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Role of Nitric Oxide in Adaptation to Hypoxia and Adaptive Defense

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Summary

Adaptation to hypoxia is beneficial in cardiovascular pathology related to NO shortage or overproduction. However, the question about the influence of adaptation to hypoxia on NO metabolism has remained open. The present work was aimed at the relationship between processes of NO production and storage during adaptation to hypoxia and the possible protective significance of these processes. Rats were adapted to intermittent hypobaric hypoxia in an altitude chamber. NO production was determined by plasma nitrite/nitrate level. Vascular NO stores were evaluated by relaxation of the isolated aorta to diethyldithiocarbamate. Experimental myocardial infarction was used as a model of NO overproduction; stroke-prone spontaneously hypertensive rats (SHR-SP) were used as a model of NO shortage. During adaptation to hypoxia, the plasma nitrite/nitrate level progressively increased and was correlated with the increase in NO stores. Adaptation to hypoxia prevented the excessive endothelium-dependent relaxation and hypotension characteristic for myocardial infarction. At the same time, the adaptation attenuated the increase in blood pressure and prevented the impairment of endothelium-dependent relaxation in SHR-SP. The data suggest that NO stores induced by adaptation to hypoxia can either bind excessive NO to protect the organism against NO overproduction or provide a NO reserve to be used in NO deficiency.

Key words

Nitric oxide • Adaptation • Hypoxia • Spontaneously hypertensive rats • Myocardial infarction

Introduction

Nitric oxide (NO) takes part in many processes occurring in the organism. Insufficient or excessive production of NO underlies many serious diseases and pathological states (Marin and Rodriguez-Martinez 1997). This is why attention has recently been increasingly focused on the possibilities of treating and preventing NO-related metabolic disorders. Of special interest are non-pharmacological approaches which differ from pharmacological ones, because they are virtually free from side effects and have considerably less contraindications. These approaches include stepwise adaptation to such environmental factors as stress, exercise, hypoxia, heat, etc. While many protective effects of adaptation to stress or exercise have been shown to be mediated by their stimulating influence on NO synthesis, the effect of adaptation to hypoxia on NO

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metabolism still remains obscure in many respects (Malyshev and Manukhina 1998).

Adaptation to hypoxia is widely used for the prevention and treatment of cardiovascular diseases in experiments and clinics (Meerson 1994). The adaptation possesses many properties which may implicate the important role of NO-dependent mechanisms in adaptive defense. For instance, adaptation to hypoxia inhibits the development of a NO-deficient condition such as hypertension in spontaneously hypertensive rats (Meerson 1984, Behm *et al.* 1986, Obrezchikova *et al.* 1997) and increases endothelium-dependent relaxation of blood vessels (Manukhina *et al.* 1995), although the relation between these events has not been demonstrated. In addition, protective effects of adaptation to hypoxia can be abolished with NO synthase inhibitors and reproduced with NO donors (Malyshev *et al.* 1999). These data have suggested that adaptation to hypoxia should stimulate NO synthesis in the organism. This hypothesis seems to be confirmed by the finding that long-term hypoxia induced the expression of the NO synthase gene (Shaul *et al.* 1995). However, quantitative measurements of NO production have yielded somewhat controversial results (Xue *et al.* 1994, McQuillan *et al.* 1994, Manukhina *et al.* 1995, Shaul *et al.* 1995, Lapshin *et al.* 1995). The reason for such disagreements may be due to intense NO storage in the vascular wall, which could „mask" a portion of NO and make accurate measurement of NO production difficult. It is known that NO can be stored in the form of dinitrosyl iron complexes (DNIC) or S-nitrosothiols and can subsequently be released from these complexes (Vanin 1998). The NO store can be detected using diethyldithiocarbamate (DETC), which reacts with NO-containing complexes to form vasoactive products (Smirin *et al.* 1999).

The aims of the present work were 1) to study the relationship between processes of NO production and storage during adaptation to hypoxia, 2) to demonstrate the protective effects of adaptation to hypoxia in the conditions of NO deficiency and overproduction, and 3) to evaluate the possible role of NO in adaptive defense.

