Diazoxide protects rat vascular endothelial cells against hypoxia and cold-induced damage

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Abstract. The present study aimed to examine the effects of hypoxia and cold on vascular endothelial cells (VECs), as well as the protective ability of novel VECs-protective drugs against these injuries. A rat model simulating exposure to hypoxia and cold at high altitude environments was established. Based on these animal experiments, rat aortic VECs were established as injury models and exposed to hypoxia and/or adrenaline (ADR) *in vitro*. The results revealed that hypoxia significantly altered the levels of nitric oxide and vascular endothelial growth factor, while the cold temperature significantly increased the release of ADR and noradrenaline. Exposure to hypoxia combined with cold temperature significantly affected all these indices. *In vitro* experiments demonstrated that hypoxia, ADR (which was used to simulate cold in the animal experiments) and the combination of the two factors resulted in damage to the VECs and endothelial dysfunction. In addition, the results also showed that diazoxide, a highly selective mitoK_{ATP} opener, protected VECs against these injuries. In conclusion, hypoxia and cold temperature induced endothelial cell dysfunction and endocrine disorders, respectively. Improving endothelial function using diazoxide may be an effective therapeutic strategy in patients with altitude-associated disorders. However, the potential for clinical application requires further study.

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Abbreviations: NO, nitric oxide; ADR, adrenaline; NADR, noradrenaline; EC, endothelial cell; VEC, vascular endothelial cell; RAEC, rat aortic endothelial cell; mitoK_{ATP}, mitochondrial ATP-sensitive potassium channel; I/R, ischemia/reperfusion; ET, endothelin; LDH, lactate dehydrogenase; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endothelial growth factor

Key words: damage effects, hypoxia, cold, rat aortic endothelial cells, diazoxide, adrenaline

Introduction

High altitude environment is a significant challenge for human survival (1,2). In recent years, high altitude regions have gained increased popularity for travel and occupational purposes. Epidemiological studies have indicated that the harsh weather conditions at these regions are a serious threat to human health (3). The special geographical conditions associated with high altitudes include low barometric pressure, low temperature (cold), hypoxia, low humidity, strong wind and high solar radiation. Hypoxia and cold are the two main factors that affect human health and capacity. With increasing altitude, the atmospheric pressure and the oxygen partial pressure are lower, resulting in deficiency of partial pressure of inspired oxygen, which in turn leads to decrease in arterial oxygen pressure and oxygen saturation, and tissue hypoxia (4). The pathophysiology of altitude-induced diseases involves a series of changes in the function, metabolism and structure of the body. Furthermore, the atmospheric temperature decreases with an increase in altitude. A rise in altitude of 1,000 meters above sea level leads to ~6˚C drop in the average temperature. At the Qinghai-Tibet Plateau in China, with an average altitude of 4,000 m above sea level, the average annual temperature is only ~5˚C (5,6). The low temperature induces systemic cold within the body, which is manifested as peripheral blood vessel contraction, decreased blood perfusion and the occurrence of severe frostbite (7). In addition, due to the low temperature, the body oxygen consumption increases, aggravating the degree of hypoxia. Hypoxia further reduces energy production and the ability to maintain body temperature, ultimately aggravating the effects of cold. Therefore, the deleterious effects of hypoxia and low temperature complement and promote each other at high altitude, resulting in frostbite and mountain sickness. Therefore, it is imperative to study the damage associated with exposure to high altitude in order to develop appropriate preventive and treatment measures.

Vascular endothelial cells (VECs) are the monolayer of flat cells adhering to the surface of intravascular space,

in direct contact with the blood circulation, and forming a barrier between the tissues and blood (8). VECs sense the initial changes in oxygen, temperature and various physico-chemical factors in the blood circulation, and therefore are affected by low temperature and hypoxia. Under various external physical and chemical stimuli, endothelial cells (ECs) can synthesize and secrete a variety of important vasoactive substances, including nitric oxide (NO), endothelin-1 and vascular endothelial growth factor (VEGF). These vasoactive factors serve an important role in the pathophysiology of a variety of diseases and injuries, affecting the vasomotor state, permeability and inflammatory response of blood vessels, and internal homeostasis. Increased vascular permeability and decreased antioxidant capacity eventually result in a variety of high altitude-associated diseases and injuries (9-11). However, the effects associated with the damage induced by hypoxia combined with low temperature on VECs have yet to be elucidated. Increasing evidence suggested that ECs are the key to prevention and treatment of altitude-associated diseases, by protecting the VECs and reversing endothelial dysfunction (12). Therefore, it is significant to study the combined effects of cold and hypoxia on VECs, and the effects of novel VEC-protective drugs against hypoxic and cold injuries.

The thiazide derivative diazoxide is a highly selective mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) opener (13,14), which is a potent antihypertensive drug for the clinical treatment of hypertensive crisis (15). mitoK_{ATP} is a voltage-independent ligand-gated potassium channel regulating the mitochondrial potassium homeostasis. Previous studies have demonstrated that diazoxide not only served as a vasodilator acting on the potassium channels of vascular intima and lowering blood pressure, but also served an important role in protecting the heart, brain, kidneys and other organs in ischemia/reperfusion (I/R) injuries (16-20). In the I/R process, diazoxide reduced the oxidative stress by scavenging the superoxide anions generated by mitochondria, thereby protecting against endothelial dysfunction, when used as a pre-treatment (21) or post-treatment (22). In addition, evidence strongly suggested that diazoxide had a direct effect on the inhibition of apoptosis of VECs (23). However, the protective effect of diazoxide against the damages induced by hypoxia and low temperature at high altitude are incompletely understood.

