ESR Techniques for the Detection of Nitric Oxide in Vivo and in Tissues

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Abstract: Plasma levels of nitrite/nitrate may not accurately reflect endothelial nitric oxide synthase (eNOS) function due to interference by dietary nitrates. Nitrosyl hemoglobin (HbNO), a metabolic product of nitric oxide ($NO^{\bullet}$), may better correlate with bioavailable $NO^{\bullet}$ but it may depend on the activity of different nitric oxide synthase (NOS) isoforms and may be affected by dietary nitrite/nitrate. This work examined the correlation between vascular endothelial $NO^{\bullet}$ release and blood levels of HbNO. We measured HbNO in mouse blood using electron spin resonance (ESR) spectrometry and also quantified vascular production of $NO^{\bullet}$ using colloid $Fe(DETC)_2$ and ESR. C57Blk/6 mice who were fed a high-nitrate diet, had levels of plasma HbNO increased 10-fold, while those fed a low-nitrate diet had decreased HbNO levels from 0.58±0.02 to 0.48±0.01 μM. Therefore, a low-nitrate diet is essential when using HbNO as a marker of eNOS activity. Treatment with L-NAME and the eNOS specific inhibitor, L-NIO, halved HbNO formation, which reflects the complete inhibition of $NO^{\bullet}$ release by aorta endothelium. Treatment of mice with the selective inducible nitric oxide synthase (iNOS) inhibitor, 1400W, or the selective neuronal nitric oxide synthase (nNOS) inhibitor, N-AANG, did not alter either blood HbNO levels or vascular $NO^{\bullet}$. The relationship between HbNO and $NO^{\bullet}$ production by the endothelium (0.23 μM HbNO to 5.27 μM/hour of $NO^{\bullet}$ per mg dry weight aorta) was found to be identical for both C57Blk/6 mice and for mice with vascular smooth muscle-targeted expression of p22phox associated with strong increase in eNOS activity. These results support the important role of eNOS in the formation of circulating HbNO, while iNOS and nNOS do not contribute to HbNO formation under normal conditions. These data suggest that HbNO can be used as a noninvasive marker of endothelial $NO^{\bullet}$ production in vivo.

I. Introduction

In the past decade, a growing interest in nitric oxide ($NO^{\bullet}$) called for reliable and sensitive techniques for its quantification both in vitro and in vivo. Developing the in vivo technique was the most challenging demand. Most in vivo studies investigating endothelium function relied on nitrite/nitrate measurements in blood plasma (Minamino et al., 1997). Nitrite and nitrate are metabolic products of $NO^{\bullet}$ and are used to quantify $NO^{\bullet}$ by biotransformation of nitrates (Salvemini *et al*., 1992; Hasegawa *et al*., 1999; Minamiyama *et al*., 1999). Unfortunately, nitrite/nitrate plasma levels are strongly affected by the dietary consumption of nitrite/nitrate, which is difficult to minimize even in laboratory conditions. Moreover, the nitrite/nitrate level does not reflect bioactive amount of $NO^{\bullet}$ since the inactivation of $NO^{\bullet}$ by superoxide and other oxidants leads to the formation of nitrite/nitrate (Pfeiffer *et al*., 1997). A method for in vivo $NO^{\bullet}$ detection based on the formation of nitrosyl hemoglobin (HbNO) in the reaction of deoxyhemoglobin (Hb) with $NO^{\bullet}$ has been described (Hall *et al*., 1996). Therefore, to overcome the limitations of nitrite/nitrate assay, we used electron spin resonance (ESR) to analyze HbNO in frozen blood (Landmesser *et al*., 2003; Jaszewski *et al*., 2003).