Method

Animal experiments

The study was conducted in conformity with the policies and procedures detailed in the „Guide for the Care and Use of Laboratory Animals".

Wistar male rats (230-250 g) were adapted to hypoxia in an altitude chamber at the air rarefaction corresponding to 5 000 m above sea level. On the 1st day, the adaptation session lasted for 10 min, on the 2nd day for 20 min, on the 3rd day for 30 min, etc., up to 5 h at the 10th session. All the other sessions lasted for 5 h. All sessions of adaptation were carried out daily, in the morning at 20±1 °C. The decompression velocity comprised 40 mm Hg/min. The complete course of adaptation consisted of 40 sessions. Animals were decapitated 24 h after the 3rd, 6th and 40th session of adaptation.

Experimental myocardial infarction induced in Wistar rats by ligation of the left coronary artery (Selye *et al.* 1960) was used as a model of endothelial NO overproduction (Vanin *et al.* 1994). Rats were adapted to hypoxia prior to acute myocardial infarction and taken into the experiment 3 h after the coronary ligation.

Stroke-prone spontaneously hypertensive rats (SHR-SP) (Charles River) at 15-16 weeks old were used as a model of endothelial NO deficiency (Vanhoutte 1996, Wu and Yen 1997). Age-matched Wistar-Kyoto rats (WKY) were used as a control. Adaptation of SHR-SP and WKY was started at 5-6 weeks of age.

Blood pressure was measured in conscious rats using the indirect tail-cuff method by Physiograph DMP-4F (Narco Bio-Systems, USA).

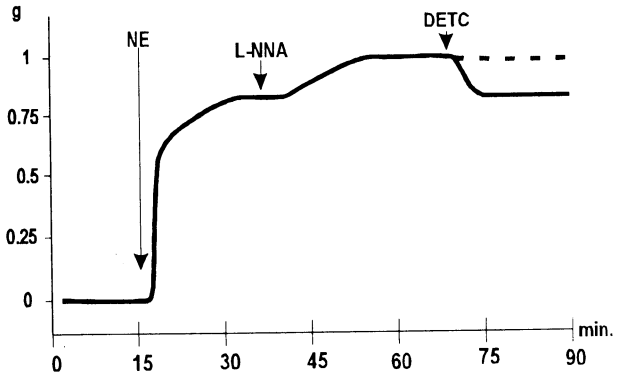


Fig. 1. *Detection of NO store in the isolated rat aorta after adaptation to hypoxia: a typical record. Abscissa: time in min; ordinate: contraction force in g. NE: norepinephrine; L-NNA: N^{ω}-nitro-L-arginine; DETC: diethyldithiocarbamate. Arrows indicate addition of the agents. Dotted line: control; solid line: adaptation.*

Determination of plasma nitrite/nitrate

NO production was evaluated by the total nitrite/nitrate level in the plasma (Moshage *et al.* 1995). Blood was obtained during decapitation using heparin as anticoagulant. Whole blood was centrifuged at 3000 x*g* for 15 min at room temperature and the plasma was deproteinized by adding 5% volume of $ZnSO_4$ (200 g/l) to give a final concentration of 15 g/l and centrifuged again at 3000 x*g* for 15 min. The supernatant was then used for nitrate/nitrite determination. Nitrates were reduced to nitrites using reactors Nitralyzer™ (World Precision Instruments, Inc., USA) in the presence of 0.5M NH_4OH buffer, pH 9.0 (plasma to buffer ratio 9:1). After the reduction, a plasma aliquot was incubated with an equal volume of Griess reagent for 10 min at room temperature. The intensity of developed color was measured spectrophotometrically at 540 nm. Each sample was assayed in duplicate. Values obtained by this procedure represented the sum of nitrite and nitrate. The nitrite/nitrate concentrations were determined by comparison with a calibration curve (0.5-50 μM) of sodium nitrite in distilled water.

