Evaluation of systemic blood NO dynamics by EPR spectroscopy: HbNO as an endogenous index of NO

Kazuyoshi Kirima,[1] Koichiro Tsuchiya,[1] Hiroyoshi Sei,[2] Toyoshi Hasegawa,[1] Michiyo Shikishima,[1] Yuki Motobayashi,[1] Kyoji Morita,[1] Masanori Yoshizumi,[1] and Toshiaki Tamaki[1]
Departments of [1]Pharmacology and [2]Integrative Physiology, The University of Tokushima School of Medicine, Tokushima 770-8503, Japan

Kirima, Kazuyoshi, Koichiro Tsuchiya, Hiroyoshi Sei, Toyoshi Hasegawa, Michiyo Shikishima, Yuki Motobayashi, Kyoji Morita, Masanori Yoshizumi, and Toshiaki Tamaki. Evaluation of systemic blood NO dynamics by EPR spectroscopy: HbNO as an endogenous index of NO. *Am J Physiol Heart Circ Physiol* 285: H589–H596, 2003. First published March 27, 2003; 10.1152/ajpheart.01010. 2002.—The measurement of hemoglobin-nitric oxide (NO) adduct (HbNO) in whole blood by the electron paramagnetic resonance (EPR) method seems relevant for the assessment of systemic NO levels. However, ceruloplasmin and unknown radical species overlap the same magnetic field as that of HbNO. To reveal the EPR spectrum of HbNO, we then introduced the EPR signal subtraction method, which is based on the computer-assisted subtraction of the digitized EPR spectrum of HbNO-depleted blood from that of sample blood using the software. Rats were treated with N^ω-nitro-L-arginine methyl ester (L-NAME; 120 mg·kg^{-1}·day^{-1}) for 1 wk to obtain HbNO-depleted blood. When this method was applied to the analysis of untreated fresh whole blood, the five-coordinate state of HbNO was observed. HbNO concentration in pentobarbital-anesthetized rats was augmented (change in [HbNO] = 1.6–5.5 μM) by infusion of L-arginine (0.2–0.6 g/kg) but not D-arginine. Using this method, we attempted to evaluate the effects of temocapril on HbNO dynamics in an L-NAME-induced rat endothelial dysfunction model. The oral administration of L-NAME for 2 wk induced a serious hypertension, and the HbNO concentration was reduced (change in [HbNO] = 5.7 μM). Coadministration of temocapril dose dependently improved both changes in blood pressure and the systemic HbNO concentration. In this study, we succeeded in measuring the blood HbNO level as an index of NO by the EPR HbNO signal subtraction method. We also demonstrated that temocapril improves abnormalities of NO dynamics in L-NAME-induced endothelial dysfunction rats using the EPR HbNO signal subtraction method.

nitric oxide; hemoglobin-nitric oxide adduct; electron paramagnetic resonance; temocapril

NITRIC OXIDE (NO), a free radical molecule, has numerous roles in various physiological functions, such as the regulation of the cardiovascular, immune, and nervous systems. An imbalance of NO results in many diseases such as hypertension, atherosclerosis, septic and hemorrhage shock, ischemia-reperfusion injury, diabetes, and neurodegenerative disorders (18, 40, 70).

Because of increasing interest in the effect of NO, an accurate method is required for the measurement of this radical. Several methods for the quantitation of NO such as chemiluminescence (46), methemoglobin formation (23), and electron paramagnetic resonance (EPR) spectroscopy of nitrosyl-metal complexes (15) have been developed (3). The Griess and cGMP methods have been widely used to measure NO production in vivo (35, 62) and in vitro (34, 61), but these values do not always reflect the concentration of NO, because of their poor specificities for blood NO. EPR spectroscopy is a technique used for the detection and measurement of free radical species. EPR methods using endogenous and exogenous spin-trapping agents, which stabilize NO as a polyatomic spin adduct, have been developed (1, 4, 29, 41, 72).

