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Effects of endothelial nitric oxide synthase uncoupling on pulmonary endothelial dysfunction in rats with decompression sickness

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Abstract

Background: To investigate the effects of unsafe decompression on rat pulmonary endothelial function and its relevant mechanisms.

Methods: Sixty male Sprague-Dawley (SD) rats were randomly divided into a control group (n=30) and a decompression sickness (DCS) group (n=30). The DCS model was established by placing the rats in the DCS group in a pressurized cabin where they were exposed to a 600 kPa compressed air environment for 60 min, and the pressure was then reduced by 100 kPa/min until it reached atmospheric pressure. After the surviving rats in the DCS group and the rats in the control group were anesthetized, their pulmonary arteries were stripped to test the in vitro pulmonary artery endothelium-dependent vasodilation capacity. Western blotting was used to measure the expression and dissociation of endothelial nitric oxide synthase (eNOS) in pulmonary artery tissues and all protein nitration levels in pulmonary artery tissues; reactive oxygen species (ROS) formation was measured via in vitro pulmonary artery superoxide anion probe dihydroethidium (DHE) staining.

Results: After experiencing unsafe decompression, 10 of the 30 rats in the DCS group died. The pulmonary artery endothelium-dependent vasodilation capacity in the surviving rats decreased significantly ($P<0.05$). The difference in eNOS expression between the DCS group and the control group was statistically insignificant ($P>0.05$), but the ratio of eNOS monomer/dimer in the DCS group was significantly higher than that in the control group ($P<0.05$). All protein tyrosine nitration levels in the pulmonary artery tissues of the DCS group were significantly higher than those of the control group ($P<0.05$). The results of DHE staining showed that the amount of ROS formation in the pulmonary arteries of the DCS group was significantly higher than that of the control group ($P<0.05$).

Conclusion: Unsafe decompression during a simulated submarine escape process can lead to eNOS dimer uncoupling in the pulmonary artery endothelium. The dissociated eNOS monomer cannot synthesize nitric oxide (NO) and thus affect the endothelium-dependent vasodilation capacity. The eNOS monomer can promote peroxynitrite ($ONOO^-$) synthesis, leading to an increase in protein tyrosine nitration levels in pulmonary artery tissues and causing disorder in cell cycle regulation. The eNOS monomer can also cause an increase in the formation of ROS and thus mediate peroxidation damage.

Key words Decompression sickness; Nitric oxide synthase type III; Superoxide anion; 3-nitrotyrosine

Background

At present, foreign and domestic scholars generally believe that unsafe decompression can lead to gas bubble formation within blood vessels and tissues and that the functional status of the blood vessels is directly related to whether intravascular gas bubble formation after decompression leads to circulatory disorders[1]. Relevant studies show that regardless of whether the diameter of the gas bubble is greater than the diameter of the blood vessel (in reality, the diameter of each visible gas bubble is greater than the microvascular diameter, or at least it must be greater than the diameter of the capillary), the gas bubble flow rate, direction, and pattern depend entirely on the functional status of the blood vessels. Furthermore, gas bubbles, which can be affected by blood flow and blood pressure, can be easily deformed to pass through various diameters of blood vessels and enter the circulation via blood. Gas bubbles will only enter a static state during blood stagnation and vasospasm and atresia, when the gas bubbles have no way out and when the force of the blood pressure is less than the force of the vessel sealing[2]; this state will cause

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decompression sickness (DCS) symptoms. Therefore, we conjecture that during the process of unsafe decompression, vasodilation capacity disorders can lead to DCS.