Experiments on isolated blood vessels

Immediately after decapitation, the thoracic aorta was excised and the connective tissue was removed. Then the aorta was cut into rings 3.5 mm in length. Two stainless steel wires were inserted into the vascular lumen, and one was connected to an isometric force transducer DY-1 (Ugo Basile, Italy). The ring was suspended in a thermostated (37 °C) chamber containing 30 ml Krebs solution (in mM): 130 NaCl, 11 glucose, 14.9 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, pH 7.4) that was bubbled with a 95 % O_2/5 % CO_2 mixture. The rings were incubated for equilibration at a resting tension of 1.2 g for 60 min, the buffer being exchanged every 30 min during this period.

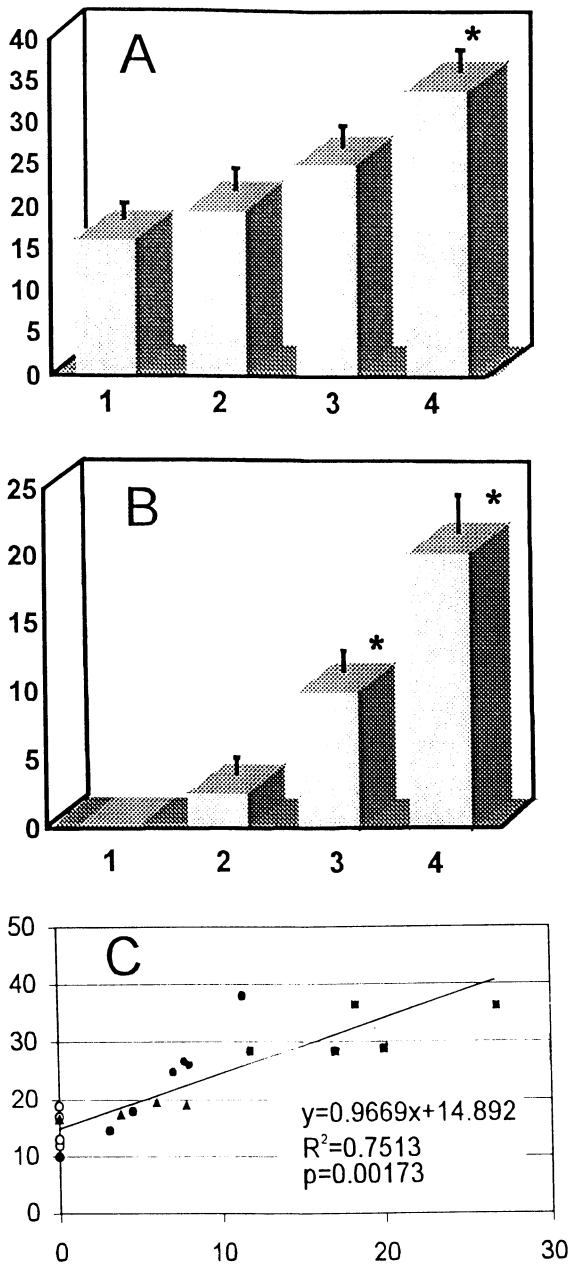


Fig. 2. *Plasma nitrite/nitrate concentration* ***[A]****, formation of NO store in vascular wall* ***[B]*** *and correlation between these two parameters* ***[C]*** *in adaptation to hypoxia.* ***[A]****: Bars show plasma nitrite/nitrate concentration in μM. 1: control; 2: three sessions of adaptation; 3: six sessions of adaptation; 4: 40 sessions of adaptation. *Significant differences (p<0.05) from the control.* ***[B]****: Bars show the magnitude of relaxation of aorta to DETC in percentage of the value of norepinephrine-induced contraction. 1: control; 2: three sessions of adaptation; 3: six sessions of adaptation; 4: 40 sessions of adaptation. * Significant differences (p<0.05) from the first appearance of NO store (three sessions of adaptation.* ***[C]****: Relationship between the size of NO store and the plasma level of nitrite/nitrate. Abscissa: relaxation of the aorta induced by DETC (% of the norepinephrine-induced contraction); ordinate: total nitrite/nitrate concentration (μM). open circles – control, full triangles – three sessions of adaptation, full circles – six sessions of adaptation, full squares – 40 sessions of adaptation.*

The recordings were performed on a Gemini two-channel recorder (Ugo Basile, Italy). The aorta was contracted with norepinephrine (0.5 μM). Endothelium-dependent relaxation was induced by acetylcholine (10^{-8} to 10^{-5} M).

