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Effect of hypoxia on nitric oxide and endothelin-1 in pikas and rats

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Abstract：The aim of the present study was to compare the effects of hypoxia on NO，ET-1 and NO/ET-1levels in Wistar rats and pikas. We determined NO and ET-1levels in the serum of 30 rats（three groups of 10 rats，each taken to 3 780 m and divided into 24 h，2 wk and 3 wk durations）and 10 pikas at 3 780 m by ELISA and Nitrate Reductase Method. We compared the values of NO，ET-1 and NO/ET-1 in rats of 24 h duration with those in rats of 2 wk and 3 wk durations. We then compared NO，ET-1 and NO/ET-1 values in the 24 h，2 wk，3 wk groups with those in pikas. In the beginning of entry to high altitude，the NO and ET-1levels in rats were markedly increased compared to those in pikas（respectively，$P < 0.01$），whereas the values of NO/ET-1in rats and pikas were very close（$P > 0.05$）. There was a decreasing trend in NO levels and an increasing trend in ET-1levels（respectively，$P < 0.01$）with passage of time at high altitude in rats，and NO was well correlated with ET-1（$r^2 = 0.2416$，$P < 0.01$）. The NO/ET-1levels in pikas was approximately 2 fold greater than in the 2 wk and 3 wk groups（$P < 0.01$）. In conclusion，NO，ET-1 and NO/ET-1 levles in Wistar rats and pikas at 3 780 m are very different，may indicate adaption or acclimitization to high altitude hypoxia.

Key words：*Endothelin-1*（ET-1）；Hypoxia rat；Nitric oxide（NO）；Pika

低氧对高原鼠兔和大鼠血液 NO 与 ET-1的影响

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摘要：将 Wistar 大鼠暴露于 3 780 m 低氧环境，分别于 24 h、2 wk 及 3 wk 后采用酶联免疫法和硝酸还原酶法测定血液中的ET-1和 NO 的含量，计算 NO/ET-1值，并与高原鼠兔比较，探讨低氧条件下大鼠与高原鼠兔血液中 NO 与 ET-1含量的变化趋势。结果表明，低氧 24 h 后，大鼠血液中 NO 和 ET-1的含量显著高于同海拔的高原鼠兔（$P < 0.01$），而 NO/ET-1值无显著差异（$P > 0.05$）。随着大鼠在高海拔停留时间的延长，血液中 NO 含量呈减少趋势，而 ET-1则有上升趋势，二者呈显著的负相关（$r^2 = 0.2416$，$P < 0.01$）。高原鼠兔 NO/ET-1值约为大鼠低氧 2 wk和 3 wk 的 2 倍（$P < 0.01$）。说明不同低氧暴露时间，高原鼠兔和大鼠的 NO、ET-1及 NO/ET-1值有显著差异，提示 NO/ET-1值可以作为有机体是否适应高原低氧环境的一个指标。

关键词：高原鼠兔；Wistar 大鼠；一氧化氮（NO）；内皮素-1（ET-1）
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Nitric oxide（NO）is a pluripotent intra- and extra-cellular messenger molecule which regulates a variety of biological processes. In the normal pulmonary circulation，NO not only mediates vasodilation in response to physical and chemical factors but also opposes vasoconstriction induced by various stimuli such as hypoxia and endothelin（ET）（Raffestin *et al.*，1991）. Loss of NO-mediated pulmonary endothelium-dependent relaxation during hypoxic pulmonary hypertension may contribute to an increase in pulmonary vascular tone，favor thrombus formation，and facilitate migration and/or proliferation of vascular smooth muscle cells（Carville *et al.*，1993；Maruyama and Maruyama，1994）. ET-1 is a kind of vascular active polypeptide，it strongly effects vessel contraction and promotes growth of vascular smooth muscle. In normal physiological condition，NO and ET-1 as endothelium-dependent vasomotion factors are homeostatic，and play a normal role in regulating vascular. The homeostasis between vasoconstriction and vasodilation can be upset by various stimuli such as hypoxia，and can affect pulmonary circulation. So far the secretion and correlation of NO and ET-1 in the adaptive mode of hypoxic pulmonary circulation has not been reported. This paper compares levels of NO and ET-1 in the blood of hypoxic Wistar rats with similar levels in acclimatized pikas in order to describe the relationship between the levels of NO，ET-1，NO/ET-1 and the hypoxic adaptation mechanism at high altitude.