Vasodilation is divided into endothelium-dependent vasodilation and non-endothelium-dependent vasodilation. Non-endothelium-dependent vasodilation might be caused by stimuli, such as acetylcholine and bradykinin. Vasodilation is mainly caused by physiological stimuli. Nitric oxide (NO) is an important factor of endothelium-dependent vasodilation[3], and it is synthesized by nitric oxide synthase (NOS), which comes in three types: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). The latter, nNOS, is a neurological type that is mostly located in neuronal cells. iNOS is an inducible type that can be induced to form under inflammatory conditions. eNOS is stably expressed in vascular endothelial cells and can synthesize NO in response to physiological demands. In the dimer form, eNOS converts the L-arginine into NO. When eNOS is dissociated into monomers, NO cannot be normally synthesized, and a large amount of the superoxide anion O_2 is generated. The reaction between O_2 and NO generates a large amount of peroxynitrite ($ONOO^-$)[4], which nitrates important proteins, changes signal pathways, and directly and/or indirectly mediates the cytotoxic effects of NO. This paper describes a preliminary study on whether the vascular damage in DCS is related to the uncoupling of eNOS, as there has been yet no reporting on this issue.

Methods

Experimental Animals, Materials, and Equipment

Ethics statement

All experiments were performed according to the German regulations for animal experimentation and approved by the Regierung von Unterfranken as the responsible authority (Permit Number 55.2-2531.01-30/09).

Sixty male Sprague-Dawley (SD) rats with body weights of 210±25g were purchased from the Animal Center of the Naval Medical Research Institute; Laboratory Animal Certification: SCXK (Shanghai)2008-0016. The anti-3-nitrotyrosine antibody (ab110282) was purchased from Abcam (Cambridge, U.K.). The eNOS antibody was purchased from BD Bioscience (Germany). The superoxide anion probe dihydroethidium (DHE) and the loading buffer without 2-mercaptoethanol were purchased from Sigma (U.S.A.). The following solutions were used. Krebs-Henseleit (K-H) solution: 119mmol/L NaCl, 25mmol/L $NaHCO_3$, 1.19mmol/L $MgCl_2$, 4.7mmol/L KCl, 1.2mmol/L KH_2PO_4, 2.5mmol/L $CaCl_2$, 11.1mmol/L D-Glucose. High-potassium K-H solution (containing 60mmol/L KCl): 63.7mmol/L NaCl, 25mmol/L $NaHCO_3$, 1mmol/L $MgCl_2{\cdot}6H_2O$, 60mmol/L KCl, 2.5mmol/L KH_2PO_4, 11.1mmol/L D-glucose. High-potassium, calcium-free solution: $CaCl_2$ was replaced with ethylene glycol tetraacetic acid (EGTA) (0.05) in the high-potassium solution, and the remaining ingredients were the same as the high-potassium solution. Radioimmunoprecipitation assay (RIPA) lysis buffer: 100ml RIPA buffer, 50mmol/L Tris-HCl (pH 7.4), 150mmol/L NaCl, 1% NP-40, 0.1% SDS. Storage: 4℃, 2μg/ml aprotinin (protease inhibitor, added prior to use). Laboratory Equipment: $0.3m^3$ compression chamber (Shandong Hongyuan Corporation), Model 610M multi-channel vascular tone detector (DMT Company, Denmark), PowerLab 8/30 biological signal acquisition and processing system (ADInstruments, Australia), Model DK-8D electric heated thermostatic water tank (Shanghai Medical Thermostatic Equipment Factory), Stemi Model DV4 stereomicroscope (Zeiss AG, Germany).

Experimental Groups and Models

The 60 male SD rats were randomly divided into two groups. Control group: Thirty rats were placed in the $0.3m^3$ compression chamber (which was used for creating DCS rat models) for 65min without any pressurization. Air was continuously passed into the chamber to maintain a carbon dioxide (CO_2) level of less than 300mg/L. The humidity in the chamber was maintained at 40%-60%, and the temperature was maintained at 27-30℃. The rats could move about freely in the chamber. The researchers could observe the rats' behavior in the chamber through closed-circuit television. DCS group: Thirty rats were placed in the same compression chamber as the control group with the same temperature and humidity settings. The air inside the chamber was pressurized at a rate of 100kPa/min to 600kPa (equivalent to the pressure at a sea depth of 60 meters), which was maintained for 60min. Air was continuously passed into the chamber to maintain a CO_2 level of less than 300mg/L. The pressure was reduced at the same rate until it reached atmospheric pressure.