In the current study, in order to explore the injury mechanisms of hypoxia and cold on VECs, a rat animal model was established following exposure to hypoxia, low temperature, or hypoxia combined with cold conditions simulating the high-altitude environment. Based on the animal experimental results, the rat aortic endothelial cells (RAECs) were established as injury models, which were respectively exposed to hypoxia, adrenaline (ADR) to simulate the cold conditions, or hypoxia combined with ADR *in vitro*. In addition, the protective effects of diazoxide, which is a highly selective mitoK_{ATP} opener, on VECs were investigated against hypoxia and cold-induced injury.

Materials and methods

Animals. A total of 32 wild-type 3 to 5 month-old male Wistar rats, weighing 180-220 g were purchased from the Experimental Animal Center of the Beijing Academy of Military Medical Sciences (Beijing, China) for use in the present study. Rats were housed individually under a 12 h light/dark cycle at a temperature of 18-24˚C and relative humidity of 56±10% and provided with food and water *ad libitum*. The care and use of animals in the experiments met the standards set forth in the guidelines of the National Institute of Health (NIH) for the Care and Use of Laboratory Animals, and closely complied with the Animal Care and Use Committee of the Tianjin Institute of Health and Environmental Medicine (Tianjin, China). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Tianjin Institute of Health and Environmental Medicine (permit no. 2013-D-3601). For surgical procedures, tissue collection and blood sample collection, anesthesia was induced and maintained with sodium pentobarbital (35 mg/kg, intraperitoneal injection; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). All efforts were made to minimize the number of animals used and to reduce animal stress.

Animal grouping and treatment. An experimental hypobaric hypoxic and cold chamber was used to mimic the plateau environment. Wistar rats were randomly divided into four groups, as follows: i) Control group, simulating normothermia and normoxia conditions (oxygen content corresponding to an altitude of 0 m; temperature, 25˚C); ii) hypoxia group, simulating normothermia and hypoxia (oxygen content corresponding to an altitude of 6,000 m; temperature, 25˚C); iii) cold group, simulating cold and normoxia (oxygen content corresponding to an altitude of 0 m; temperature, -5˚C); and iv) hypoxia combined with cold group, simulating cold and hypoxia conditions (oxygen content corresponding to an altitude of 6,000 m; temperature, -5˚C). Exposure to the different conditions was performed for 1 h, with 8 rats in each group.

Biochemical assays of serum NO, VEGF, ADR and noradrenaline (NADR). Rats were immediately anaesthetized after exposure to hypoxia and/or cold conditions as described earlier, and blood was drawn from the abdominal aorta. The blood sample was placed at room temperature for 30 min and then centrifuged at 250 x *g* and 4˚C for 20 min to separate the serum. The NO Detection kit (nitrate reductase method, catalog number, ab65328; Abcam, Cambridge) was used to measure the level of NO in the serum, and the experimental procedure was in accordance with the manufacturer's instructions. The absorbance of each tube was measured using a microplate reader at 550 nm following coloration. In addition, the levels of ADR and NADR in the serum were determined using test kits, catalog number, KA1877 from Abnova (Taipei, China) according to the manufacturer's instructions. A VEGF Detection kit obtained from OriGene Technologies, Inc. (Rockville, MD, USA) was also used to detect the VEGF serum level. The final results are represented as the concentration of each parameter in the serum.

Reagents for in vitro study of damage induced by hypoxia and ADR. Dulbecco's modified Eagle's medium (high glucose) and trypsin were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The fetal bovine serum (FBS) was purchased from Hyclone (GE Healthcare, Chicago, IL,

USA); dimethyl sulfoxide and MTT were purchased from Sigma-Aldrich (Merck KGaA); lactate dehydrogenase (LDH) and NO detection kits were obtained from Abcam; and the VEGF, endothelin (ET) and hypoxia-inducible factor-1α (HIF-1α) detection kits were from Cell Signaling Technology, Inc., Danvers, MA, USA.

Isolation, culture and identification of RAECs. A total of 5 rats were sacrificed by cervical dislocation. The chest was opened under sterile conditions, and the rat aortas were quickly removed and washed twice using the D-Hanks solution (pH 7.2; Beijing Leagene Biotechnology Co. Ltd., Beijing, China) to wash away the blood. The connective tissues surrounding the blood vessels were excluded, and the blood vessels were longitudinally cut open and sliced into 1-mm^2 sections, which were affixed to the bottom of a Petri dish. Next, M199 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20%, 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.) was slowly added to the Petri dish, and then the Petri dish was transferred to an incubator at 37˚C and 5% CO_2. Half of the medium was replaced when a small amount of RAECs expanding from the surrounding tissue was visible after 72 h. The medium was replaced after 5 days, when a large number of cells expanding from the surrounding tissue. Finally, RAECs were identified using an antibody against CD31 labeled with FITC (ab33858; 1:200; Abcam) according to the manufacturer's protocol.

Grouping and treatment. The cells from generations 4-10 were selected for experimental use, seeded in 96-well cell culture plates at a density of $1x10^4$ cells/ml and synchronized in serum-free medium for 2 h. The cells were randomly divided into four groups as follows: i) Control group; ii) hypoxia group; iii) ADR group; and iv) hypoxia combined with ADR group. The control and hypoxia groups were cultured in the serum-free M199 medium, while the ADR and hypoxia combined with ADR groups in serum-free M199 medium supplemented with ADR (Tianjin KingYork Pharmaceuticals Co., Ltd., Tianjin, China), which was used to model the low temperature condition, to the final concentration of 0.1 mmol/l. The control and ADR groups were placed in a normoxic incubator containing 5% CO_2 at 37˚C, and the other two groups were cultured in a 37˚C incubator containing 1% O_2, 92% N_2 and 5% CO_2. All the experimental groups were cultured under the different conditions for 27 h.