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1 Materials and methods

1.1 Experimental animal

Ten male pikas and 30 male Wistar rats were used for this study. The Wistar rats, which were born and raised in Xining (2 300 m altitude, Barometric pressure is 77.4 kPa), Qinghai, China, weighed 120 – 180 g, and were provided by the Qinghai Experimental Animals Center. Three groups of 10 rats were each taken from Xining to Guoluo Dawu Township (3 780 m altitude, Barometric pressure is 65.7 kPa) where the study was conducted and were divided into 24 h, 2 wk and 3 wk groups, by random screening. Pikas, weighing 100 – 180 g, were captured at an altitude of 3 780 m and were involved in the experiment after 2 days.

1.2 Determination of NO and ET-1

After measuring body weight, the animals were anesthetized with abdominal administration of pentobarbital sodium (30 – 50 mg/kg). The right external jugular vein was isolated, then a curved-tip polyvinylene catheter (ID 0.28 mm) was inserted and advanced to both the right ventricle and main pulmonary artery. Correct placement was confirmed by the typical pressure wave tracing seen on an oscilloscope, and blood samples were drawn from the catheter. 2 ml blood samples were put into a tube containing 10% 30 μl disodium edetate (EDTA-2Na) and 40 μl trasylol and amalgamated. The plasma was centrifugally separated (4℃, 3 000 r/min) for the assay of ET-1. Additional, 5 ml blood samples were centrifugally separated (4℃, 3 000 r/min) and withdrawn from the serum for the assay of NO. 1 ml whole blood containing heparin was used to determinate the concentration of hemoglobin (Hb) and hematocrit (Hct) with a hemoglobinometer and microcentrifugation, respectively.

The measuring kit for rats was produced by American LifeKey Inc. and provided by Tientsin Qingxing Science & Technology Co., Ltd. The level of ET-1 in rat and pika plasma was determined using ELISA. The optical density at the wavelength of 450 nm was measured through EIX-800 of BIO-PEK Instruments Inc (USA) in order to calculate the level of ET-1. The level of NO in serum of rat and pika was determined used Nitrate Reductase Method, in which the optical density of 550 nm was measured by ultraviolet spectrophotometer (UV-200, made in Japan) in order to calculate the level of NO. The ratio of NO/ET-1 was calculated also.

1.3 Statistical analysis

Data were expressed as the mean ± standard deviation (SD) and were analyzed by a one-way analysis of variance (ANOVA) using SPSS 10.0 software. Linear regression analysis and correlation coefficients were used to assess the relationships between variables. Comparisons and correlations were considered significant when the P value was less than 0.05.

2 Results

2.1 Comparison of NO, ET-1, NO/ET-1 in hypoxia rats and pikas

Table 1 shows the changes of NO, ET-1 and NO/ET-1 in rats and pikas at 3 780 m.

Table 1 Changes of NO, ET-1 and NO/ET-1 in Wistar rats and pikas ($\bar{x} \pm SD$)

Group	Hypoxia time	n	Hb (g/L)	Hct (%)	NO (μmol/L)	ET-1 (pg/ml)	NO/ET-1
Pika	Native	10	131.8 ± 6.55	47.5 ± 3.59	36.52 ± 7.35	1.09 ± 0.12	34.24 ± 6.92
Wistar rat	24 h	8	139.8 ± 8.00	51.38 ± 2.82	47.03 ± 4.12 *	1.25 ± 0.44 * *	37.77 ± 3.05
	2-week	10	156.7 ± 10.00 * * ▲▲	60.95 ± 4.60 * * ▲▲	23.04 ± 5.04 * * ▲▲	1.35 ± 0.75 * * ▲	17.18 ± 4.24 * * ▲▲
	3-week	10	179.1 ± 15.50 * * ▲▲	61.9 ± 4.95 * * ▲▲	20.90 ± 5.24 * * ▲▲	1.31 ± 0.92 * *	16.13 ± 4.34 * * ▲▲

Note: compared with plateau pikas * $P<0.05$, * * $P<0.01$; compared with rats of 24 hours ▲ $P<0.05$, ▲▲ $P<0.01$

ANOVA results indicated that there was a decreasing trend in NO during the total 3-weeks- hypoxia ($P<0.01$) in the serum of rats. In 24 h groups, the level of NO was significantly higher and was approximately 2 fold than that of the 2-wk groups ($P<0.01$), but there was no significant difference between 2-wk and 3-wk groups ($P>0.05$). The level of ET-1 ascended steadily during the total 3-wk-hypoxia in rats ($P<0.01$). While there was a significant difference between 24 h and 2 wk groups ($P<0.05$), there was no significant difference between the 2 wk and the 3 wk groups ($P>0.05$). The changes in the ratio of NO/ET-1 were similar to the changes of the NO level.