The process of $NO^{\bullet}$ transfer into erythrocytes is of critical biological importance because it controls plasma $NO^{\bullet}$ bioavailability and diffusional distance of endothelial-derived $NO^{\bullet}$**.** It has been suggested that $NO^{\bullet}$ under physiological conditions it is consumed rather than conserved by reaction with oxyhemoglobin (Joshi *et al.,* 2002*).* Indeed, rate constants imply that most of the $NO^{\bullet}$ will react with oxyhemoglobin-producing nitrate and methemoglobin (Scheme 1), while only a minor fraction will form HbNO detectable by ESR (Henry et al., 1997). Our experiments with mouse blood show that only 10% of $NO^{\bullet}$ forms HbNO while most of $NO^{\bullet}$ is oxidized to nitrite.

$NO^{\bullet}$ is a well-known ligand of deoxygenated hemoglobin (Keilin *et al*., 1937). Paramagnetic properties of HbNO have been previously studied with the use of ESR (Ingram *et al*., 1955) and recent in vitro and in vivo ESR studies demonstrated the presence of HbNO in vivo during inflammation, drug metabolism, and treatment with statins (Kosaka *et al*., 1994; Takahashi *et al*., 1998; Glover *et al*., 1999; Ongini *et al*., 2004). The lifetime of HbNO ranges from 12 minutes to 20 hours with an average lifetime of 4 hours, therefore, it can be accumulated in the blood in substantial concentrations (Henry *et al*., 1997). While an electrochemical assay for measurement of HbNO was recently developed (Palmerini *et al*., 2004), ESR remains the most direct and unambiguous method for HbNO measurement. Development of high-sensitivity ESR spectrometers has made possible the quantification of 5-coordinate HbNO

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in blood (Hall et al., 1996; Jaszewski *et al*., 2003). A growing body of evidence supports the idea that HbNO can be used as a marker of bioavailable $NO^{\bullet}$ in vivo. ESR analysis of 5-coordinate HbNO permits the highly specific detection of low levels of HbNO during hypertension and other diseases (Glover *et al*., 1999, Datta *et al*., 2004).

$NO^{\bullet}$ production in vivo can also be analyzed ex vivo in various tissues. Previously, $NO^{\bullet}$ production in rabbit and mice aorta has been measured by colloid iron diethyldithiocarbamate, $Fe(DETC)_2$ and ESR spectroscopy (Kleschyov *et al*., 2002; Khoo *et al*., 2004). This chapter details the ESR techniques for measuring blood HbNO and $NO^{\bullet}$ production in tissues with colloid $Fe(DETC)_2$. It shows the association of blood HbNO with $NO^{\bullet}$ production in the tissue.

II. Materials and Procedures

$FeSO_4$, DETC, and A23187 were obtained from Sigma-Aldrich (St. Louis, MO). L-NAME, L-NIO, 1400W, and N-AANG were purchased from EMD Biosciences (San Diego, CA). The modified Krebs-HEPES buffer (KHB) for vessel studies was composed of 99.01 mmol/liter NaCl, 4.69 mmol/liter KCl, 2.50 mmol/liter $CaCl_2$, 1.20 mmol/liter $MgSO_4$, 25 mmol/liter $NaHCO_3$, 1.03 mmol/liter K_2HPO_4, 20 mmol/liter Na-HEPES, and 5.6 mmol/liter D-glucose, pH 7.35. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) in the highest grade available.

Animals: C57Blk/6 (wild-type) and eNOS KO mice were obtained from Jackson Laboratories (Bar Harbor, ME). Studies were performed on 12- to 18-week-old male mice. Mice overexpressing the NADPH oxidase $p22^{phox}$ subunit in vascular smooth muscle cells (VSMC) (Tg^{p22smc} mice) were created by cloning the $p22^{phox}$ cDNA downstream of these cells (K. Laude et al., 2004). Investigation of in vivo effects of selective eNOS, iNOS, and nNOS inhibitors on HbNO formation and $NO^{\bullet}$ production in the aorta of C57Blk/6, $p22^{phox}$ overexpressed and eNOS KO mice were performed using subcutaneously injection of L-NIO (L-N^5-(1-Imunoethyl)ornitine), 1400W (N-(3-aminomethyl)benzylacetamidine), or N-AANG ((4S)-N-(4-Amino-5[aminoethyl]aminopentyl)-N′-nitroguanidine) (1 mg/kg in 09.% NaCl) twice a day. On the day of study, mice were injected with 100 U heparin/25 g BW i.p. 5 minutes before euthanization with CO_2. The renal and iliac arteries were cut, and the aorta was flushed gently two times with 1 ml of cold KHB. The aorta was rapidly removed and dissected free of adherent tissues. During preparation, the vessels were maintained in chilled KHB (6°C) using a thermo-stabilized cold plate (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany).

