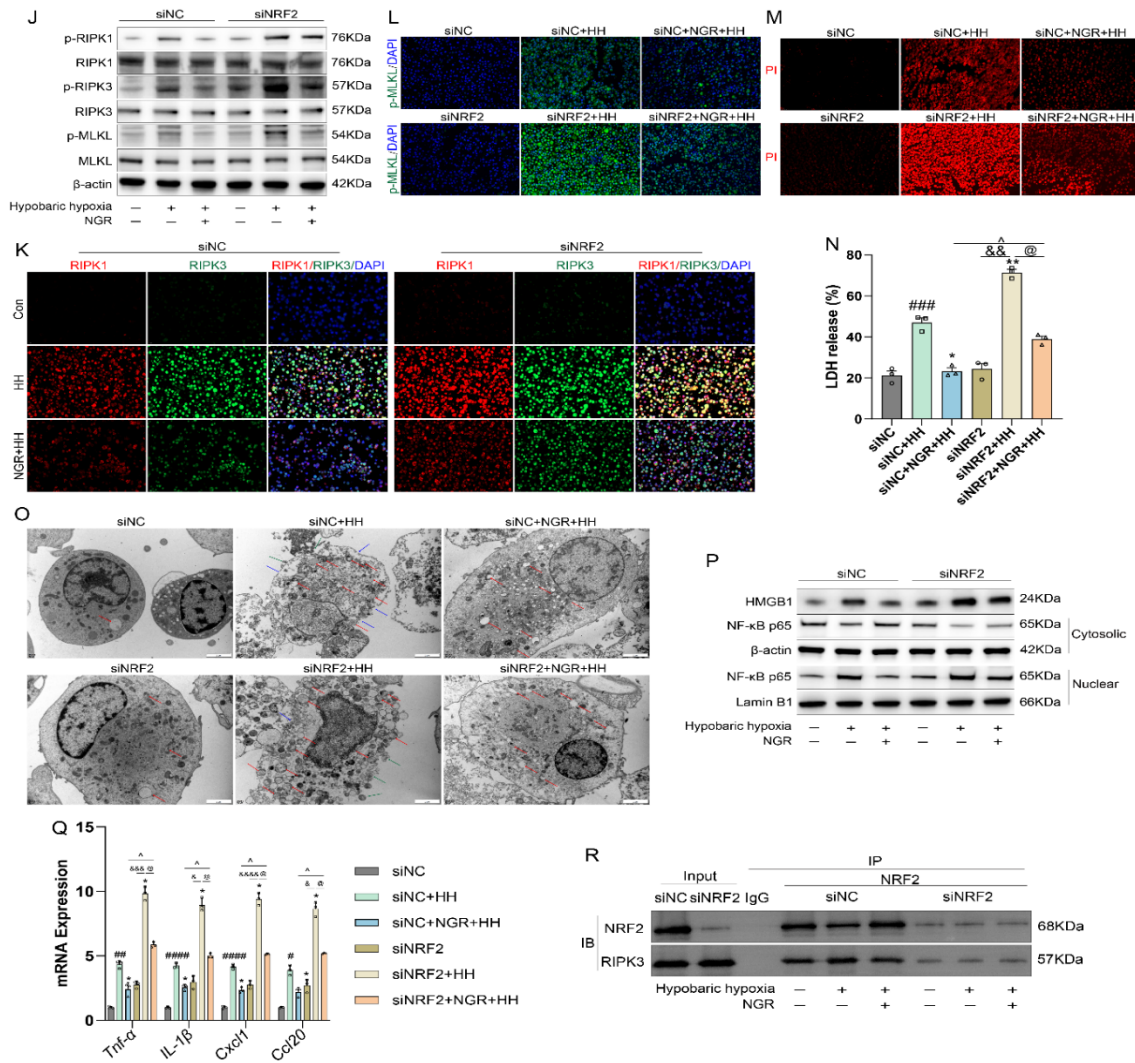


Fig. 6 NGR inhibited hypobaric hypoxia-induced necroptosis by regulating NRF2 in NR8383 cells. (A) Western blot analysis evaluating RIPK1, RIPK3, and MLKL proteins after ML385 treatment. (B) Immunofluorescence staining shows co-localization of RIPK1 and RIPK3 in NR8383 cells treated with ML385. Scale bars: 20 μ m. (C) Immunofluorescence analysis of p-MLKL was conducted following treatment with ML385. Scale bars: 20 μ m. (D) Cell death was assessed by PI staining after ML385 treatment. Scale bars: 20 μ m. (E) LDH release levels following ML385 treatment. (F) TEM details ultrastructural changes in NR8383 cells after ML385 treatment. Scale bars: 2 μ m. Red arrows, swollen and vacuolated mitochondria; blue arrows, ruptured plasma membrane; green arrows, leakage of cytosolic content. (G) Western blot analysis was performed to assess HMGB1 levels and the nuclear and cytoplasmic distribution of NF- κ B p65 after ML385 treatment. (H) Quantitative RT-PCR for Tnf- α , IL-1 β , Cxcl1, and Ccl20 mRNA levels post ML385 treatment. (I) Coimmunoprecipitation to detect the interaction between NRF2 and RIPK3. (J) Western blot analysis evaluating RIPK1, RIPK3, and MLKL proteins after NRF2 knockdown. (K) Immunofluorescence staining shows co-localization of RIPK1 and RIPK3 in NR8383 cells post-NRF2 knockdown. (L) Immunofluorescence analysis of p-MLKL was performed after NRF2 knockdown. (M) Cell death was assessed by PI staining following NRF2 knockdown. (N) LDH release levels post-NRF2 knockdown. (O) TEM details ultrastructural changes in NR8383 cells after NRF2 knockdown. Scale bars: 2 μ m. Red arrows, swollen and vacuolated mitochondria; blue arrows, ruptured plasma membrane; green arrows, leakage of cytosolic content. (P) Western blot analysis was performed to assess HMGB1 levels and the nuclear and cytoplasmic distribution of NF- κ B p65 after NRF2 knockdown. (Q) Quantitative RT-PCR for Tnf- α , IL-1 β , Cxcl1, and Ccl20 mRNA levels after NRF2 knockdown. (R) Co-immunoprecipitation was performed to detect the interaction between NRF2 and RIPK3 after NRF2 knockdown. Data represent means $\pm$ SEM (n=3). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared with HH group; & P < 0.05, && P < 0.01, &&& P < 0.001 compared with NGR+HH group; @ P < 0.05, @@ P < 0.01, @@@ P < 0.0001 compared with ML385+HH group. See also **Fig. S3**. In NRF2 knockdown cells, # P < 0.05, ## P < 0.01, ### P < 0.001, #### P < 0.0001 compared with siNC group; * P < 0.05, ** P < 0.01, *** P < 0.0001 compared with siNC+HH group; & P < 0.05, && P < 0.01, &&& P < 0.001, &&&& P < 0.0001 compared with siNRF2 group; @ P < 0.05, @@ P < 0.01 compared with siNRF2+HH group; ns presents no statistical significance. See also **Fig. S4**.