Analysis of the RAECs survival rates. The survival rates of RAECs treated under different conditions were measured. Cells in the logarithmic growth phase were digested into a single cell suspension and plated in 96-well plates with ~$1x10^4$ cells per well. Cells were cultured in the 37˚C incubator for 48 h, and the survival rates were detected by MTT arrays.

Measurement of the RAECs apoptosis rates. The apoptosis rate of RAECs treated under different conditions was determined by propidium iodide (PI) staining (Sigma-Aldrich; Merck KGaA) and flow cytofluorometry. Cells were collected by trypsin digestion, washed twice with PBS and re-suspended with 70% ethanol pre-cooled to 4˚C. The cells were incubated at 4˚C overnight, and then PI, a DNA-specific dye, was added. Samples were stored at 4˚C for 30 min and subsequently cellular apoptosis was detected by flow cytometry.

Determination of LDH activity in the cell culture supernatant. The activity of extracellular (released) LDH was measured using an LDH Detection kit. Briefly, cells at the logarithmic growth phase were digested and plated into 96-well plates with 10^2-10^4 cells/well. Cells were normally cultured until they adhered to the bottom of the well, and the medium was replaced with serum-free medium. The LDH activity in the cell culture supernatant was immediately detected using the LDH detection kit according to the kit instructions, after the cells were treated at different conditions described earlier.

Determination of the release of NO in the cell culture supernatant. The release of NO in the cell culture supernatant was quantified using the NO Detection kit (ab65328; Abcam), which was based on the capability of the nitrate reductase enzyme to convert nitrate into nitrite. The Griess reaction was then used to detect the level of nitrite (24), and the absorbance was measured with a microplate reader at 540 nm.

Determination of VEGF, ET and HIF-1α levels in cultured supernatant. Subsequent to culturing RAECs treated at different conditions as described earlier, the culture supernatant without serum was collected by centrifugation for 10 min at 250 x *g* and 4˚C. The levels of VEGF, ET and HIF-1α in the supernatant were respectively measured using commercial quantified ELISA kits (OriGene Technologies, Inc.) according to a standard enzyme immunoassay procedure (25,26).

In vitro experiments to examine the protective effect of diazoxide on RAECs. In order to examine the protective effect of diazoxide against hypoxia damage in RAECs, the cells were divided into the following groups: i) Control group; ii) hypoxia group; iii) hypoxia combined with diazoxide group; and iv) hypoxia combined with diazoxide and 5-HD group. In addition, the protective effect of diazoxide against damage from hypoxia combined with ADR in RAECs was examined by adding 0.1 mmol/l ADR treatment to obtain i) hypoxia combined with ADR group, ii) hypoxia combined with ADR and diazoxide group and iii) hypoxia combined with ADR and diazoxide and 5-HD group. The control groups were cultured at 37˚C in a normoxic incubator containing 5% CO_2, while all other groups were incubated at 37˚C in 1% O_2, 92% N_2 and 5% CO_2. Serum-free M199 medium was used for culturing, supplemented with 0.1 mmol/l diazoxide, 0.1 mmol/l 5-HD and 0.1 mmol/l ADR in the corresponding groups. All experimental groups were cultured under the different conditions for 27 h, and then RAECs survival and apoptosis rates, LDH activity and NO, VEGF, ET and HIF-1α levels were respectively detected with the methods described earlier.

Statistical analysis. Statistical analyses were performed using the SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). All data are expressed as the mean ± standard error of the mean. Unpaired Student's t-test and 2x2 factorial analysis

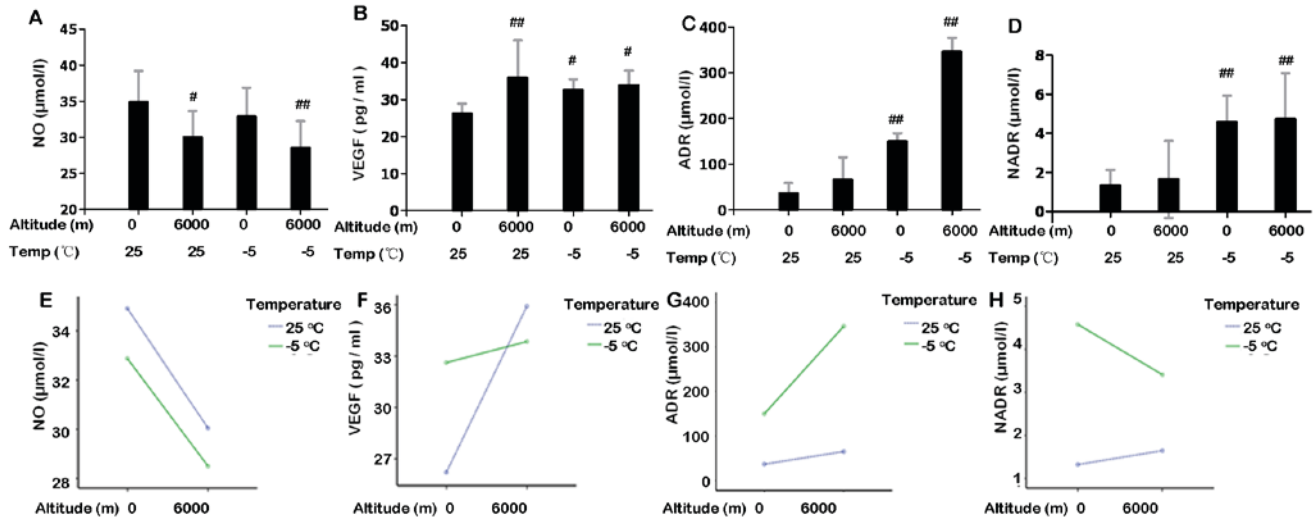

Figure 1. Effects of hypoxia, cold, and hypoxia combined with cold on the rat vascular endothelial function and endocrine system. The effects of the different conditions on the release of (A) NO, (B) VEGF, (C) ADR and (D) NADR are shown, as measured in the rat serum. The interaction between hypoxia and cold in conjunction with the serum levels of (E) NO, (F) VEGF, (G) ADR and (H) NADR are also demonstrated. Data presented as the mean ± standard error (n=8 in each experiment). $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the control group (0 m and 25˚C). NO, nitric oxide; VEGF, vascular endothelial growth factor; ADR, adrenaline; NADR, noradrenaline.