Preparation of blood for measurements of HbNO: Heparinized blood (0.7 ml) was collected by a 26G needle from the right ventricle of the CO_2 euthanized mice. The blood was then frozen in liquid nitrogen in 1 ml syringes and kept at –80 ºC before measurements. The frozen blood was transferred from syringes into ESR dewar flasks filled with liquid nitrogen. ESR measurements of HbNO were obtained at the temperature of liquid nitrogen (77 ºK). The amount of detected $NO^{\bullet}$ was determined from the calibration curve for intensity of the ESR signal of erythrocytes treated with known concentrations of nitrite (1-25 μM) and $Na_2S_2O_4$ (20 mM). Erythrocytes were prepared by immediate centrifugation at 3,000 rpm for 5 minutes. Erythrocytes were separated from plasma and resuspended in deoxygenated PBS buffer.

The three-line hyperfine spectrum of the 5-coordinate complex of $NO^{\bullet}$ with hemoglobin (Hall et al., 1996; Jaszewski *et al*., 2003) was recorded with an X-band EMX ESR spectrometer (Bruker Instruments Inc., Billerica, Massachusetts, USA) using a high sensitivity SHQ microwave cavity in finger dewar filled with liquid nitrogen. ESR spectrometer settings were as follows: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; field center, 3320 G; sweep width, 320 G; microwave frequency, 9.39 GHz; conversion time, 655 milliseconds; time constant, 5.24 seconds; number of scans, 2; sweep time, 336 seconds.

Preparation of colloid $Fe(DETC)_2$: To prepare 0.8 mM $Fe^{2+}(DETC)_2$ stock solution, DETC (7.2 mg) and $FeSO_4$ $7H_2O$ (4.45 mg) were separately dissolved under nitrogen flow in two volumes (10 ml) of filtered and deoxygenated ice-cold 0.9% NaCl. A cold solution of DETC was mixed with $FeSO_4$ in the ratio of 1:1 in either a microcentrifuge tube (1.5 ml) without air bubbles or in a 10 ml plastic tube in the nitrogen flow. The formed 0.8 mM $Fe(DETC)_2$ colloid solution was yellow-brown in color and was used immediately after preparation.

Incubation of aorta with colloid $Fe(DETC)_2$: Blood vessels were carefully and thoroughly cleaned of adhering fat and cut into 2-mm rings. An ice-cold KHB was used for cleaning and storing vessels. Four aortic rings were placed in 1.5 ml of KHB on a 12-well plate. Incubation of aortic rings was started by the addition of 0.5 ml colloid $Fe(DETC)_2$ to tissue samples with 1.5 ml KHB in each well. Samples can be treated with the various stimuli for $NO^{\bullet}$ production (H_2O_2, A23187) before addition of $Fe(DETC)_2$. In some studies, aortic segments were preincubated with PEG-SOD (100 U/ml), PEG-catalase (100 U/ml) for 4 hours, or tetrahydrobiopterin (20 □M), apocynin (50 □M) for 15 min before treatment with $Fe(DETC)_2$. Aortic segments of vessels were incubated at 37 °C for 60 minutes in the presence of 200 □M colloid $Fe(DETC)_2$. Due to its high lipophilicity, the formed NO-$Fe(DETC)_2$ complex was exclusively localized in the vascular tissue and not in the medium. The vessels were then placed in the center of a 1-ml syringe with 0.6 ml KHB and snap-frozen in the liquid nitrogen. Samples were stored for up to six months at -80°C before ESR analysis. The syringe was briefly rubbed between the palms and frozen sample column was removed from the syringe by gentle push from the slightly warmed plastic. Sample was chilled with liquid nitrogen and loaded into a finger dewar, fixed by cotton ball, filled with liquid nitrogen on the top and then analyzed with a Bruker EMX ESR spectrometer. The ESR spectrometer settings were as follows: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; field center, 3290 G; sweep width, 90 G; microwave frequency, 9.39 GHz; conversion time, 328 milliseconds; time constant, 5.24 seconds; number of scans, 4; sweep time,