Next, we investigated the regulatory impact of NRF2 on the progression of secondary inflammation triggered by necroptosis in NR8383 cells subjected to hypobaric hypoxia. Our analyses revealed that inhibiting NRF2 further increased the protein expression of HMGB1 and nuclear NF- κ B p65, while concurrently reducing cytoplasmic NF- κ B p65 expression (**Fig. 6G and Fig. S3I**). Furthermore, the mRNA levels of *Tnf- α* , *IL-1 β* , *Cxcl1*, and *Ccl20* were elevated (**Fig. 6H**). Additionally, the presence of ML385 attenuated the mitigating effects of NGR-mediated inflammation (**Fig. 6G, H**). Co-immunoprecipitation assays further confirmed that exposure to hypobaric hypoxia induced the formation of NRF2-RIPK3 complexes in NR8383 cells (**Fig. 6I**). Notably, pre-treatment with NGR and the NRF2 inhibitor ML385 significantly reduced the formation of these complexes (**Fig. 6I**).

To further elucidate the regulatory role of NRF2 in NGR-mediated attenuation of hypobaric hypoxia-induced necroptosis, we performed NRF2 knockdown using siRNA. The results obtained were consistent with those observed following NRF2 inhibition with ML385 (**Fig. 6J-R and Fig. S4A-L**).

Based on these observations, we speculated that hypobaric hypoxia exposure impedes NRF2 translocation into the nucleus, resulting in its accumulation in the cytoplasm. This cytoplasmic accumulation may facilitate the interaction between NRF2 and RIPK3. In contrast, pre-administration of NGR appears to promote NRF2 translocation into the nucleus, thereby reducing NRF2 binding to RIPK3. This nuclear entry of NRF2 may enable the protein to exert its antioxidant and anti-inflammatory effects, ultimately attenuating necroptosis induced by hypobaric hypoxia.

3.7 NGR promoted nuclear translocation of NRF2 in vivo experiments

To comprehensively understand the critical role of NRF2 in NGR attenuating hypobaric hypoxia-induced HAPE, we conducted *in vivo* experiments. Nuclear and cytoplasmic proteins were extracted from lung tissue and subjected to immunoblot analysis. Consistent with *in vitro* results, in the HH group, the expression of NRF2 was elevated in the cytoplasm and diminished in the nucleus relative to the Con group (**Fig. 7A-C**). Likewise, it is reassuring to observe that intraperitoneal administration of NGR promotes nuclear translocation of NRF2, reduces the level of ROS, and activates the activity of the antioxidant SOD and GSH (**Fig. 7A-F**). However, the NGR-induced nuclear translocation of NRF2 and attenuation of oxidative stress damage were abolished by ML385 (**Fig. 7A-F**).