In a separate experiment, the preparation was incubated with the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 1 mM) for 20 min and again contracted with norepinephrine (0.5 μM). After stabilization of the response, DETC (0.3 mM) was added to the chamber. The NO store was calculated as the percentage of DETC-induced relaxation relative to the value of precontraction to norepinephrine plus L-NNA (Fig. 1).

Statistical analysis

The results are presented as means ± S.E.M. from at least 5 experiments. For statistical analysis Student's t test for paired and unpaired observations was used. The regression line for the relation between the size of NO store and the plasma level of nitrite/nitrate was determined by the least-squares method. Values of $p<0.05$ were considered to be significant.

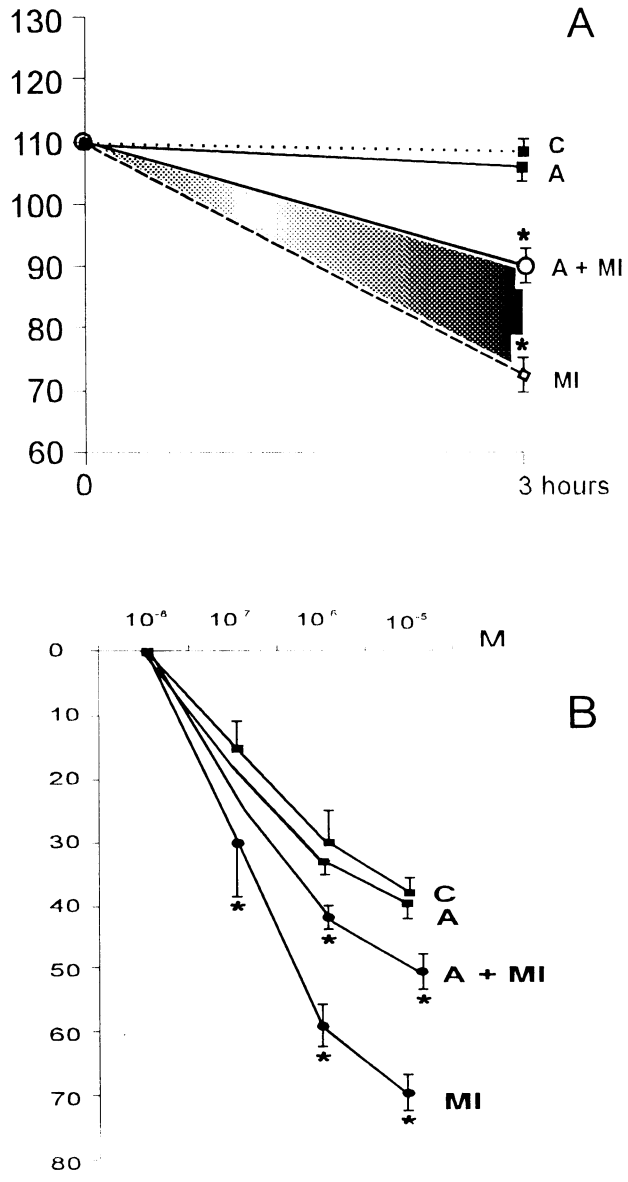
Results

Determination of plasma nitrite/nitrate

Figure 2A demonstrates the changes in the plasma levels of the stable NO metabolites (nitrite/nitrate concentration) in the course of adaptation to hypoxia. It can be seen that adaptation induced a gradual increase in the nitrite/nitrate level which indicated a corresponding potentiation of NO synthesis and/or NO release from the NO stores. After 3 and 6 days of adaptation, a consistent but non-significant increase in nitrite/nitrate level was already observed. By the end of complete adaptation there was a twofold increase in the concentration of NO metabolites as compared to the control (from 18.8±1.7 μM to 32.3±2.0 μM, $p<0.001$).