168 seconds. The amount of detected $NO^{\bullet}$ was determined from the calibration curve for integral intensity of the ESR signal of NO-$Fe^{2+}(MGD)_2$ prepared at various concentrations (1-20 μM) of the NO-donor MAHMA-NONOate (Morley *et al*., 1993).

III. Results and Discussion

Electron spin resonance analysis of HbNO: Nitrosyl hemoglobin (HbNO) is stable and can be measured in the blood by ESR (Fig. 1). The ESR spectrum of mouse blood consists of three major components: ceruloplasmin, free radical component and nitrosyl hemoglobin (Fig. 1). The amount of HbNO was quantified after subtracting ceruloplasmin and the free radical components from the ESR spectrum of the blood (Fig. 1D). HbNO can be analyzed either in the blood (Fig. 1A-D) or in the isolated erythrocytes (Fig. 1E). Unlike other **$NO^{\bullet}$** assays, the formation of HbNO is proportional to the bioavailable amount of $NO^{\bullet}$ (Ongini *et al*., 2004). This allowed us to study the effect of hypertension on HbNO content in the red blood cells, showing significant decrease in HbNO in DOCA-salt treated mice compared with control (Landmesser *et al*., 2003).

ESR spectroscopy provides convenient quantification of HbNO by double integration of ESR spectrum or by analysis of ESR amplitude (Fig. 1E), which has an advantage due to better resolution of HbNO even in the ESR spectrum of the blood (Fig. 1A,D). Quantification of HbNO demonstrates that only 10% of $NO^{\bullet}$ produces HbNO in the aerobic samples (Fig. 1E). Therefore, HbNO calibration probes must be prepared in air-free blood treated with $Na_2S_2O_4$.