Specimen Collection

After the two groups of animals were taken out of the chamber, changes in animal behavior and mortality were observed. After one hour, the surviving rats were anesthetized by intraperitoneal injection of 50mg/kg of 1% sodium pentobarbital. The rats were fixed on an operating platform in a supine position. Their chests were opened for the removal of cardiopulmonary tissues. The stripping of pulmonary artery tissues was performed under ice bath conditions. Eight sections of pulmonary artery tissues from each group were retained and placed in the K-H solution. An in vitro vascular tone test and DHE staining were performed immediately. The

remaining tissues were kept frozen at –80℃ in preparation for Western blotting.

Determination of Artery Endothelium-dependent Vasodilation Function

The vascular specimens were quickly placed in a K-H nutrient solution (pH 7.4, 4℃) containing a pumped-in gas mixture [95% oxygen (O_2)+5% CO_2]. Vascular rings (3-4mm) were prepared and suspended in a physiological chamber with built-in 10ml nutrient solution containing indomethacin (10^{-5}mol/L, to inhibit the endogenous prostacyclin system). The gas mixture was pumped in at a constant speed. The constant temperature was 37.0±0.5℃. The resting load was 4g. The collected signals were imported into the computer system through the tension transducer to record the tension changes of the vascular rings. First, a high-potassium solution containing 60mmol/L KCl was used to stimulate the blood vessels to test the vascular tone reactivity. The K-H solution in the chamber was then replaced with a high-potassium K-H solution. The high-potassium solution produced a contraction reaction in the coronary artery circle. After the contraction was sustained for five minutes and the tension had stabilized, the activated tension at the end of five minutes (unit was mN/mm) was used as 100%, maximum to baseline. First, norepinephrine was used for the precontraction of vascular rings, and acetylcholine (Ach) was added after the tension had stabilized. Changes in vascular tone were recorded, and its endothelium-dependent vasodilation response to Ach (10^{-9}-10^{-4}mol/L) was measured. To determine whether the Ach-induced relaxation response in this experiment was mediated by the endothelium-derived NO, some of the vascular rings were wiped off the endothelia or were pretreated with the NOS inhibitor L-NAME (3×10^{-4}mol/L) for 15min prior to being tested.

Measurement of Pulmonary Artery 3-Nitrotyrosine and eNOS Monomer and Dimer

The Western blotting method was used to measure the level of 3-nitrotyrosine in pulmonary artery tissues. An equal amount of pulmonary artery tissues was taken from each rat, RIPA (0.1ml/10mg) was added, and an electric homogenizer was used for homogenization. A low temperature was maintained during this process by burying the sample in ice while homogenizing quickly. The sample was transferred to a 1.5ml centrifuge tube for centrifugation at 4℃and 12,000g for two to three minutes. The supernatant was collected, the loading buffer was added, and the sample was then heated to 98℃ for five minutes. SDS-polyacrylamide gel electrophoresis was performed, and the sample was transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). A reaction solution containing the primary antibody (rabbit anti-mouse at 1:1,000 dilution) was added, followed by the addition of secondary antibody with goat anti-rabbit IgG (1:4,000 dilution). For the eNOS monomer and dimer test, a loading buffer without mercaptoethanol was added during the tissue sample processing. The mixtures were not heated and SDS-polyacrylamide gel electrophoresis was performed at 4℃. After the transfer to a membrane, a reaction solution containing the primary antibody (rabbit anti-mouse, dilution proportion was 1:500) was added for incubation overnight, and the follow-up procedure was the same as before. A domestic Model HP IAS21000 image analyzer was used to determine the average optical density of the samples.

Pulmonary Artery ROS Determination

Six surviving rats were taken from each group, and they were each given tail vein injections of DHE (100μmol/L, 0.1ml/10g) fluorescent dyes 30 min before being sacrificed. After 30 min, they were anesthetized for the removal of pulmonary artery tissues, which were embedded in a frozen section embedding agent (optimal cutting temperature compound OCT), frozen at –20℃, sliced into 10μm slices using a microtome, washed with phosphate-buffered saline (PBS) three times, and sealed with anti-fluorescence quencher. The ROS content in the pulmonary artery tissues of the rats was evaluated under a fluorescence microscope (red florescence). Image-Pro Plus 6.0 image analysis software was used to quantitatively analyze the fluorescence intensity. The results are expressed in units of relative fluorescence intensity.