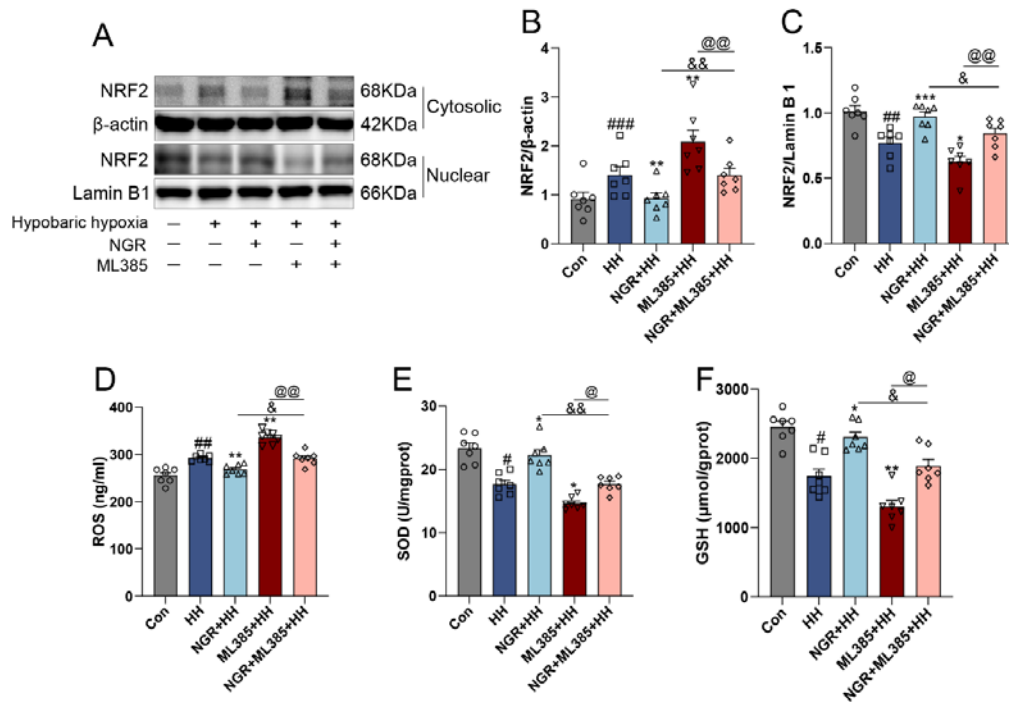


Fig. 7 NGR promoted nuclear translocation of NRF2 in HAPE rats. (A-C) Western blot analysis shows NRF2 distribution in nuclear and cytoplasmic fractions. (D-F) Analysis of ROS, SOD and GSH activities. Data represent means $\pm$ SEM (n = 7). # P < 0.05, ## P < 0.01, ### P < 0.001, compared with Con group; * P < 0.05, ** P < 0.01, *** P < 0.001 compared with HH group; & P < 0.05, && P < 0.01, compared with NGR+HH group; @ P < 0.05, @@ P < 0.01 compared with ML385+HH group.

3.8 NGR attenuated hypobaric hypoxia-induced necroptosis and ameliorated subsequent inflammation *in vivo* experiments

Next, we further validated the *in vitro* findings in *in vivo* experiments. Unsurprisingly, exposure to hypobaric hypoxia induced necroptosis in rat lung tissues, as manifested by elevated phosphorylation of RIPK3 and MLKL (**Fig. 8A-C**), positive immunofluorescent staining for p-RIPK3 and p-MLKL (**Fig. 8D, E**), and ultrastructural alterations, including organelle swelling and plasma membrane rupture in lung tissues (**Fig. 8F**). In addition, we conducted Western blot analysis to assess inflammation following hypobaric hypoxia-induced necroptosis. Our results demonstrated a significant increase in HMGB1 and nuclear NF- κ B p65 protein expression in lung tissues exposed to hypobaric hypoxia. Conversely, cytoplasmic NF- κ B p65 expression was notably reduced under these conditions (**Fig. 8G-J**).

Encouragingly, consistent with the results of the *in vitro* experiments, pre-administration of NGR reversed these changes (**Fig. 8A-J**).

Next, to further validate the involvement of RIPK3-mediated necroptosis in the pathogenesis of HAPE *in vivo*, we administered the RIPK3 inhibitor GSK'872 intraperitoneally to rats prior to hypobaric hypoxia exposure. As shown in **Fig. 8A-F**, pre-administration of GSK'872 effectively inhibited necroptosis and inflammation induced by hypobaric hypoxia. Specifically, GSK'872 reduced phosphorylation of RIPK3 and MLKL proteins, attenuated immunofluorescence intensity of p-RIPK3 and p-MLKL, and improved lung tissue ultrastructural changes. Moreover, GSK'872 decreased HMGB1 and nuclear NF- κ B p65 protein expression, while increasing cytoplasmic NF- κ B p65 expression (**Fig. 8G-J**).