Evaluation of NO store in adaptation to hypoxia

Figure 2B shows the magnitude of DETC-induced aortal relaxation corresponding to the size of NO store. It can be seen that the adaptation resulted in the accumulation of NO stores in the vascular wall. Control preparations of the aorta isolated from untreated rats did not respond to DETC. This suggests that the NO store is either absent or below the detection limit. The NO store was found in about 50 % of animals as early as after the first three sessions of adaptation. After five adaptation sessions, the NO store was detected in all the rats tested and the NO store size consistently increased with developing adaptation.



Fig, 3. *Effect of prior adaptation to hypoxia on blood pressure of conscious rats* ***[A]*** *and endothelium-dependent relaxation of the isolated rat aorta* ***[B]*** *in acute myocardial infarction.* ***[A]****: abscissa: time after ligation of the coronary artery in hours; ordinate: blood pressure in mm Hg. * Significant difference ($p<0.05$) from the pre-infarction value.* ***[B]****: abscissa: acetylcholine concentration in M; ordinate: endothelium-dependent relaxation in %. * Significant difference ($p<0.05$) from the pre-infarction value. C: control; MI: myocardial infarction; A: adaptation to hypoxia; A+MI: myocardial infarction against the background of adaptation to hypoxia.*

Relation between NO store and plasma nitrite/nitrate

Relation between the size of NO store and the plasma nitrite/nitrate level is shown in Figure 2C. The accumulation of NO store occurred in parallel with the increase in the NO stable metabolites. In all experimental series, the size of the NO store correlated significantly with the plasma nitrite/nitrate concentration.

Protective effects of adaptation to hypoxia in acute myocardial infarction

As can be seen in Figure 3A, blood pressure of the rats fell from 109±1 to 73±2 mm Hg ($p<0.05$) within 3 h after experimental myocardial infarction. Adaptation itself did not influence the blood pressure but significantly restricted the acute hypotension induced by myocardial infarction, because blood pressure fell only to 90±2 mm Hg ($p<0.05$).

Figure 3B demonstrates the endothelium-dependent relaxation of norepinephrine-precontracted rat aorta induced by acetylcholine. The endothelium-dependent relaxation was sharply increased 3 h after coronary ligation as compared to the controls. For instance, the maximum endothelium-dependent relaxation induced by acetylcholine at the concentration of 10^{-6} M comprised 39.0±3.2 % in controls, while it increased to 70.1±4.0 % ($p<0.05$) after myocardial infarction. Adaptation to hypoxia did not significantly affect the endothelium-dependent relaxation but restricted the post-infarction increase in this parameter to 53.0±3.3 % ($p<0.05$).