In the circulation, NO exists as relatively stable hemoglobin (Hb)-NO adduct (HbNO) (48), which means that the amount of HbNO may reflect the blood NO concentration.

Hb is known to bind to NO, resulting in the formation of HbNO, which has a characteristic EPR spectrum, and hence is used as a spin-trapping agent for NO in lipopolysaccharide (LPS)-treated (67, 69) and NO donor-administered (10, 28) animals.

In addition, HbNO signal has been reported to be successfully detected in untreated animals and human blood using the EPR method (39, 51, 55, 68). However, there were still some difficulties in obtaining a fine HbNO signal, because of existing of paramagnetic compounds that give a strong EPR signal overlapping the same region to HbNO (5, 7, 10, 13, 19, 42). In the present study, we developed an improved method to detect this HbNO signal in whole blood by EPR spectroscopy. To overcome the difficulties mentioned above, we introduced the EPR signal subtraction method, which is based on the subtraction of the EPR spectrum

of HbNO-depleted whole blood from that of each sample. In addition, changes of blood NO levels induced by angiotensin-converting enzyme inhibitor (ACEI) treatment were assessed to confirm the relevance of this method.

MATERIALS AND METHODS

Materials

L-Arginine hydrochloride (L-arginine) and D-arginine hydrochloride (D-arginine) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). N^ω-nitro-L-arginine methyl ester (L-NAME) was from Nacalai Tesque (Kyoto, Japan). Temocapril was kindly provided by Sankyo (Tokyo, Japan). Other reagents were of the highest grade available from Wako Pure Chemical Industries. NO gas was obtained commercially from Sumitomo Seika Chemicals (Osaka, Japan), and higher oxides such as NO_2 and NO_3 (NO_x) were removed by passing through a trap containing 1 M KOH. An NO-saturated aqueous solution was prepared by bubbling NO gas for 15 min through water that had been previously deoxygenated by bubbling with purified argon gas for 30 min (32). Temocapril (0.5 or 5 mg/ml) was suspended in carboxyl methyl cellulose (0.5%) and orally given to the animals.

Animals

Male Sprague-Dawley rats (12 wk old) were obtained from Japan SLC (Shizuoka, Japan) and kept in plastic cages at a controlled temperature (25°C) under controlled lighting condition (12:12-h light-dark cycle). The animals were fed a commercial diet and had access to tap water ad libitum until the day of the experiments. All animal care and treatments were conducted in accordance with the guidelines of the animal use and care committee of the University of Tokushima.

Animal Experiments

Protocol 1: preparation of HbNO for calibration curves. Rats were anesthetized with pentobarbital sodium (40 mg/kg body wt ip). Venous blood was collected from the vena cava, and aliquots (1 ml) were mixed with various amounts of the NO-saturated solution at 0°C. Immediately, the sample was sucked into a disposable 1-ml plastic syringe and then immersed and stored in liquid nitrogen until the EPR measurement.

Protocol 2: arginine infusion. Rats were anesthetized with pentobarbital sodium as in *protocol 1*. The femoral vein and artery were cannulated with polyethylene catheters for administration of the drug and the recording of systolic blood pressure (SBP), respectively. SBP was measured using an Amplifier Case 7746 (NEC San-ei Instruments; Tokyo, Japan) equipped with a pressure transducer and recorded using a WT-6856 (Nihon Koden; Tokyo, Japan). After surgery, rats were kept for 30 min to stabilize the hemodynamic parameters, and the drug infusion was then started using a syringe pump (model 100, KD Scientific) from the femoral vein. Rats were divided into five groups, and one group ($n = 4$) each received saline, 0.2 g/kg L-arginine, 0.2 g/kg D-arginine, 0.6 g/kg L-arginine, or 0.6 g/kg D-arginine at a rate of 0.1 ml/min for 10 min. During experiments, rats were kept at 37°C by a water jacket. One minute after cessation of the infusion, venous blood was collected from the vena cava with a 1-ml plastic syringe and stored in liquid nitrogen until use.