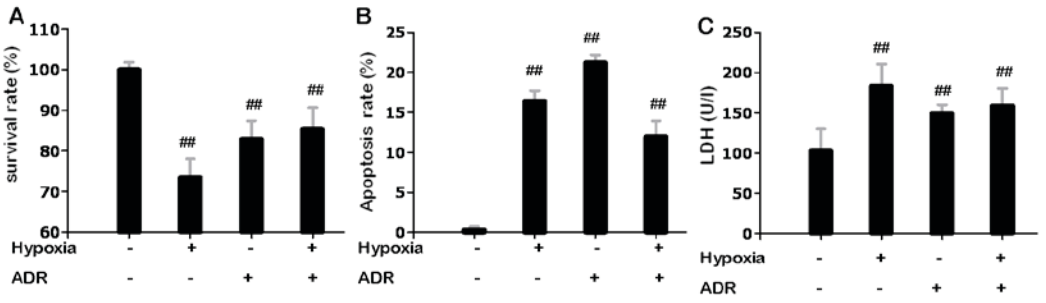

Figure 2. Effects of hypoxia, ADR (simulating cold), and hypoxia combined with ADR on the rat aortic endothelial cells. (A) Survival rate determined by MTT assay, (B) apoptosis rate and (C) the release of lactate dehydrogenase. Data are presented as the mean ± standard error (n=10 in each experiment). $^{\#\#}$P<0.01 vs. the control group (without hypoxia and ADR).

method were used to compare the differences between two groups and among multiple groups, respectively. In all instances, P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of hypoxia, cold, and hypoxia combined with cold on the vascular endothelial function and endocrine system. An animal injury model was initially established that simulated the conditions of hypoxia, cold, and hypoxia combined with cold at high altitudes, respectively. As shown in Fig. 1, compared with the control group (0 m, 25˚C, 1 h), the release of NO in the hypoxia group (6,000 m, 25˚C, 1 h) was significantly decreased (P<0.05). In addition, the serum VEGF levels in the hypoxia group were significantly increased (P<0.05), whereas the serum levels of ADR and NADR were partially increased, but not significantly (P>0.05). Compared with the effects of hypoxia, the NO levels in the cold group (0 m, -5˚C, 1 h) were slightly decreased, but not significantly, compared with the control group. Nevertheless, the levels of VEGF, ADR and NADR in the blood were significantly increased in the cold group (P<0.01). The differences in the release of NO, VEGF, ADR and NADR were also significant (P<0.01) between the hypoxia combined with cold group (6,000 m, -5˚C, 1 h) and the control group, with a concomitant decrease in NO and an increase in VEGF, ADR and NADR levels (Fig. 1A-D). The results suggested that the release of NO and VEGF, but not ADR and NADR, was significantly influenced by hypoxia. Furthermore, cold significantly influenced the levels of VEGF, ADR and NADR in the blood; however, its effect on the release of NO was minimal. Hypoxia combined with cold significantly changed the levels of NO, VEGF, ADR and NADR.

Using factorial analysis, hypoxia alone was found to decrease NO release; however, cold conditions had a minimal effect on the NO release, with no interaction between the two factors, suggesting that hypoxia was the major factor underlying the decrease in NO release. The effects of the two factors on VEGF release were synergistic, in that hypoxia and cold led to significant increase in VEGF expression. In addition, the release of the catecholamine hormones ADR and NADR at low temperature was greater compared with that at normal temperature, and their secretion was also affected by hypoxia. The effects of hypoxia and cold on the release

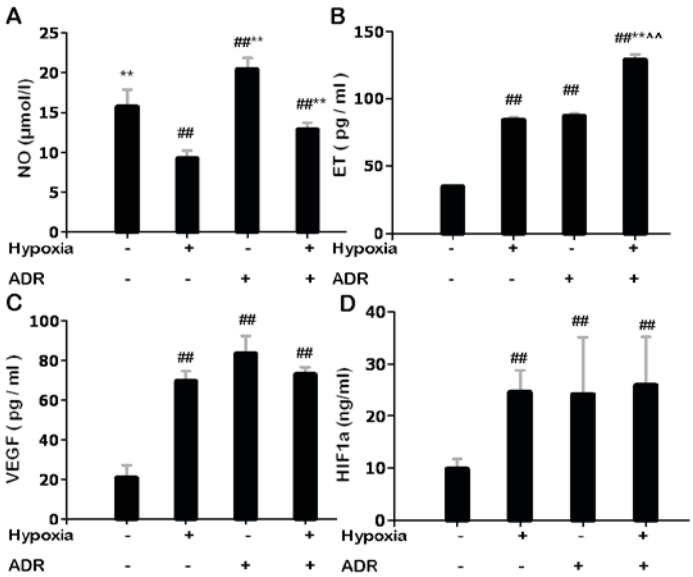

Figure 3. Effects of hypoxia, ADR, and hypoxia combined with ADR on the release of (A) NO, (B) ET, (C) VEGF and (D) HIF-1α of rat aortic endothelial cells. Data presented as the mean ± standard error (n=10 in each experiment). ##P<0.01 vs. control group; **P<0.01 vs. hypoxia group; ^^P<0.01 vs. ADR group. ADR, adrenaline; NO, nitric oxide; ET, endothelin; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α.

of catecholamine hormones were thus interactive. However, the effect of cold was more prominent, indicating that endocrine changes were mainly caused by the low temperature environment, and hypoxia altered the endocrine functions significantly (Fig. 1E-H).