At different hypoxia times, the NO、ET-1 levels and the NO/ET-1 ratio in rats were significantly different compared to those in pikas (respectively, $P<0.01$). In 24 h groups, the NO and ET-1 levels in rats were markedly increased compared to those in pikas ($P<0.05$, and $P<0.01$ respectively), whereas the values of NO/ET-1 in rats and pikas were very close ($P>0.05$). In other groups (2 wk and 3 wk), there were significant differences in both NO and ET-1 between rats and pikas ($P<0.01$). The NO/ET-1 levels in pikas was approximately 2 fold greater than that in the 2 wk

and 3 wk groups ($P < 0.01$).

2.2 Correlations of NO and ET-1 in rats and pikas during the process of hypoxia

The NO level was well correlated with ET-1 during the process of hypoxia in rats ($r^2 = 0.2416$, $P < 0.01$, linear regression equation was y (NO) $= -75.302x$ (ET-1) $+ 127.48$, see Fig. 1), but there was no correlation in pikas ($r^2 = 0.0046$, $P > 0.05$)

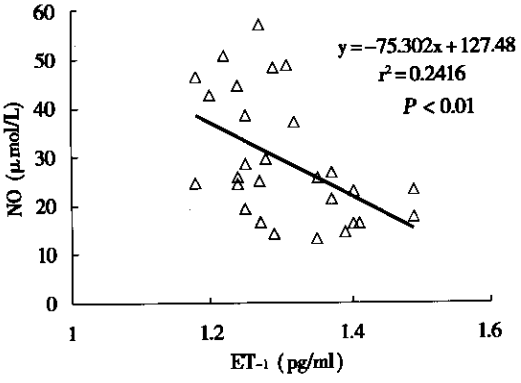

Fig. 1 The correlation of NO and ET-1 in rat

3 Discussion

NO and nitrovasodilators cause pulmonary artery (PA) vasodilatation by activating soluble guanylate cyclase and increasing cGMP levels in vascular smooth muscle (VSM). Increases in cGMP precede relaxation, and inhibition of guanylate cyclase blocks both cGMP synthesis and relaxation, indicating a central role for cGMP in NO and nitrovasodilator-induced relaxation. In the cell of vascular smooth muscle, cGMP could inhibit protein kinase phosphorylation, leading to a decrease in Ca^{2+}. The concentration of Ca^{2+} in cells was low, indicating the myosin light chain was dephosphorylated and the pulmonary smooth muscle was diastolated. Resistance of circulation was also lowered. Davidge *et al.* (1995) have indicated that acute anoxia can increase the expression of nitric oxide synthase (NOS), increasing the level of NO. The results in this paper show a significant difference in NO levels between rats and pikas after 24 h of acute hypoxia, suggesting that the acute hypoxia could stimulate endothelial cells to secrete NO in order to diastolate the smooth muscle in bronchi, and avoid the damnification caused by the excess contraction of lung blood vascular. This reaction is an important recovery mechanism. Our results are consistent with those of Chen (2001). Because NO can combine with Hb into NO-Hb and NO-Hb can be oxidized into MetHb, the oxygen carrying function of Hb can be lost. As a result, NO may increase in response to Hb to enhance the oxygen carrying capacity of the cell in acute anoxia. This result is one of the compensatory protective mechanisms in rats to adapt to acute hypoxia.