Statistical Analysis

SPSS 13.0 software was used for statistical analysis. The measurement data are represented as $\bar{x}\pm s$. The LSD-*t* test was used to compare the mean between the two groups. $P<0.05$ indicated that the difference was statistically significant.

Results

Endothelium-dependent Vasodilation Function

For the vascular rings that were precontracted using 10^{-5}mol/L norepinephrine, the percentage of the maximum relaxation response (12.5%±3.2%) in the Ach-induced blood vessels of the DCS group was significantly lower than that of the control group (48.6%±4.3%, $P<0.05$), suggesting that the endothelium-dependent vasodilation function of the DCS model rats was significantly impaired. After endothelium removal or 3×10^{-4}mol/L L-NAME preprocessing, Ach no longer produced a significant relaxation effect on the vascular rings, suggesting that Ach-induced blood vessels relaxation effect was mediated by endothelium-derived NO (Figure 1).

Expression of 3-Nitrotyrosine in Pulmonary Artery Tissues

The relative expression level of 3-nitrotyrosine in the

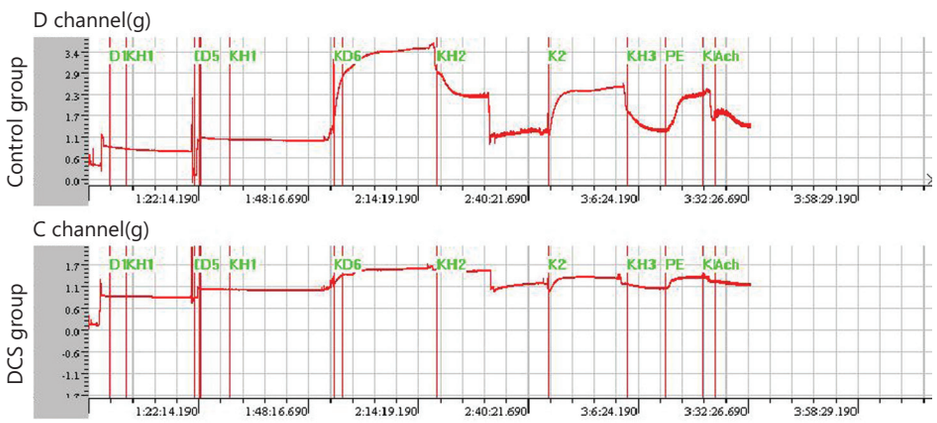

Figure 1. Changes in the pulmonary artery endothelium-dependent vasodilatation function of rats.

KD6 and K2. High-potassium solution was added; KH2 and KH3. K-H elute was added; PE. Norepinephrine was added; KAch. Acetylcholine was added.

pulmonary artery tissues of the DCS group (256±7) was significantly higher than that in the control group (178±7). The difference was statistically significant (P<0.05, Figure 2).

Measurement of eNOS Monomers and Dimers

No significant difference (P>0.05) was observed in the total quantity of eNOS between the control and DCS groups, indicating the constant expression of eNOS in vascular endothelial cells. The eNOS monomer/dimer ratio was significantly higher in the DCS group, and the difference between the DCS group and the control group was statistically significant (P<0.05, Figure 3).

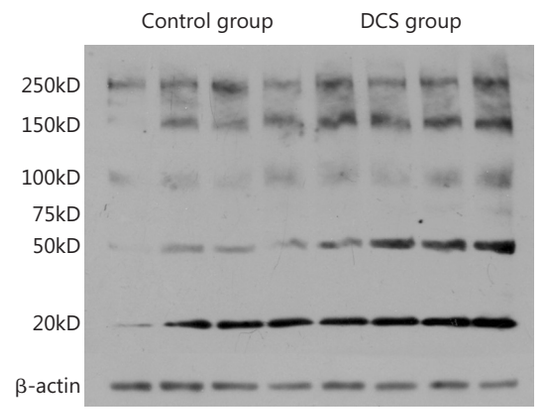

Figure 2. Expression of 3-nitrotyrosine in the pulmonary artery tissues of rats ($\bar{x}\pm s$, n=8).