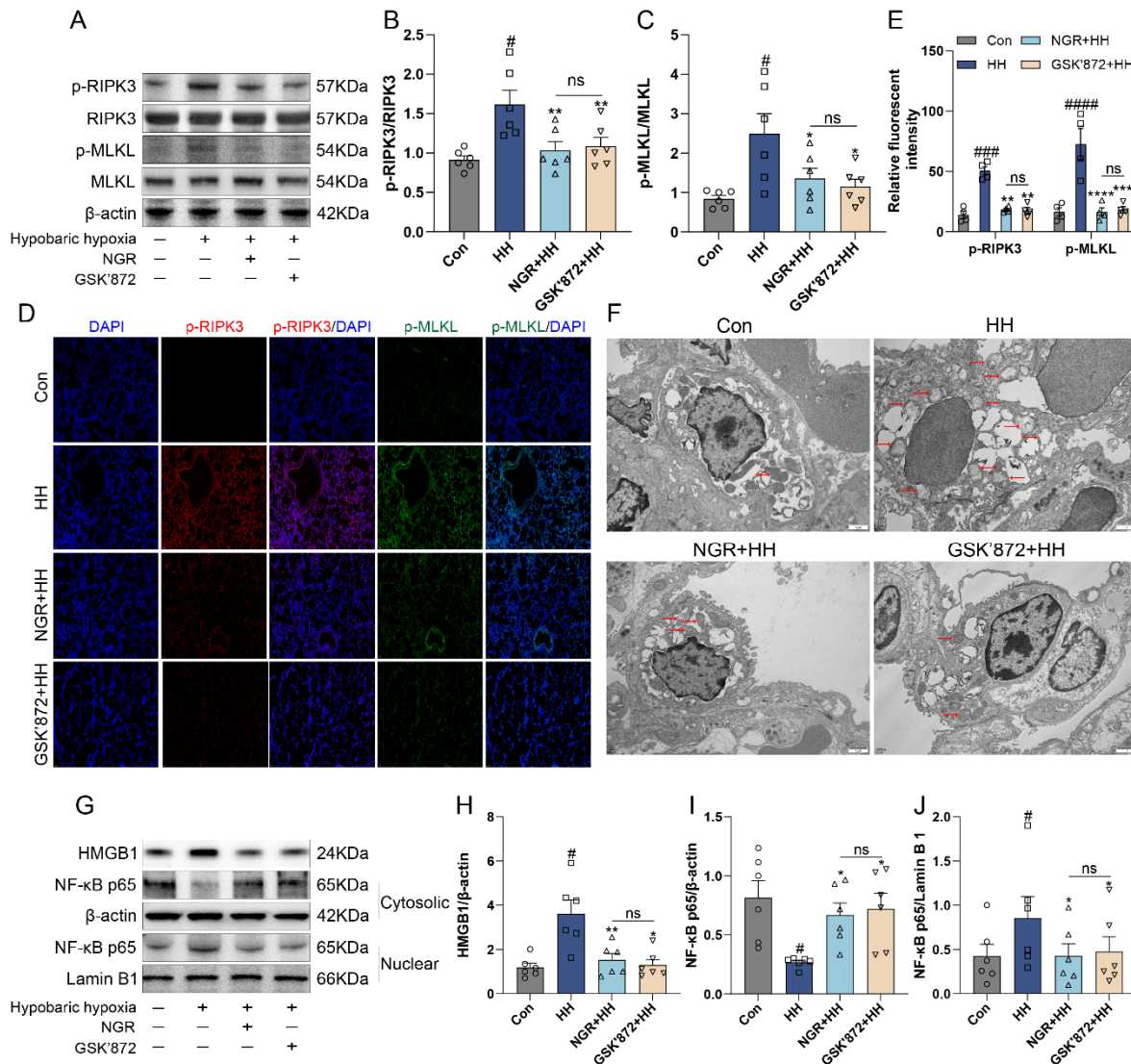


Fig. 8 Effects of NGR on necroptosis and inflammation in HAPE rats. (A–C) Western blots were used to analyze the protein levels of RIPK3 and MLKL ($n = 6$). (D, E) Immunofluorescence analysis of p-RIPK3 and p-MLKL. Scale bars: 20 μm . (F) TEM showing ultrastructural changes in lung tissues. Scale bars: 1 μm . Red arrows, swollen and vacuolated mitochondria. (G–J) Western blot analysis of HMGB1 and NF- κB p65 in nuclear and cytoplasmic fractions ($n = 6/7$). Data represent means $\pm$ SEM. $\#P < 0.05$, $###P < 0.001$, $####P < 0.0001$ compared with Con group; $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$ compared with HH group; ns presents no statistical significance.

Taken together, results from both *in vitro* and *in vivo* experiments demonstrated a potential association between the pathogenesis of hypobaric hypoxia-induced HAPE and the induction of necroptosis. Additionally, the observed mitigation of HAPE development following pre-treatment with the natural active compound NGR and the RIPK3 inhibitor GSK'872 may be attributed to the suppression of necroptosis. Moreover, it is noteworthy that the effects of NGR and GSK'872 were similar across most of the evaluated metrics, with no statistically significant differences observed between the two groups.

3.9 NGR inhibited hypobaric hypoxia-induced necroptosis by regulating NRF2 in vivo experiments

Furthermore, *in vivo* results revealed that inhibition of NRF2 by ML385 led to increased phosphorylation of RIPK3 and MLKL (**Fig. 9A**), as well as heightened immunofluorescence intensity of p-RIPK3 and p-MLKL in rat lung tissues (**Fig. 9B–C**). Additionally, we examined necroptosis at the subcellular level using

TEM, which revealed organelle swelling, including mitochondria and nuclei, and rupture of cell membranes (Fig. 9D). These data are consistent with the results obtained in *in vitro* studies.