Effects of hypoxia, ADR (simulating cold) and hypoxia combined with ADR on the survival rates, apoptosis and the permeability in RAECs. To observe the endothelial cell damage associated with hypoxia and cold, RAECs were isolated and cultured. Subsequent to culture and passaging, the green fluorescent antibody against CD31 labeled with FITC was used to identify RAECs. VECs cannot attain a temperature <30˚C except in organ transplantation, implying that low temperature affects VECs indirectly by altering the body's endocrine levels, which damage the ECs. Therefore, ADR, one of the catecholamine hormones that are severely affected by low temperature, was used to simulate cold exposure in order to study the damaging effects of low temperature on ECs.

First, the survival and apoptosis rates of RAECs were investigated in the different treatment groups. Compared with the control group, the survival rates in the hypoxia, ADR, and hypoxia combined with ADR group were significantly decreased (P<0.01; Fig. 2A). Furthermore, compared with the hypoxia and ADR groups, the survival rate of cells treated with 0.1 mmol/l ADR was elevated following culture in hypoxic conditions (1% O_2) for 27 h; however, this change was not statistically significant (P>0.05).

The apoptosis rates of the three treatment groups were significantly increased compared with the control group (P<0.01; Fig. 2B). When the ECs treated with 0.1 mmol/l ADR were exposed to hypoxia (1% O_2), the apoptosis rate was lower in comparison with cells in the hypoxia or ADR alone groups, indicating that the combined effect of hypoxia and ADR resulted in the apoptosis of ECs, but had a lower effect compared with that of each of the two factors alone.

To study the effects of different factors on the permeability of RAECs, the LDH activity in the cell culture supernatant was determined, which was originally localized in the cytoplasm. As shown in Fig. 2C, the LDH release in the three groups treated with different factors was significantly increased compared with the control group (P<0.01). However, there was reduced LDH release in the group treated with the combined factors compared with the group treated with hypoxia alone, and increased LDH compared with the ADR alone group.

Effects of hypoxia, ADR, and hypoxia combined with ADR on the release of the vasoactive factors NO and ET, as well as VEGF and HIF-1α, in RAECs. Compared with the control group, NO release in the hypoxia group and in the group with hypoxia combined with ADR was significantly decreased (P<0.01), whereas NO release in the ADR group was significantly increased (P<0.01). In addition, NO release in the ADR and in the hypoxia combined with ADR groups was significantly increased compared with the hypoxia alone group (P<0.01; Fig. 3A).

The release of ET, an important vasoactive peptide participating in blood vessel contraction, was also investigated. Compared with the control group, ET release in the three treatment groups was significantly increased (P<0.01). Furthermore, the release of ET in the hypoxia combined with ADR group was significantly increased compared with the hypoxia alone and the ADR alone groups (P<0.01), indicating that ADR and hypoxia had overlapping effects on the increased release of ET (Fig. 3B).

Finally, the effects of the different conditions on the expression levels of VEGF and HIF-1α were investigated. The results revealed that the expression levels of VEGF (Fig. 3C) and HIF-1α (Fig. 3D) were significantly upregulated in the hypoxia, ADR and the combination groups (P<0.01). There were no significant differences among the expression levels in the three intervention groups.