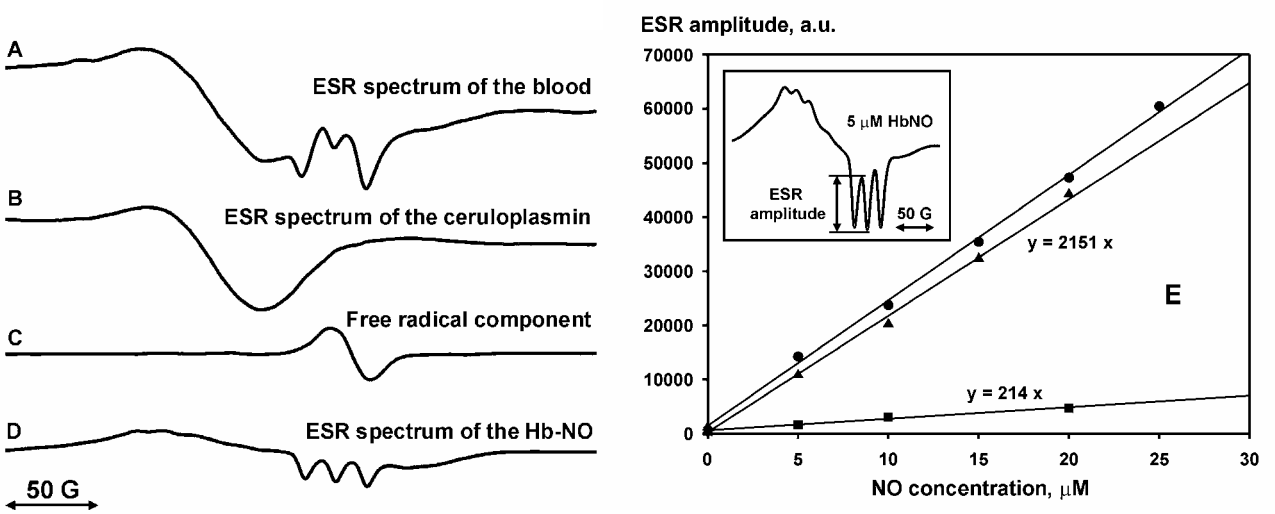


Figure 1. Measurements of nitrosyl hemoglobin (HbNO) in mouse blood by subtraction of ESR spectra of ceruloplasmin and the free radical component from the ESR spectrum of the blood (A-D). Calibration curve for HbNO (E). Insert shows ESR spectrum of HbNO in erythrocytes. ESR signal HbNO was determined after incubation of washed erythrocytes with nitrite (•), MAHMA NONOate (▲) in the presence of 20 mM of $Na_2S_2O_4$ or in the absence of $Na_2S_2O_4$ (■).

Effect of dietary supplementation of nitrates: It has been reported that nitrite can be reduced to nitric oxide by deoxyhemoglobin and cause vasodilatation in the human circulation under hypoxic condition (Cosby et al., 2003). Various intracellular compartments, such as endoplasmic reticulum, and mitochondria are also involved in bioconversion of nitrates (Kozlov *et al*., 2003). Therefore, we tested the effect of dietary supplementation of nitrate with the drinking water (100 mg/l) of animals fed by conventional labor diet #5001 containing 33 mg/kg nitrate and 5 mg/kg nitrite, compared with basic low nitrate/nitrite diet containing 12 mg/kg nitrate and 3 mg/kg nitrite. Our results demonstrated a significant 20% decrease (from 0.58±0.02 to 0.48±0.01 μM) in HbNO in animals fed the low-nitrite diet, while supplementation with the nitrate in the drinking water led to a 10-fold increase in HbNO content (Fig. 2A). These data suggest minimal interference of plasma nitrate with HbNO formation; however, a controlled low nitrate/nitrite diet was very useful for consistent HbNO data (Fig. 2B).

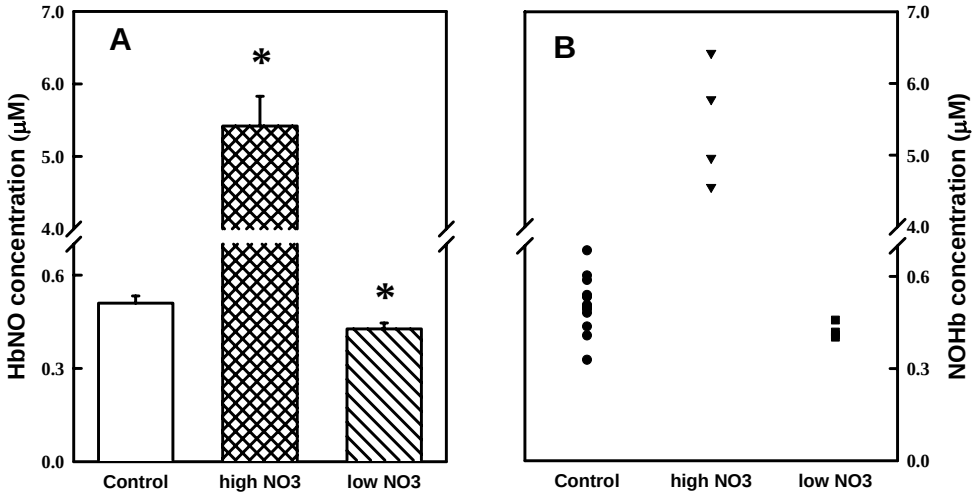


Figure. 2. Influence of dietary nitrite/nitrate on HbNO formation in mice. C57Blk/6 mice were investigated after feeding with a conventional diet containing 33 mg/kg nitrate and 5 mg/kg nitrite (Control), with 100mg/L nitrate in the drinking water (high NO_3), or with a basic low nitrite/nitrate diet containing 12 mg/kg nitrate and 3 mg/kg nitrite (low NO_3). Data presented as mean ± SEM (A) and as scatter plot (B).