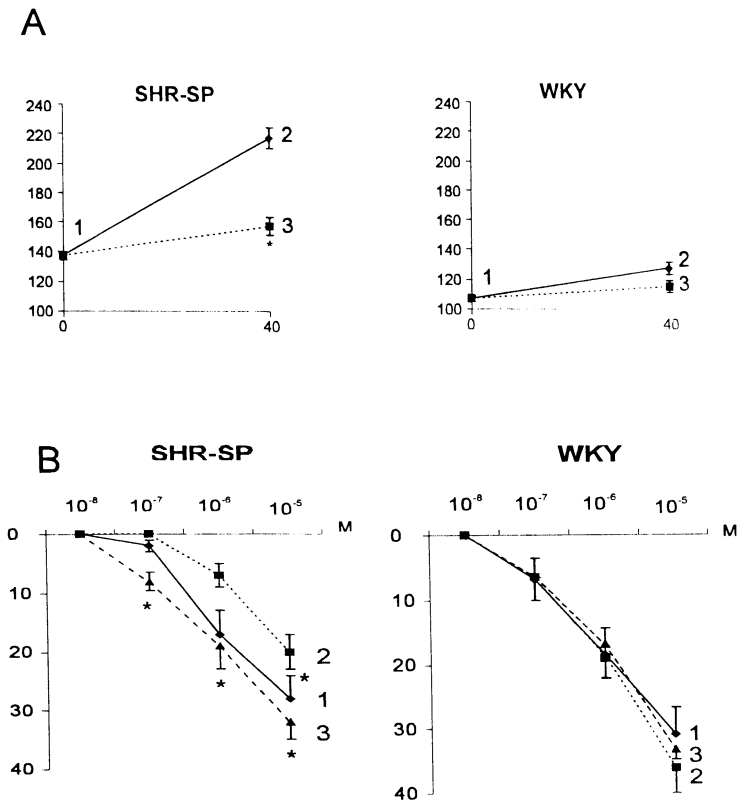


Fig. 4. *Effect of adaptation to hypoxia on blood pressure* ***[A]*** *and endothelium-dependent relaxation of the isolated aorta* ***[B]*** *in stroke-prone spontaneously hypertensive rats (SHR-SP) and Wistar-Kyoto rats (WKY).* ***[A]****: abscissa: duration of adaptation to hypoxia in days; ordinate: blood pressure in mm Hg. * Significant difference ($p<0.05$) from the pre-adaptation value.* ***[B]****: abscissa: acetylcholine concentration in M; ordinate: endothelium-dependent relaxation in %. * Significant difference ($p<0.05$) from the pre-adaptation value. 1 – before adaptation (5-6 weeks old rats), 2 – non-adapted rats, 3 – adaptation to hypoxia.*

Protective effects of adaptation to hypoxia in spontaneous hypertension

In the course of hypertension development, blood pressure of non-adapted SHR-SP increased from 136±3 mm Hg in 5-6 weeks old rats to 216±7 mm Hg in 15-16 weeks old rats ($p<0.05$) (Fig. 4A). Adaptation to hypoxia significantly decelerated the increase in blood pressure in SHR-SP but BP was unaffected in age-matched WKY. It is evident that in 15-16 weeks old SHR-SP adapted to hypoxia, blood pressure increased to 156±4 mm Hg only.

Endothelium-dependent relaxation of the aorta isolated from prehypertensive SHR-SP was already slightly decreased as compared to age-matched WKY (28.0±4.2 % vs. 38.5±5.2 %, respectively, $p>0.05$) (Fig. 4B). Adaptation to hypoxia did not influence the

endothelium-dependent relaxation of the aorta from normotensive rats. At the same time, adaptation of hypertensive rats not only prevented further attenuation of endothelium-dependent relaxation but even normalized this parameter to a considerable extent.