Results of the Pulmonary Artery ROS Test

The DHE staining results showed that in the control group, comparatively less ROS was formed in the pulmonary artery tissues, and the fluorescence intensity was comparatively weaker. The DCS group, which experienced unsafe decompression, had a significantly higher amount of ROS formation in the pulmonary artery tissues, and the red fluorescence intensity of the pulmonary arteries was significantly increased. Image analysis results showed that compared with the control group, the DCS group had significantly greater ROS formation in the pulmonary artery tissues (P<0.05, Figure 4).

Discussion

DCS is caused by the formation of gas bubbles inside and

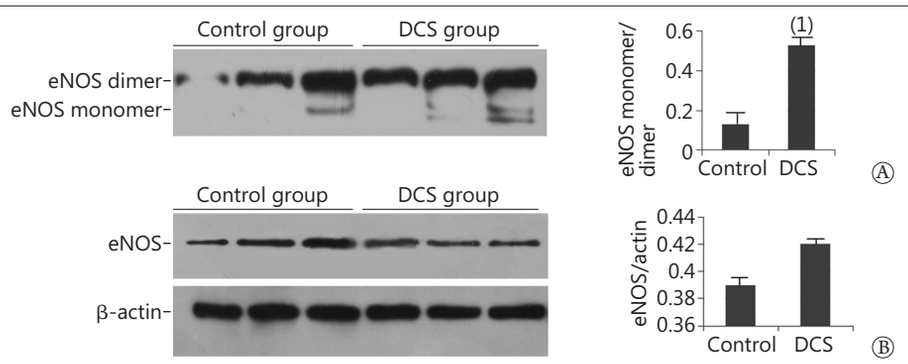

Figure 3. Expression of eNOS and its monomer/dimer ratio in the pulmonary artery tissues of rats ($\bar{x}\pm s$, n=8).

A. eNOS monomer/dimer; B. Total amount of eNOS. (1)P<0.05 compared with the control group.

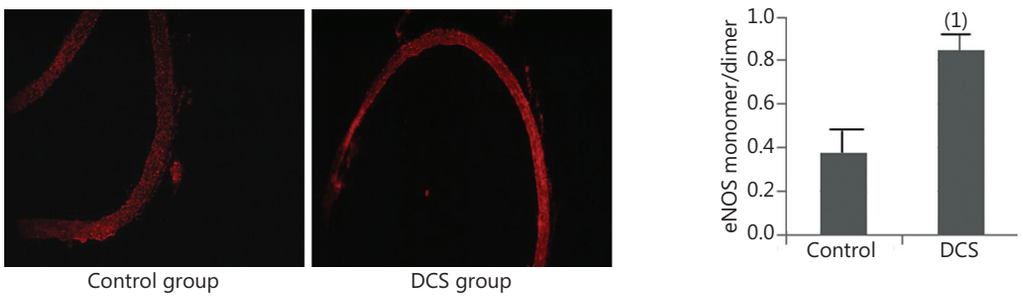

Figure 4. The amount of ROS formation in the pulmonary artery tissues of rats (DHE staining).
(1)P<0.05 compared with control group.

outside blood vessels due to a sudden drop in environmental pressure; this situation can happen to divers, astronauts, and other individuals[5]. When the gas bubbles enter the pulmonary circulation along with the blood, some of the gases can escape through the pulmonary capillary beds. The gas bubbles that have not been eliminated reach the left ventricle with the blood and spread with the arterial blood to the blood vessels in all parts of the body, including organs such as the heart and the lung. The pathogenicity of gases in the circulatory system is due to the vascular embolism caused by the gases (gas bubbles) on the one hand[6]. On the other hand, pathogenicity is also caused by the extremely large changes in the heart chamber and pulmonary vascular bed composition that result from the large amount of gases that enter the circulatory system, which cause the heart to change from a blood pump to a gas pump; this difference leads to myocardial ischemia and hypoxia and myocardial contraction weakness or heart failure, thereby causing secondary ischemia and hypoxia in the respiratory and circulatory centers[2]. Insufficient decompression can also cause vascular dysfunction in the body, leading to disorders of the blood flow rate, direction and pattern and to gas bubbles. Severe cases can even cause blockage of the flow of blood and gas bubbles, blood stagnation, and a static state of internal gas bubbles. Improving vascular function can promote gas bubble elimination and lessen the incidence and mortality rate of DCS to a certain extent.