Detection of NO in aorta: Colloid $Fe(DETC)_2$ has been recently used to study $NO^\bullet$ production in mouse aorta (Khoo *et al*., 2004; K. Laude et al., 2004). Similar to HbNO, $Fe(DETC)_2$ detects only bioactive $NO^\bullet$, does not interfere with nitrite/nitrate, and does not inhibit vascular Cu/Zn SOD (Kleschyov *et al*., 2002). The NO-$Fe(DETC)_2$ was found to be exclusively associated with blood vessels, suggesting a "one-way delivery" of the trap into the cellular hydrophobic compartments. A time-dependent linear increase of the ESR signal was observed during two-hour incubation, which implies high availability of $Fe(DETC)_2$ and stability of NO-$Fe(DETC)_2$ complex.

Stimulation of the vascular tissue with Ca-ionophore (A-23187) sharply increased $NO^\bullet$ production in the aorta, showing characteristic triplet EPR signal (g=2.035; A_N=12.6 G) of NO-$Fe(DETC)_2$ (Fig. 3A,B). Treatment of the mice with the irreversible NOS inhibitor, L-NAME, or the addition of L-NAME in vitro completely blocked $NO^\bullet$ production (Fig. 3 C,D). The use of a highly selective reversible eNOS inhibitor L-NIO led to incomplete inhibition of $NO^\bullet$ production (Fig. 3E) in the aorta due to the washing out of L-NIO from the tissue during aorta preparation. Indeed, in vitro addition of L-NIO completely blocked $NO^\bullet$ production (Fig. 3 F). Therefore, we used in vitro supplementation of reversible NOS inhibitors (L-NIO, N-AANG, 1400W) in the concentration of 10 μM to test their effects on aorta $NO^\bullet$ production.

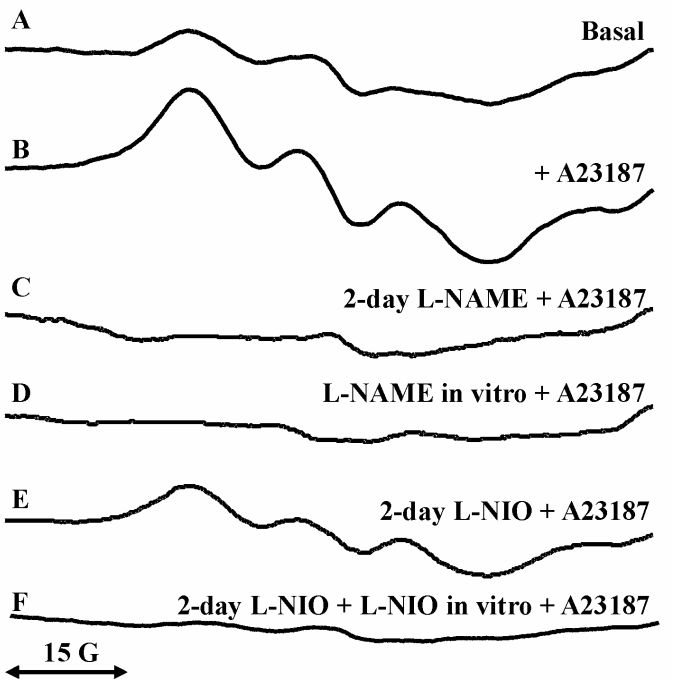


Figure 3. ESR spectra of NO-$Fe(DETC)_2$ in aortic segments of C57Blk/6 mice under non-stimulated (basal) conditions (A); after stimulation with 10 μM A-23187 (B); in vivo treatment of C57Blk/6 mice with L-NAME (100mg/L in drinking water) and additional stimulation with A-23187 (C); following in vitro treatment with 100 μM L-NAME and stimulation with A-23187 (D); after a two-day treatment with selective eNOS inhibitor L-NIO and stimulated with A-23187 (E); after additional pretreatment with 10 μM L-NIO (F).