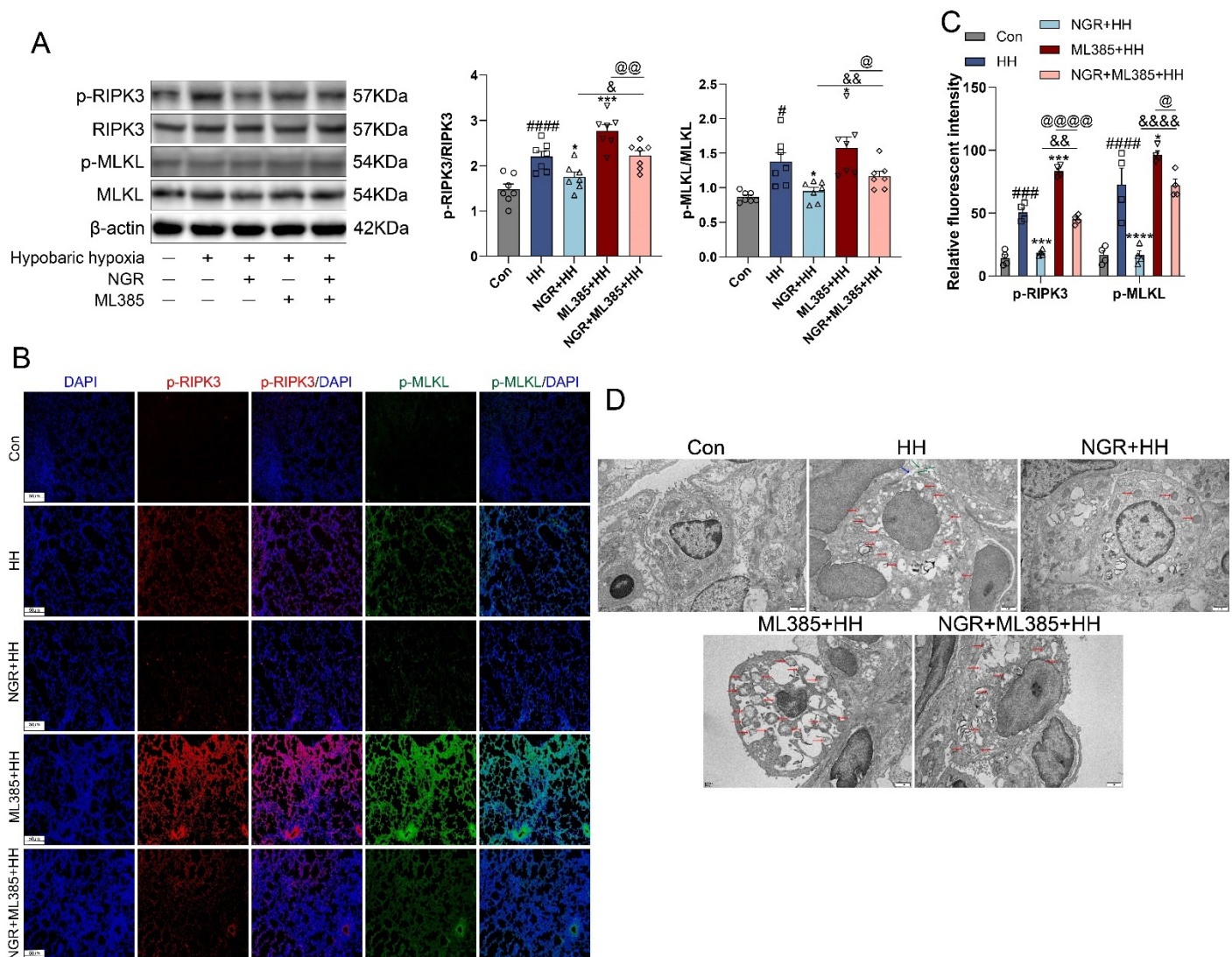


Fig. 9 NGR inhibited hypobaric hypoxia-induced necroptosis by regulating NRF2 in *in vivo* experiments. (A) RIPK3 and MLKL protein levels following treatment with NGR alone or in combination with ML385 (n = 6/7). (B, C) Immunofluorescence analysis of p-RIPK3 and p-MLKL treated with NGR alone or in combination with ML385. Scale bars: 20 μ m. (D) TEM depicting ultrastructural changes in lung tissues treated with NGR alone or in combination with ML385. Scale bars: 1 μ m. Red arrows, swollen and vacuolated mitochondria; blue arrows, ruptured plasma membrane; green arrows, leakage of cytosolic content. Data represent means $\pm$ SEM. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with Con group; $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with HH group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.0001$ compared with NGR+HH group; $^@P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.0001$ compared with ML385+HH group.