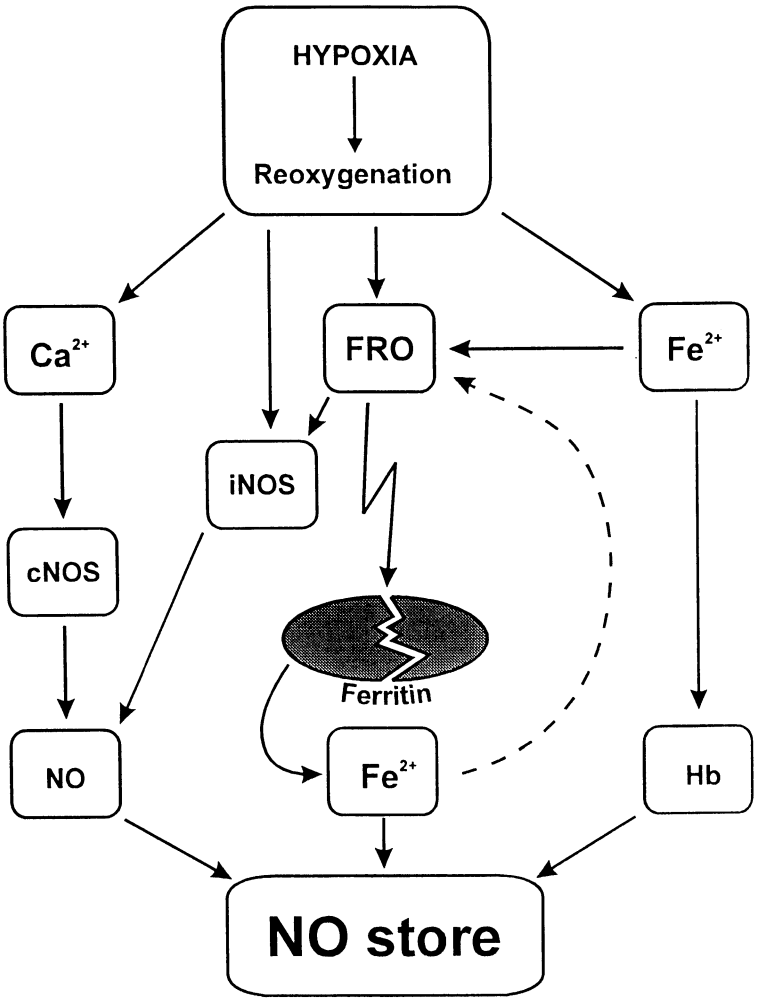


Fig. 5. *Tentative mechanism of increased synthesis and storage of NO in adaptation to hypoxia. See the text for explanations. FRO: free-radical oxidation; iNOS: inducible NO synthase; cNOS: constitutive NO synthase; Hb: hemoglobin.*

Discussion

This study has provided the following principal results: 1) adaptation to intermittent hypobaric hypoxia stimulates NO production in the organism, 2) the excessive NO synthesized in the course of adaptation is stored in the vascular wall, and 3) adaptation to hypoxia prevents both NO overproduction and NO deficiency as well as related disorders of endothelium-dependent relaxation and blood pressure.

The present results indicated that the plasma levels of nitrite/nitrate increased as adaptation to hypoxia developed. The accumulation of nitrite/nitrate indicates enhanced NO synthesis and/or release of additional NO from the NO stores. Available data about the effect of adaptation to hypoxia on NO synthesis are rather controversial, probably due to the use of different models of hypoxic exposure. In the majority of instances, these models represent chronic hypoxia rather than adaptation to hypoxia (Xue *et al.* 1994, Mcquillan *et al.* 1994, Shaul *et al.* 1995). Therefore a comparison of such models with the adaptation to hypoxia used in our study is of doubtful value. The results of physiological studies obtained on the same or a similar model of antihypertensive adaptation (Meerson 1984, Obrezchikova *et al.* 1997) as well as endothelium-stimulating effects of adaptation to

hypoxia (Manukhina *et al.* 1995) indirectly support the idea on the adaptive activation of NO production.

In the present study, we observed the formation of NO stores in the vascular wall already after first few days of adaptation and there was a permanently increasing trend in the course of adaptation. According to current ideas, NO forms a store by incorporation into DNIC with thiol ligands or S-nitrosothiols. These two types of NO-storing complexes can be transformed into each other and both serve for stabilization and transportation of NO to target cells (Vanin 1998). The increase in NO levels stimulates the process of NO storage and it becomes possible to estimate this NO storage from the response of blood vessels to DETC (Smirin *et al.* 1999). The direct relationship between NO production and storage is confirmed by the significant correlation between the plasma nitrite/nitrate level and the size of NO store formed in the vascular wall.