***Association of blood HbNO with vascular $NO^\bullet$ production*:** To determine the association of circulating HbNO with the production of $NO^\bullet$ in endothelium, we treated the mice with either L-NAME or with the eNOS specific inhibitor, L-NIO, and measured HbNO concentration in the blood and $NO^\bullet$ production in the aorta endothelium. Of note, IC_{50} for targeted enzyme can be a thousand-fold

lower than IC_{50} for nontargeted enzyme. For example, 1400W inhibits iNOS with K_d = 7nM, which is 5000-fold lower than one of eNOS (Parmentier *et al*., 1999). This treatment decreased HbNO formation by half (Fig. 4A). Furthermore, both L-NAME and L-NIO completely inhibited the release of NO• by aorta endothelium (Fig. 4B). The treatment with selective iNOS inhibitor 1400W or selective nNOS inhibitor N-AANG did not alter either blood HbNO levels or vascular NO• production (Fig. 4A,B).

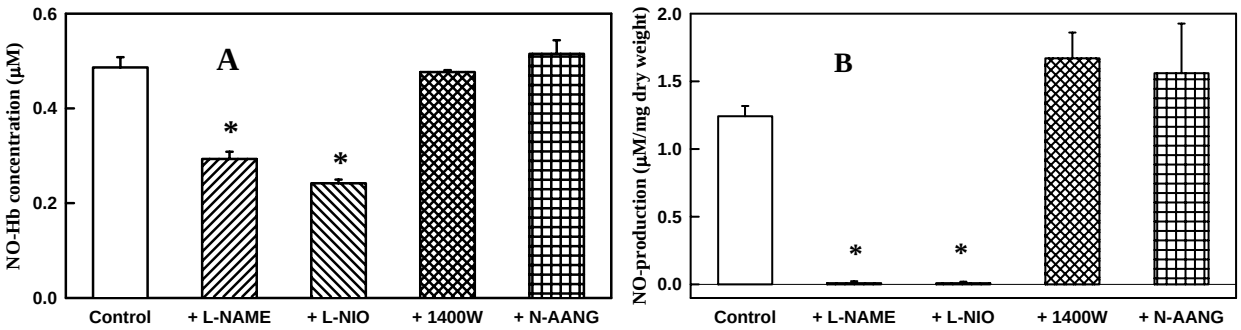


Figure 4. (A) HbNO concentration in the blood of C57 Blk/6 mice after a two-day treatment (s.c. 1mg/kg, twice a day) with highly selective inhibitors of eNOS – L-NIO, nNOS – N-AANG, iNOS – 1400W, or with a nonselective inhibitor of NOS isoforms – L-NAME (100 mg/L in drinking water); (B) NO• production in aortic sections of the same animals.

Finally, we determined the relationship between HbNO and endothelial NO• production as a ratio of L-NAME inhibitable HbNO (Fig. 5A) to NO• production in aorta (Fig. 5B). This ratio (0.23μM HbNO to 5.37 μM/h of NO• per mg dry weight aorta) was found to be identical for both C57Blk/6 mice and for mice with a vascular smooth muscle-targeted expression of p22phox (Khatri *et al*., 2004) in which vascular NO• production was markedly increased due to H_2O_2-mediated increase in eNOS expression (Laude *et al*., 2004).

Of note, the HbNO level in eNOS KO mice was similar to the C57 Blk/6 mice (Fig. 5A). However, the HbNO in eNOS KO mice was inhibited by the iNOS inhibitor 1400W which did not affect HbNO in the C57 Blk/6 mice (Fig. 4, 5). These data imply that eNOS KO mice have increased activity of iNOS, making iNOS a source of HbNO which is likely a compensatory effect of the lose of eNOS in this transgenic mice. Our results suggest that HbNO can be used not only as an index of in vivo NO• production by eNOS under normal conditions but also as an evidence of increased activity of iNOS under inflammation.