3.10 NGR exerted protective effects against HAPE by inhibiting necroptosis *in vivo* experiments

The flowchart depicting the experimental design is presented in Fig. 10A. Our previous study has thoroughly validated the pharmacodynamics of NGR *in vivo*.^[4] Briefly, NGR restored hypobaric hypoxia-induced acid-base imbalance (Fig. 10B), mitigated lung pathologic damage (Fig. 10C), lowered pathology scores (Fig. 10D), reduced lung water content (Fig. 10E), and decreased total protein concentration in BALF (Fig. 10F). Consistently, NGR decreased the high expression of HIF-1 α and VEGF induced by hypobaric hypoxia, while also elevating the low expression of AQP1 and AQP5 in lung tissues (Fig. 10G-K). Dex and

NGR had similar effects on SaO₂, lung water content and VEGF protein expression ($P < 0.05$) compared to Dex+HH group, although NGR exhibited slightly less efficacy.

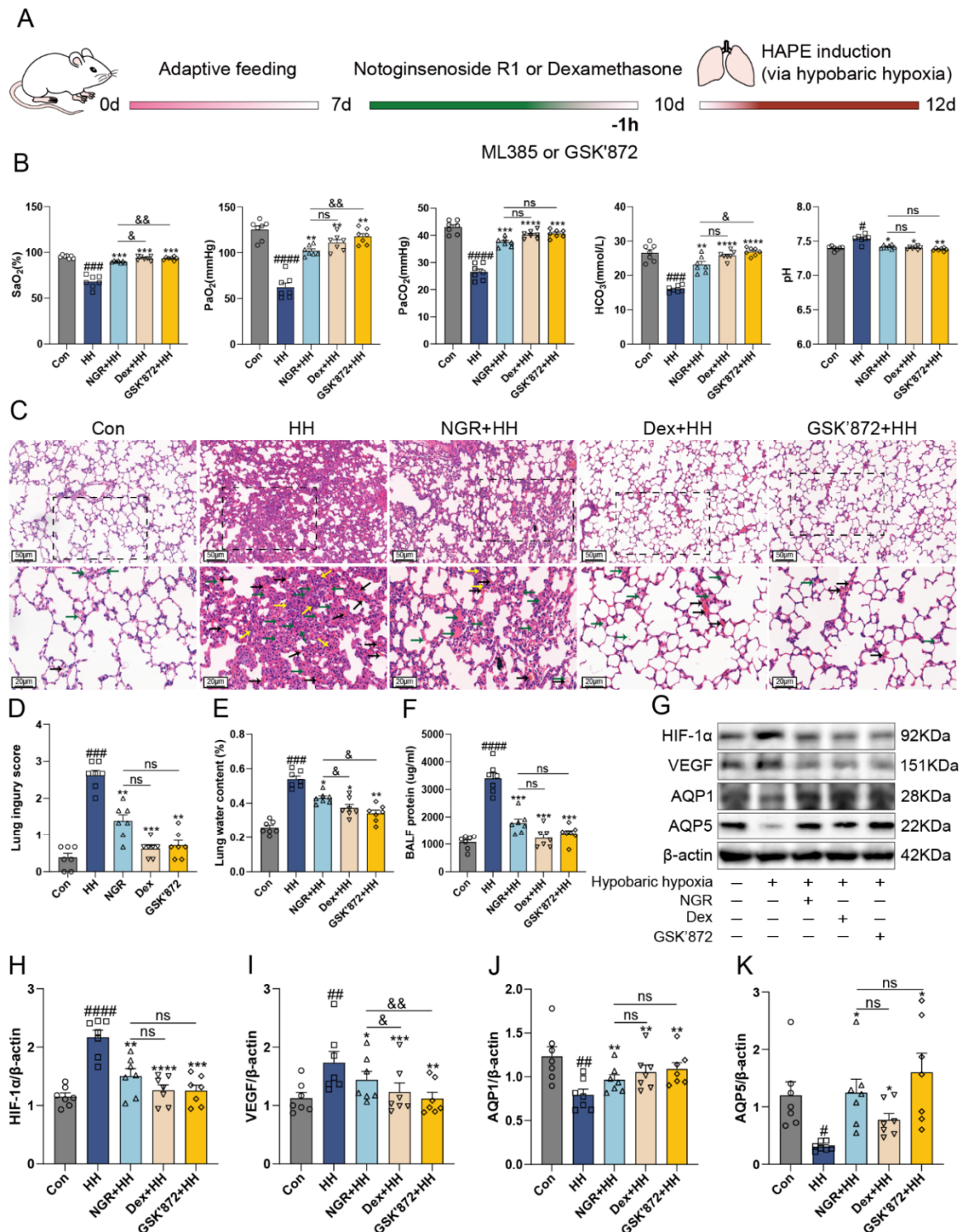


Fig. 10 Effects of NGR on pulmonary edema in HAPE rats. (A) The flowchart depicting the experimental design. (B) Effect of NGR on arterial blood gas in HAPE rats. (C) The staining of lung tissues with H&E. Magnification 200 $\times$ for the upper row (bar = 50 μ m) and 400 $\times$ (bar = 20 μ m) for the lower row. Green arrows indicate inflammatory exudation; black arrows indicate hemorrhage; yellow arrows indicate inflammatory cells infiltration. (D) Scoring lung injury semi-quantitatively. (E) Effect of NGR on lung water content. (F) Effect of NGR on total protein concentration in BALF. (G-K) Western blot analysis was conducted to detect the expression of HIF-1 α , VEGF, AQP1 and AQP5. Data represent means $\pm$ SEM ($n=7$). # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ compared with Con group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with HH group; & $P < 0.05$, && $P < 0.01$ compared with NGR+HH group; ns presents no statistical significance.