A possible mechanism of the increased synthesis and storage of NO in adaptation to hypoxia is shown in Figure 5. Intermittent short-term hypoxic exposures of moderate intensity are accompanied by transient increases in intracellular Ca^{2+} which activates endothelial NO synthase (Hampl *et al.* 1995). As adaptation develops (approximately after two weeks), the NO synthase gene expression progresses in blood vessels to make the adaptation more reliable and long-lasting (Xue *et al.* 1994). At the same time, hypoxia stimulates the supply of free iron to the organism and hemoglobin formation (Simpson 1992). It is known that iron strongly stimulates NO storage in the form of DNIC (Vanin *et al.* 1997), whereas hemoglobin may also bind NO to its heme and thiol groups form an additional NO store (Jia *et al.* 1996). In addition, every hypoxic session ends with the recovery of normal oxygen supply to the organism. This reoxygenation involves some activation of free-radical forming processes which lead, in particular, to accumulation of superoxide radicals. Superoxide radicals release Fe^{2+} from the iron-containing protein ferritin, and Fe^{2+} serves for the formation of additional DNIC (Lipinski and Drapier 1997). It has been demonstrated that Fe^{2+} not only facilitates NO storage but can also transiently activate the NO synthesis due to the enhancement of free-radical processes (Kubrina *et al.* 1993).

Hence, the adaptation to intermittent hypoxia induces a number of processes in the organism, which may underlie the increased production and storage of NO observed in the present report. Under these conditions, the NO storage should prevent cytotoxic and hypotensive effects of excessive NO. At the same time, the efficient NO storage can offer a physiologically active „reserve" of NO to be used in case of need. If this suggestion is true, then this adaptation to hypoxia should prevent detrimental effects of both NO overproduction and deficiency.

Acute myocardial infarction is associated with NO overproduction in the vascular wall (Vanin *et al.* 1994). The NO overproduction can reduce the vascular tone and result in a fall of blood pressure and excessive endothelium-dependent relaxation of blood vessels. In our experiments, adaptation to hypoxia prevented both the acute hypotension and excessive increase in endothelium-dependent relaxation. Apparently, prior stimulation of NO synthesis is precisely the protective factor that is involved in the conditions of acute myocardial infarction. Such protection may be based on a negative feedback mechanism which provides restriction of excessive NO synthase activity by NO itself (Assreuy *et al.* 1993).

It is known that both activity and expression of endothelial NO synthase are decreased in spontaneously hypertensive rats, though there also exist conflicting opinions (Vanhoutte 1996, Wu and Yen 1997). It is believed that the endothelial dysfunction observed in hypertensive blood vessels is a consequence rather than the cause of the pathological process (Vanhoutte 1996). Indeed, in arteries of young SHR, the relaxation to acetylcholine is similar (Radaelli *et al.* 1998) or even increased (Onda *et al.* 1994) as compared to blood vessels from young normotensive controls, but it becomes progressively impaired with increasing blood pressure (Chou *et al.* 1998). In this study, we observed a non-significant trend to decreased endothelium-dependent relaxation of the isolated aorta from prehypertensive SHR-SP. This decrease became more pronounced in 15-16 weeks old SRH-SP. Adaptation to hypoxia has long been known to slow down the development of hypertension (Meerson 1984, Behm *et al.* 1986, Obrezchikova *et al.* 1997) but we have demonstrated for the first time that this protection is associated with normalization of the endothelium-dependent relaxation of blood vessels. This observation could be useful for clinical practice because antihypertensive therapy in hypertensive patients does not always restore a normal endothelium-dependent vasodilator response (Panza *et al.* 1993).

Therefore, adaptation to hypobaric hypoxia exerts protective effects under the conditions of both NO overproduction and deficiency. In either case, the

adaptive defense is associated with increased NO synthesis and storage. The NO storage is apparently an adaptive response which provides protection against the detrimental effects of excessive NO at the expense of NO binding to the stores during both pathological NO overproduction and adaptive stimulation of NO synthesis. At the same time, the NO release from the stores can compensate for the NO deficiency and prevent the NO overproduction by a negative feedback mechanism. Further study of NO-dependent mechanisms of adaptation would allow to use adaptation for prevention and treatment of pathologies related to NO excess or deficiency.

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