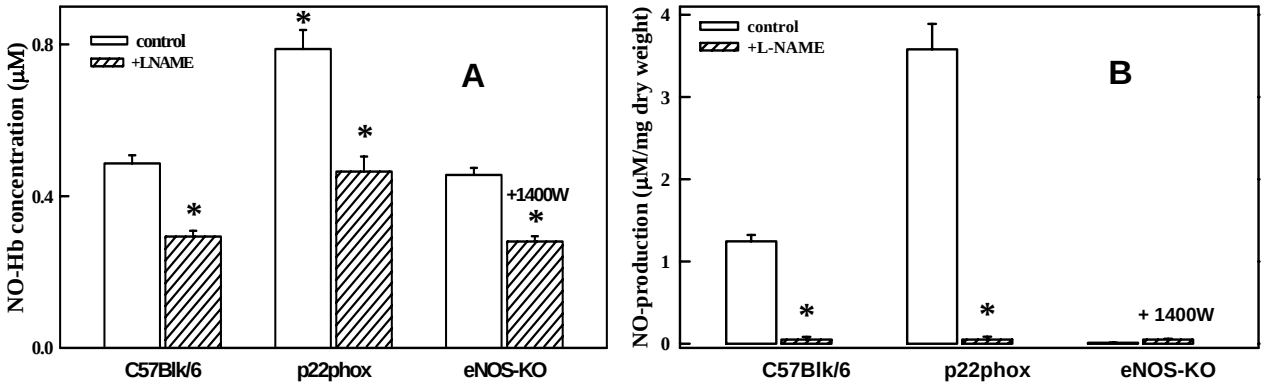
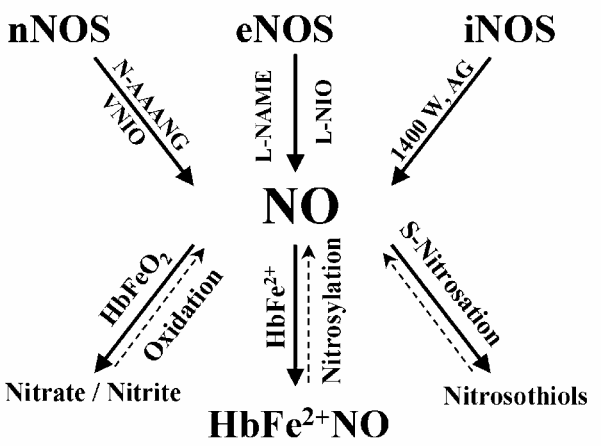


Figure 5. HbNO concentration in blood (A) and NO• production in aortic sections (B) of C57 Blk/6, p22phox or eNOS KO mice after a two-day treatment with NOS inhibitor L-NAME or with iNOS inhibitor 1400W.

***Pathways for HbNO formation*:** Our results describe the role of eNOS and iNOS in the HbNO formation. Preliminary data suggest that expression of nNOS in the vasculature of transgenic mice may significantly contribute to HbNO production. nNOS and iNOS are not significant sources of NO• in the vasculature under normal physiological condition. Inflammation, however, does greatly increases NO• production by iNOS, which may result in a dramatic increase in HbNO (Kozlov *et al*., 2003b).

Our experiments showed that NOS inhibitors do not completely block HbNO formation. It is possible that a part of the residual amount of HbNO can be derived from either endogenous or exogenous of nitrite/nitrate (low-nitrite diet had 12 mg/kg nitrate and 3

mg/kg nitrite). Alternatively, NOS inhibition may increase in vivo reduction of exogenous and endogenous nitrite to NO•, which was recently demonstrated in hypoxic tissues (Gladwin *et al*., 2004). In our analysis we considered the residual amount of HbNO as a NOS independent background, which was the same in both the C57 Blk/6 and eNOS KO mice (Fig. 5A).



Scheme 1. Possible pathways for HbNO formation.

IV. Conclusion

It is clear that HbNO is strongly associated with vascular NO• production. Our results support an important role of eNOS in the formation of HbNO. Under normal physiological conditions iNOS and nNOS do not contribute to HbNO formation. Although a low nitrate diet is essential for analysis of HbNO, the fact that HbNO reflects bioavailable levels of vascular NO• makes HbNO useful as a noninvasive marker of endothelial function.

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