ET, as a vascular regulatory polypeptide, neurotic medium and a growth factor is released via paracrine and endocellular secretions. ET regulates the function of the circulatory, respiratory, alimentary, urinary and neuroendocrine systems. In vitro tests show that ET can contract the smooth muscles of arteries and veins. ET-1's strong biological activity can lead to great concentration-dependent contractions in pulmonary vessels (Sheng, 1999). Our study indicated that there was a significant difference in ET-1 level between rats and pikas at 24 h acute hypoxia. One possible reason is that acute anoxia stimulated the artery chemoreceptor, which excited the sympathetic nervous system, and resulted in the secretion of ET-1. Another reason could be that acute anoxia lead to a morphological change within endothelial cells. As the cytomembrane's permeability increases, cells rupture and a large amount of ET-1 may be released into blood. This would result in an increased ET-1 level in plasma and in the pulmonary vasotonia.

While both NO and ET-1 increased in rats at acute hypoxia, the ratio of NO/ET-1 in rats was not significantly different compared to pikas. This result suggests that the compensatory irritated response is reasonable, the ratio of NO/ET-1 is fit, and the rats is still in a compensatory adapting phase. What, then, is the relationship between NO and ET-1 with passage of time at high altitude in rats?

NO and ET-1 are a pair of antagonistic systaltic-factors present in endothelial cells that are regulated by a negative feedback mechanism. ET can promote the release of NO by ET-receptors, whereas NO can inhibit the production of ET by a GMP channel. A dynamic synthesis-release equilibrium exists between NO and ET so that they naturally regulate vascular activity. The synthesis-release of ET-1 and NO is changed when endothelial cells in pulmonary vessels are damaged. Therefore the pulmonary circulation resistance is heightened causing pulmonary hypertension (Rizvi and Myers, 1997; Fuchgott, 1999; Diluozzo *et al.*, 2000). We observed an decreasing trend in NO and ET-1 (respectively, $P < 0.01$) with passage of time at high altitude in rats and a high correlation between NO and ET-1 ($r^2 = 0.2416$, $P < 0.01$). This indicates that the secreting action of endothelial cells may be damaged in chronic hypoxia. Under this scenario, productive mechanism of NO is inhibited, the remaining NO does not inhibit the increase of ET-1, and the ratio of NO/ET-1 decreases. ET-1 can double and redouble the multiplication rate of normal vascular smooth mus-

cle cells (VSMC), promote the hyperplasia of fibroblasts, cardiac muscle cell, and increase the protein synthesis of VSMC. When 10.9 mol/L ET was added to a culture of smooth muscle cell for 4 days, the cell number increased 2 fold and the synthesis of DNA increased 7 fold (Hirata *et al.*, 1989). Therefore, the dysequilibrium of NO and ET-1 could result in the continuous contraction of pulmonary vessels, and promote the multiplication of VSMC, leading to the structural remolding of pulmonary vascular cells. Finally the pathologic change of the serious hypoxia pulmonary hypertension would occur. Previous studies showed (Meyrick and Reid, 1980; Song and Cai, 1993) that non-vascularized arterioles in pulmonary acinus became distincty vascularized-arterioles in rats 7 days after experiencing hypoxia. The increasing degree of the percent of vascularized-arterioles was due to affinity with the pulmonary hypertension caused by hypoxia. The thickness of the medial pulmonary was increased, the smooth muscle cell was converted from a contractile to a synthetic phenotype, and the synthetic phenotype was obviously hyperplasia. Hence, in the present study, we regarded that the decrease of NO and NO/ET-1 and the elevation of ET-1 to be a marker of maladaptation to high altitude in rats raised at low altitude. This decrease may also be one of the consequences of the change to hypoxia pulmonary hypertension.

Ge *et al.* (1998) reported that in pikas that lose hypoxic pulmonary vasoconstriction, the wall of the small pulmonary arteries of the pikas was extremely thin and without smooth muscle cells. The smaller pulmonary arteries (<100 μm) have a wall composed of only a single elastic lamina in pikas. In agreement with previous observations at high altitude, we also observed that the levels of Hb and Hct of pikas were lower than those of rats. Lower Hb and Hct values at high altitude, which can be a feature of residence at high altitude and may be regarded as evidence of genetic adaptation to high altitude, may function to maintain blood viscosity and pulmonary vascular resistance at low levels (Wang *et al.*, 2001). In the present study, the ratio of NO/ET-1 in pikas was not significantly different compared to those in 24 h hypoxic rats ($P > 0.05$), but was significantly higher than those in 2 wk and 3 wk hypoxic rats ($P < 0.01$). These results suggest that the pika has a ratio of NO/ET-1 that suggests adaptation to life at high altitude.

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