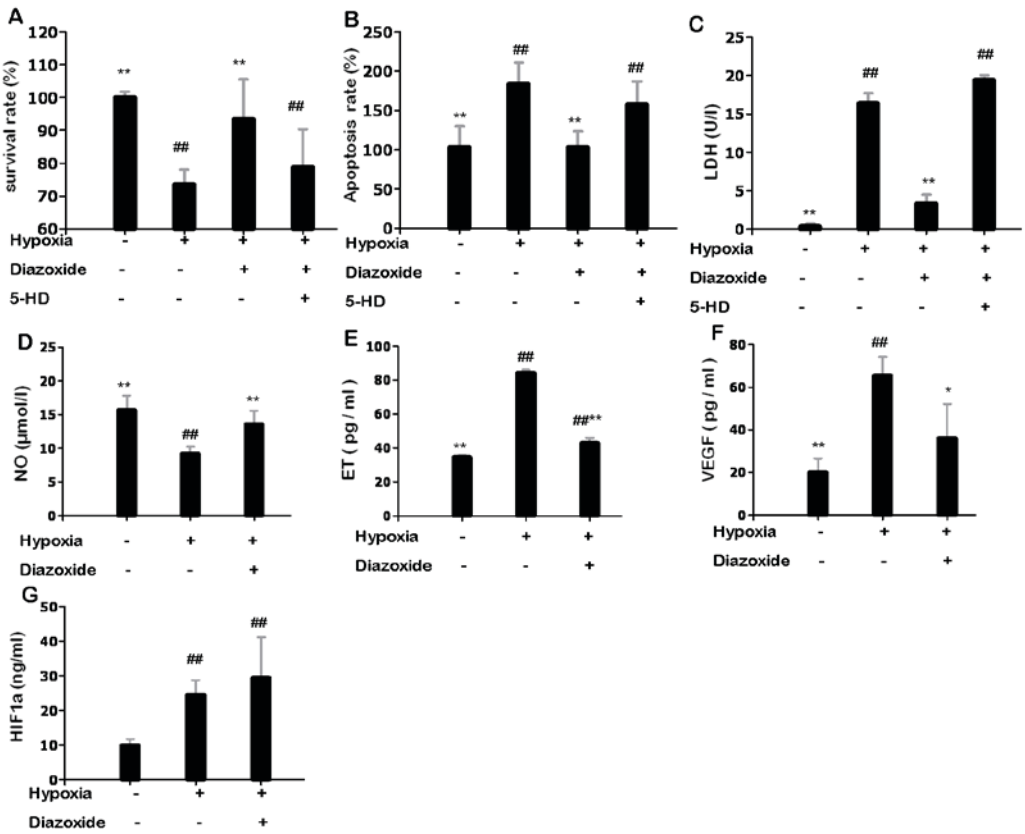

Figure 4. Diazoxide mediates protection against hypoxia-induced damage. Variations in (A) survival rates, (B) apoptosis rates, and the release of (C) LDH, (D) NO, (E) ET, (F) VEGF and (G) HIF-1α in the rat aortic endothelial cells are shown. Data are presented as the mean ± standard error (n=10 in each experiment). $^{\#\#}$P<0.01 vs. control group; *P<0.05 and **P<0.01 vs. hypoxia group. LDH, lactate dehydrogenase; NO, nitric oxide; ET, endothelin; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α.

Diazoxide protects against damage induced by hypoxia and by hypoxia combined with ADR. The protective effect of diazoxide, a potent antihypertensive drug and a highly selective mitoK$_{ATP}$ opener, against the damage induced by hypoxia and by hypoxia combined with cold (simulated by ADR) was investigated. When the cells were placed under hypoxia conditions (1% O_2) for 27 h and simultaneously treated with 0.1 mmol/l diazoxide, the survival rates were significantly increased compared with the hypoxia group (P<0.01; Fig. 4A), whereas the apoptosis rates were significantly decreased (P<0.01; Fig. 4B). Furthermore, the LDH release was significantly decreased compared with the hypoxia group (P<0.01; Fig. 4C). When the cells were simultaneously treated with 0.1 mmol/l diazoxide and 0.1 mmol/l 5-HD, no significant differences in survival rate, apoptosis rate or LDH were observed compared with the hypoxia group (Fig. 4A-C); however, all other indicators were significantly improved (P<0.01; Fig. 4D-F), with the exception of HIF-1α expression, which was not significantly improved compared with the hypoxia group (Fig. 4G). More specifically, the NO release was significantly increased (P<0.01), and the expression levels of VEGF and ET were significantly decreased (P<0.05). The results indicated that the protection of diazoxide against the damage induced by hypoxia was mediated by the mitoK$_{ATP}$ opening.

Upon ADR addition to simulate cold, the majority of the indicators were significantly improved by diazoxide treatment (Fig. 5). For instance, compared with the hypoxia combined with ADR group, the survival rate in the hypoxia combined with ADR and diazoxide group was significantly increased (P<0.05). By contrast, there was a significant decrease in apoptosis rates (P<0.01), LDH levels (P<0.05), VEGF expression (P<0.01) and ET expression (P<0.01) in the hypoxia combined with ADR and diazoxide group compared with the hypoxia combined with ADR group (Fig. 5). NO release was significantly decreased in the hypoxia combined with ADR and diazoxide group compared with the hypoxia combined with ADR group (P<0.01; Fig. 5). The results demonstrated that diazoxide does not completely improve the endothelial function upon exposure to hypoxia combined with cold.

Discussion

The present study focused on two key environmental factors, hypoxia and low temperature, which are observed at high altitude. According to the preliminary experimental results, a model was established using short-term exposure to an oxygen content corresponding to an altitude of 6,000 m and -5˚C to simulate the injury caused by complex environmental factors of hypoxia and cold, respectively, at high altitudes. Simultaneously, the combined effect of hypoxia and cold was investigated in comparison with the injuries caused by either factor alone.