A great deal of foreign and domestic research has focused on the injury from gas bubbles generated during decompression processes, such as during submarine escapes and diving. Nossum et al.[7] injected 0.01ml/(min·kg) of air into the jugular veins of domestic rabbits. After one hour, they found a decrease in the endothelium-dependent vasodilation capacity of the domestic rabbits in the experimental group, but the non-endothelium-dependent vasodilation capacity was unchanged. In addition, the vascular endothelial layer did not appear abnormal when observed under an optical microscope; this observation confirmed that gas bubbles can cause a disorder in the biochemical levels of the endothelial layer, lowering the endothelium-dependent vasodilation capacity. That study obliquely verified the correctness of the results of the present study. In addition, the study by Wang et al.[8] mentioned that using nitrates as an exogenous NO donor five days or 30min before diving can significantly reduce the gas bubbles in the bodies of rats and pigs and lower the incidence of DCS and mortality rate; however, the administration of non-selective NOS inhibitors (L-NAME) can increase the incidence of DCS and the mortality rate. NO can mediate the endothelium-dependent vasodilation capacity; therefore, we speculate that the vascular dysfunction in DCS is related to NO synthesis disorders. The NO that mediates the endothelium-dependent vasodilation capacity is primarily synthesized by the eNOS in the vascular endothelium[9,10]. The eNOS dimer is formed by two monomers, and the following chemical reaction occurs when eNOS enters the dimer state: L-arginine+$O_2 \xrightarrow{\text{eNOS dimer}}$ L-arginine+NO. If eNOS dissociates into monomers, then the mediating reaction is changed to L-arginine+$O_2 \xrightarrow{\text{eNOS monomer}}$ $ONOO^- + H_2O_2 + O_2$[11]. $ONOO^-$ can cause tyrosine nitration, change signal pathways, and initiate a series of cytotoxic reactions.

Conclusion

The endothelium-dependent vasodilation capacity of pulmonary artery tissues in the DCS model is significantly lower than that in the control group. The eNOS uncoupling situation exists in the pulmonary artery tissues of rats in the DCS model; the protein tyrosine nitration and the ROS formation in the tissues are significantly higher than those in the control group. These results demonstrate that the pulmonary artery endothelial eNOS uncoupling situation in DCS causes a decrease in NO synthesis and a reduction in endothelium-dependent vasodilation capacity, thereby

aggravating vascular occlusion, blocking gas bubble passage, reducing the filtering out of gas bubbles from within the lung, and intensifying DCS injury. The $ONOO^-$ generated after eNOS uncoupling causes protein tyrosine nitration and the synthesis of superoxide ions, mediating a series of peroxidation damage processes. The uncoupling of eNOS may be a target, but it is not the only target in DCS gas bubble injury management. Whether interfering with this process can improve the overall incidence and mortality rate of DCS still requires further studies.

Abbreviations

Ach: Acetylcholine; CO_2: Carbon dioxide; DCS: decompression sickness; DHE: Dihydroethidium; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase; K-H: Krebs-Henseleit; nNOS: Neuronal nitric oxide synthase; NO: Nitric oxide; OCT: Optimal cutting temperature compound; PBS: Phosphate-buffered saline; RIPA: Radioimmunoprecipitation assay; ROS: Reactive oxygen species; SD: Sprague-Dawley.

Competing interests

The authors declare that they have no competing interests to report.

Authors' contributions

HSL carried out the endothelium-dependent vasodilation capacity tests and superoxide anion probe dihydroethidium (DHE) stain, participated in the Western blotting and drafted the manuscript. YQF participated in the design of the study and performed the statistical analysis. MO conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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