Furthermore, building on evidence from our *in vitro* studies that inhibition of necroptosis effectively attenuates HAPE, animals were pre-treated with the RIPK3 inhibitor GSK'872. This pre-administration demonstrated to be effective in mitigating hypobaric hypoxia-induced HAPE, as evidenced by restored acid-base balance, attenuated lung pathological injuries, reduced lung injury scores, decreased lung water content, and lowered protein concentration in BALF (**Fig. 10B-F**). Additionally, GSK'872 reduced HIF-1 α and VEGF protein expression but raised AQP1 and AQP5 (**Fig. 10G-K**), aligning with NGR's efficacy. Statistical analysis showed minimal differences between the NGR+HH and GSK'872+HH groups, except that NGR demonstrated a lesser impact on elevating SaO₂ ($P < 0.01$), PaO₂ ($P < 0.01$), and HCO₃ ($P < 0.05$) as well as on reducing lung water content ($P < 0.05$) and VEGF ($P < 0.01$) protein expression (**Fig. 10B, E, G and I**). These results collectively demonstrated that the protective effect of NGR against HAPE is associated with the inhibition of necroptosis.

4. Discussion

Notoginsenoside R1, a natural compound known for its potent antioxidant, anti-inflammatory, and anti-apoptotic properties, shows promise in treating high-altitude pulmonary edema, yet its mechanisms remain poorly understood. According to previous research from our laboratory, NRF2, an antioxidant-responsive transcription factor, regulates cell survival under acute hypobaric hypoxia conditions.^[9] In our current study, we demonstrated for the first time both *in vitro* and *in vivo* that NGR inhibits necroptosis and attenuates secondary inflammation by reducing oxidative stress and suppressing necrosome complex formation through NRF2 modulation. However, pretreatment with ML385, an NRF2 inhibitor, reversed NGR's protective effects against oxidative stress, necroptosis, and inflammation. Furthermore, inhibiting hypobaric hypoxia-induced necroptosis with the RIPK3 inhibitor GSK'872 attenuated inflammation and HAPE, mirroring the effects observed with NGR treatment. This underscores that necroptosis inhibition may be a critical mechanism through which NGR mitigates HAPE.

HAPE occurs in individuals acutely exposed to high altitudes without sufficient acclimatization, typically within 2-5 days.^[3, 47] This condition is characterized by hypobaric hypoxia, caused by reduced oxygen partial pressure. Exposure to such conditions rapidly induces oxidative stress, leading to elevated production of ROS.^[48] NRF2 is a crucial transcription factor that regulates genes involved in antioxidant defense mechanisms. In response to ROS, NRF2 translocates to the nucleus and binds to AREs, thereby stimulating the synthesis and utilization of antioxidants to scavenge excessive ROS.^[49] However, high levels of ROS can turn off NRF2 signaling and instead lead to cellular necrosis, apoptosis, or ferroptosis.^[50]

Our analysis, performed using the GSE260910 high-throughput transcriptomics dataset from the GEO database, has revealed a significant correlation between the NFE2L2 gene and the onset of HAPE. A previous study demonstrated that acute exposure to hypobaric hypoxia for as little as 6 h significantly increased ROS.^[51] Our previous study showed that hypobaric hypoxia for 48 h increased ROS levels and lipid peroxidation, while activating NRF2 attenuated hypobaric hypoxia-induced HAPE through antioxidant responses.^[4, 9] In addition, inhibition of NRF2 exacerbated ROS production, thereby worsening HAPE.^[9] In line with previous research,

we observed an increase in ROS levels in NR8383 cells and lung tissues upon exposure to hypobaric hypoxia. Interestingly, there was a decrease in nuclear translocation of NRF2 alongside an accumulation of NRF2 in the cytoplasm. This abnormal NRF2 distribution suggests that hypobaric hypoxia led to excessive ROS production without effectively triggering NRF2's antioxidant defense via nuclear translocation. However, NGR treatment facilitated the nuclear translocation of NRF2, enhancing the transcription of antioxidant genes and thereby alleviating oxidative stress injury, ultimately ameliorating HAPE.

A growing body of evidence indicates that the accumulation of ROS triggers necroptosis^[18, 52], a regulated mechanism of cell death involving the activation of RIPK3 kinase and subsequent phosphorylation of its downstream substrate, MLKL.^[53] Activation of RIPK3 is governed by cysteine aspartate-specific proteases and serves as a crucial junction in necroptosis induction and inflammatory responses^[54]. MLKL acts as the executor of necroptosis. When apoptosis is suppressed, RIPK3 forms a complex with RIPK1, leading to MLKL phosphorylation. Phosphorylated MLKL then undergoes oligomerization, facilitating the translocation of necrosomes from the cytoplasm to the cell membrane. This process compromises plasma membrane integrity, ultimately contributing to the onset of necroptosis. Our previous study demonstrated that exposure to hypobaric hypoxia increased the phosphorylation level of MLKL.^[9] Furthermore, both WGCNA and network pharmacology analyses have demonstrated that RIPK3-mediated necroptosis is implicated in the pathogenesis of HAPE. In this study, we observed elevated phosphorylation of RIPK1, RIPK3, and MLKL following hypobaric hypoxia exposure, along with increased fluorescence co-localization and protein interactions of RIPK1 and RIPK3 and enhanced fluorescence intensity of p-MLKL. Additionally, positive PI staining and increased LDH release were observed in macrophage NR8383. Moreover, TEM results confirmed distinct necroptotic features in NR8383 cells and lung tissues. Importantly, these effects were mitigated upon pre-administration of NGR and the RIPK3 kinase inhibitor GSK'872.