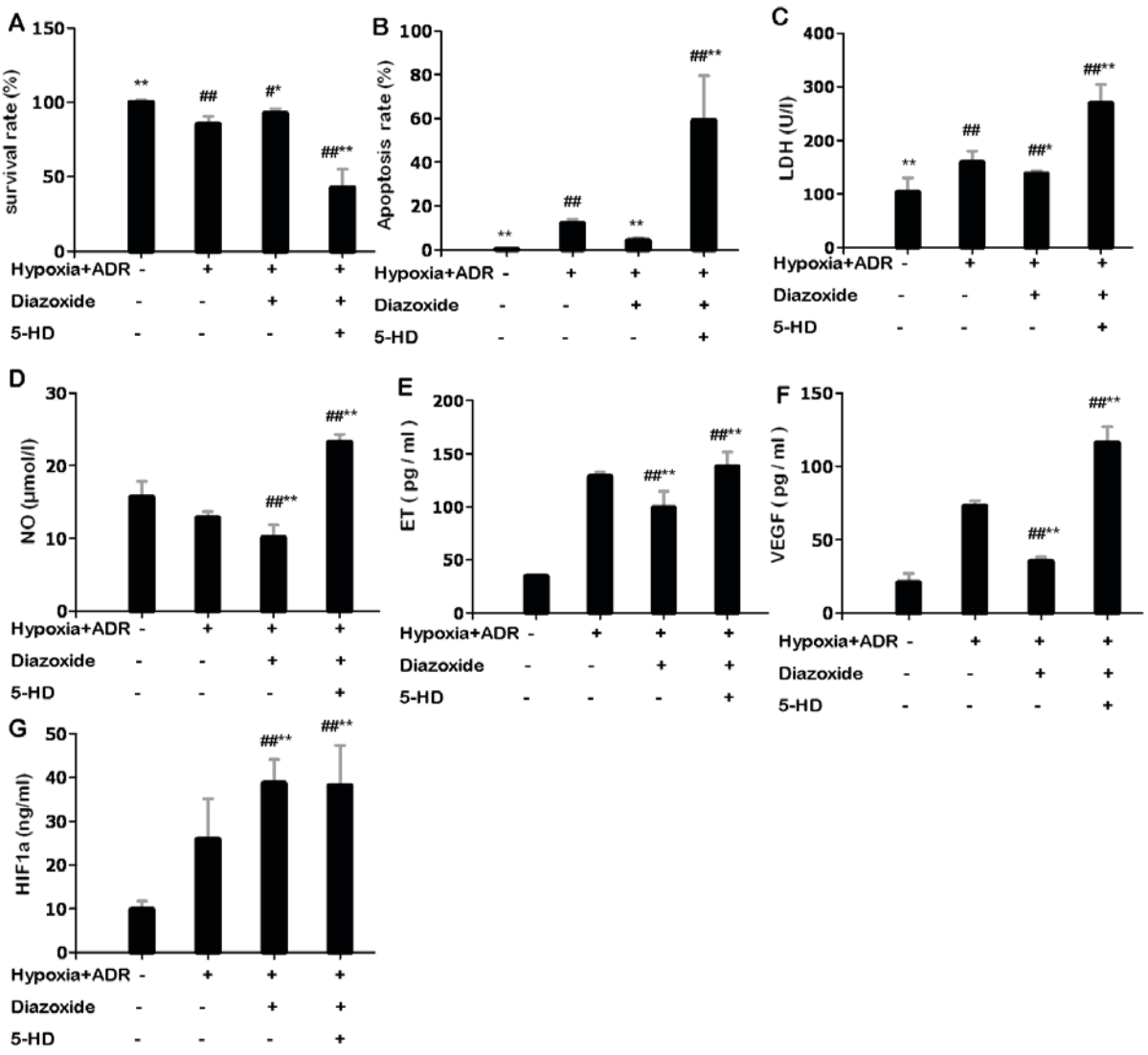

Figure 5. Diazoxide mediates protection against damage induced by hypoxia combined with ADR. Variations in (A) survival rate, (B) apoptosis rates, and the release of (C) LDH, (D) NO, (E) ET, (F) VEGF and (G) HIF-1α in the rat aortic endothelial cells are shown. Data are presented as the mean ± standard error (n=10 in each experiment). $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. control group; *P<0.05 and **P<0.01 vs. hypoxia and ADR groups. ADR, adrenaline; LDH, lactate dehydrogenase; NO, nitric oxide; ET, endothelin; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α.

The endocrine system mediates the key response to altered environment, and integrates various functional activities. Therefore, dysregulation of the endocrine system has a negative impact on the regulation of the biochemical activity. ADR and NADR are catecholamines secreted by the adrenal medulla chromaffin cells. They mobilize fat, increase oxygen consumption and heat production in the body, and subsequently increase the basal metabolic rate. Catecholamines serve a vital role in environmental stress.

In the current study, the results from the animal experiments demonstrated that, compared with the control group, the ADR and NADR levels were partially increased in the hypoxia group. Compared with the rats in the hypoxia group, the ADR and NADR release of those exposed to acute cold environment were significantly increased, indicating that low temperature significantly altered the catecholamine system. The current results involving cold or hypoxia are consistent with those observed by other studies (27-30). Furthermore, the results in rats exposed to hypoxia combined with cold environment revealed that ADR and NADR concentrations were significantly increased compared with the control group. In addition, the ADR release in the combination group was significantly higher compared with the control, hypoxia alone and cold alone groups. These findings suggested that the complex effects of hypoxia and low temperature had a significant impact on the endocrine system of the laboratory animals, and significantly increased the release of ADR and NADR. When the results were analyzed using factorial analysis, acute hypoxia and cold exposure were found to potentially have overlapping effects in altering the catecholamine system. However, acute cold was the main factor disrupting the endocrine system, while hypoxia aggravated endocrine disorders caused by acute cold exposure.

Considering that endothelial dysfunction serves a key role in altitude-associated diseases, the current study mainly focused on endothelial dysfunction under hypoxia, cold, and hypoxia combined with cold simulating high-altitude environments. The endothelial function is mediated by a series of

vasoactive substances, such as NO and ET, and endothelial dysfunction is characterized by reduction in NO bioavailability. NO is one of the most important vasoactive substances and endothelium-derived factors released by ECs. In the present study, it was hypothesized that the endothelium is damaged directly by acute hypoxia, but not by acute cold exposure. In the animal experiments of the present study, the NO release was significantly decreased under acute hypoxia, but not under acute cold exposure. This indicates that acute cold exposure does not damage ECs directly, but disturbs the secretion of endocrine hormones and damages the cells indirectly. Under a hypoxic and cold environment, the content of NO declined significantly compared with the control group. Furthermore, the degree of decrease was greater than under hypoxia alone. The results from the factorial analysis revealed an interaction between the effects of cold and hypoxia on the NO levels. ET is a potent vasoconstrictor that serves an important role in maintaining basic vascular tension and cardiovascular homeostasis. ET also serve a key role in hypertension, heart failure, kidney failure and other diseases (31).

The results from the animal experiments of the present study showed that the complex environmental factors at high altitude not only resulted in endothelial dysfunction, but also in endocrine disorders in the experimental animals. In the current study, under acute hypoxic and cold exposure, the vascular endothelial injuries caused by hypoxia were accompanied with a decreased endothelial function, and low temperature was associated with endocrine disorder. Therefore, cell experiments were also performed using ADR, one of the catecholamine hormones severely affected by low temperature, to simulate the endocrine changes under cold exposure based on the animal experiments, and to further explore the effects of hypoxia and cold on VECs.

The present *in vitro* results demonstrated that the cell viability and release of NO were significantly decreased when the cells were placed in a hypoxic environment (1% O_2) for 27 h. By contrast, the ET content in the cell culture supernatant was significantly increased. Under physiological conditions, NO and ET released by ECs maintain the normal vascular tension in a dynamic balance. EC dysfunction decreases the NO release and increases ET release, which is consistent with the study results. NO levels were significantly reduced in both the animal and the cell culture experiments, while ET levels were substantially increased in the cell culture experiment. A previous study reported that hypoxia damaged the cellular aerobic respiration and disrupted the mitochondrial oxidative phosphorylation, leading to reduced or even inhibited ATP generation, resulting in pathological changes (32).

LDH is mainly distributed in the cytosol, and is identified as a common marker of cell damage (33). In the present study, the LDH content in the cell culture supernatant was significantly increased, which suggested that VECs were damaged under hypoxia (1% O_2) lasting 27 h. In addition, VEGF serves a key role in promoting the proliferation of ECs. Due to its effects on increasing vascular permeability, it is also known as the vascular permeability factor (34). HIF-1α is the nuclear factor closely associated with the regulation of the expression of hypoxia-sensitive genes (such as VEGF) (35,36). The present study results revealed that the expression levels of VEGF and HIF-1α were significantly upregulated under hypoxia. Combined with the results of LDH activity described earlier, it can be concluded that high-altitude exposure to hypoxia abnormally increases VEGF and HIF-1α levels, as well as the microvascular permeability, leading to leakage of large plasma protein and resulting in endothelial injuries.

The animal experiments of the current study revealed that low temperature significantly induced the secretion of ADR, which was accompanied by vascular endothelial damage. Therefore, ADR was then used to simulate the effects of low temperature and to observed the effects of ADR on the vascular endothelium in RAECs. The results of the cell experiments demonstrated that hypoxia and ADR significantly increased apoptosis and the levels of LDH, VEGF, ET and HIF-1α in the cell culture supernatant, while it significantly decreased the cell survival rate. This suggested that ADR induced the damage of VECs, and the enhanced secretion of ADR *in vivo* mediated the acute cold exposure-induced VECs injuries. However, unlike in the animal model, administration of ADR in the cell model significantly induced NO release from VECs. It is suggested that the strong vasoconstrictor effects of ADR induced the increase in NO levels in order to ensure the balance of systolic and diastolic blood vessels; however, the underlying causes remain to be further studied. Nonetheless, the results of the animal experiments indicated that low temperature not only significantly promoted ADR secretion, but also caused a decline in NO. The mechanism may involve NO synthesis driven by elevated ADR secretion, leading to the consumption of more NO synthase (NOS). When NOS is depleted, NO synthesis also declines (37). Therefore, the impact of acute cold exposure on VECs function may be mediated by high ADR release, resulting in the decrease of NOS catalytic activity and injury to VECs.

Several studies have demonstrated that the K_{ATP} of ECs serves an important role in regulating endothelial function (38-42). The K_{ATP}, first discovered by Noma in 1983 in guinea pig myocardial cells (43), is a voltage-independent ligand-gated channel, and its opening results in vascular dilatation and protection of the heart and nerve cells. The surface K_{ATP} channel is located on the cell membrane, and the mitoK_{ATP} channel is located on the mitochondrial membrane. The main physiological functions of mitoK_{ATP} channel include mitochondrial potassium homeostasis, regulation of mitochondrial volume, maintenance of transmembrane potential in mitochondrial energy production, protection of mitochondrial function and stability of cellular energy metabolism (41,42). The mitoK_{ATP} channel may mediate the protective mechanism against hypoxia injuries.

Diazoxide is a selective mitoK_{ATP} channel opener (44), which has strong effects on the K_{ATP} channel in the mitochondrial membrane, and relatively weak effects on the surface K_{ATP} channel. Numerous studies have demonstrated that diazoxide has a protective effect against oxidative stress injuries in I/R, thus improving the ability of cells to withstand oxidative stress and resulting in organ protection (16-20). In the current study, the protective effects of diazoxide on ECs were investigated under conditions of hypoxia or hypoxia combined with cold. It was found that diazoxide significantly increased the survival rate of cells damaged by hypoxia or by hypoxia combined with cold, and significantly decreased the apoptosis rate, with a significant improvement in the ET, VEGF and LDH. Diazoxide attenuated the VEC damage induced by hypoxia

or by hypoxia combined with ADR, and protected the cells, which was antagonized by 5-HD. However, diazoxide treatment failed to reduce the elevated expression of HIF-1α caused by hypoxia or the composite of hypoxia and ADR, indicating that the protective effects of diazoxide may not be achieved by inhibiting HIF-1α expression, but by enhancing ATP activity. Under the combined effect of hypoxia and ADR, diazoxide significantly decreased the NO levels, which indicated that diazoxide does not completely improve the endothelial function following exposure to hypoxia combined with ADR. Additional studies are required to elucidate the mechanisms involved.

In conclusion, hypoxia and cold, the two main environmental factors at high altitudes, were found to induce EC dysfunction and cause endocrine disorders, respectively. Improving the endothelial dysfunction using the mitoK_{ATP} opener diazoxide may be an effective strategy to treat patients with altitude-associated disorders. However, the application of these findings in a clinical setting requires further study.

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