Additionally, during necroptosis, the plasma membrane ruptures, releasing cellular content and various DAMPs, which in turn triggers a localized inflammatory response.^[55] HMGB1, a widely expressed functional protein and a prototype DAMP, acts as a crucial mediator of inflammation upon its extracellular release. This encompasses the induction of nuclear translocation of NF- κ B p65, as well as the secretion of pro-inflammatory cytokines and chemokines.^[56] Several studies have documented that induction of necroptosis activates NF- κ B p65 and augments the levels of pro-inflammatory mediators, such as TNF- α , IL-1 β , IL-6, as well as chemokines like CXCL1.^[18, 57, 58] In our study, we found that treatment with NGR and GSK'872 significantly attenuated the expression of HMGB1 protein, suppressed the nuclear protein levels of NF- κ B p65, and reduced the mRNA levels of *Tnf- α* , *IL-1 β* , *Cxcl1*, and *Ccl20*.

These results above fully indicate that necroptosis, as a regulated form of inflammatory cell death, is involved in the pathogenesis of hypobaric hypoxia-induced HAPE. Beyond that, the inflammatory response accompanying HAPE development appears to be triggered by the release of various pro-inflammatory mediators from cells following plasma membrane rupture due to necroptosis.

NRF2 is recognized as a pivotal cytoprotective factor and has been found to possess anti-inflammatory and antioxidant effects.^[7] In normal physiological circumstances, the transcription factor NRF2 is kept in the cytoplasm through interaction with the Kelch-like ECH associated protein 1 (KEAP1), which leads to its ubiquitination and subsequent degradation.^[59] In response to oxidative stress, Keap1 is modified by ROS, leading to a conformational change and a loss of affinity for NRF2. This allows NRF2 to dissociate from Keap1 and translocate into the nucleus. There, NRF2 binds to specific DNA sequences known as antioxidant response elements (AREs). This binding initiates the transcription of genes implicated in defense mechanisms against ROS and antioxidant gene expression.^[60-62] Recent studies have shown that heightened NRF2 activity reduces the incidence of necroptosis.^[18, 63] However, the mechanisms by which NRF2 regulates necroptosis and inflammation in hypobaric hypoxia-induced HAPE remain unclear. Our findings reveal that NGR abrogates the binding between NRF2 and RIPK3 and promotes NRF2 upregulation in the nucleus, leading to a reduction in the RIPK1/RIPK3 necrosome complex formation and attenuation of necroptosis. Strikingly, activation of NRF2's transcriptional activity also alleviates the inflammatory response triggered by necroptosis. Intriguingly, inhibition of NRF2 activity with ML385 exacerbates hypobaric hypoxia-induced necroptosis and inflammation, while ML385 diminishes the pharmacodynamic effects of NGR. These results suggest that NGR's pharmacodynamic effects against HAPE may be mediated through NRF2-mediated regulation of necroptosis and inflammation.

While this study provides pharmacological evidence supporting the role of NRF2 in necroptosis regulation, several limitations remain. Although the present work clarifies the general association between NRF2 transcriptional activation and the inhibition of necroptosis, it does not delve into the specific molecular cascade underlying this regulation. The precise mechanism by which NRF2 exerts this inhibitory effect, whether through direct transcriptional regulation or as an indirect consequence of alterations in the cellular redox state, metabolic environment, or inflammatory microenvironment, remains unclear. These complex molecular mechanisms have yet to be fully explored. In addition, future studies integrating transcriptomic analysis, together with further validation of NRF2 downstream target genes, will be required to more comprehensively elucidate the molecular mechanisms by which NGR activated NRF2 regulates necroptosis in HAPE. Moreover, we have only validated this mechanism in NR8383 cells. Future studies will extend this investigation to other relevant cell models, such as HPMECs or A549 cells, to further validate this mechanism, thereby enhancing the generalizability and clinical relevance of the findings.

5. Conclusion

In summary, this study demonstrates that NGR inhibits necroptosis and inflammation by modulating NRF2 transcription activity. NGR facilitates NRF2 nuclear translocation, enhances antioxidant gene expression, and disrupts the RIPK1/RIPK3 necrosome complexes, ultimately suppressing necroptosis and inflammation. These effects attenuate hypobaric hypoxia-induced HAPE in both NR8383 cells and rats. Our findings underscore the NRF2-RIPK3/MLKL axis as pivotal in mitigating HAPE induced by hypobaric hypoxia, highlighting NGR's therapeutic potential as a natural compound for HAPE management.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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