Protocol 3: L-NAME-induced endothelial dysfunction model. Rats were divided into four groups [*group 1*, control; *group 2*, L-NAME; *group 3*, low-dose temocapril (an ACEI);

and *group 4*, high-dose temocapril; Fig. 1]. Each group had 12 rats except for the control group ($n = 8$), and the animals received tap water containing distilled water (*group 1*) or L-NAME (1 g/l, *groups 2–4*) from *day 0* to *day 13*. Water consumption was ~35 ml·rat^{-1}·day^{-1} in *group 1* and ~25 ml·rat^{-1}·day^{-1} in the L-NAME-treated groups (*groups 2–4*), and these values were constant throughout the experiment. On the basis of the drug solution intake, the effective daily dose of L-NAME was estimated to be ~65 mg·kg^{-1}·day^{-1}. Temocapril was administered twice a day by oral gavage (*group 3*, 2 mg·kg^{-1}·day^{-1}; *group 4*, 20 mg·kg^{-1}·day^{-1}) from *day 7* to *day 15*. *Groups 1* and *2* were given vehicle instead of temocapril. Body weight, SBP, and heart rate (HR) were measured at *days 0*, *7*, *14*, and *16*. SBP and HR were measured by the tail-cuff method using a BP-98A (Softron; Tokyo, Japan). To evaluate the endothelial function for maintenance of blood NO levels, we stopped the L-NAME treatment 2 days before the experiment. Rats were anesthetized with pentobarbital sodium, and venous blood was obtained on *day 16* (see *Protocol 2: arginine infusion*).

Protocol 4: preparation for NO-depleted whole blood. To deplete NO in blood, eight rats were administered tap water containing L-NAME (2 g/l) for 1 wk. The daily dose of L-NAME was ~120 mg·kg^{-1}·day^{-1}, and it was constant throughout the experiment. Rats were euthanized, and venous blood was taken (see *Protocol 2*: *arginine infusion*).

EPR Measurement and Data Processing

All EPR measurements were carried out in liquid nitrogen. The frozen sample was directly transferred into a liquid nitrogen-filled quartz finger dewar, which was placed in the cavity of the EPR measurement device. A JES TE 300 ESR spectrometer (JEOL; Tokyo, Japan) with an ES-UCX2 cavity (JEOL) was utilized to collect EPR spectra at the X band (9.5 GHz). Each sample was measured four times and normalized using ESPRIT 432 software (JEOL) to improve the signal-to-noise ratio. Typical EPR conditions were as follows: power, 20 mW; frequency, 9.045 GHz; field, 3,200 ± 250 gauss; mod width, 6.3 gauss; sweep time, 60 min; time constant, 1 s; and amplitude, 250. Spectra were stored on an IBM personal computer for analysis.
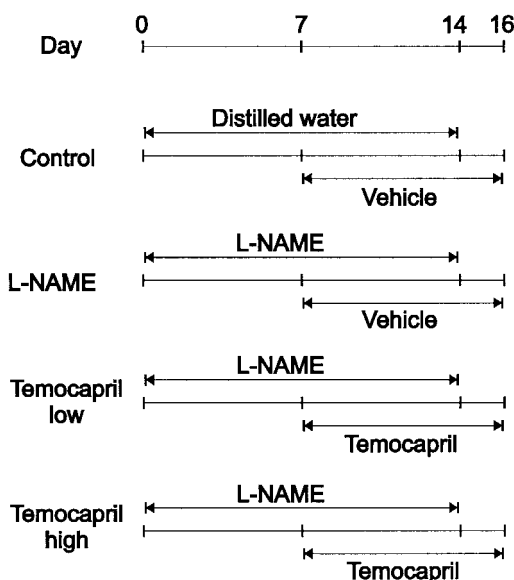

Fig. 1. Experimental design. L-NAME, N^ω-nitro-L-arginine methyl ester.
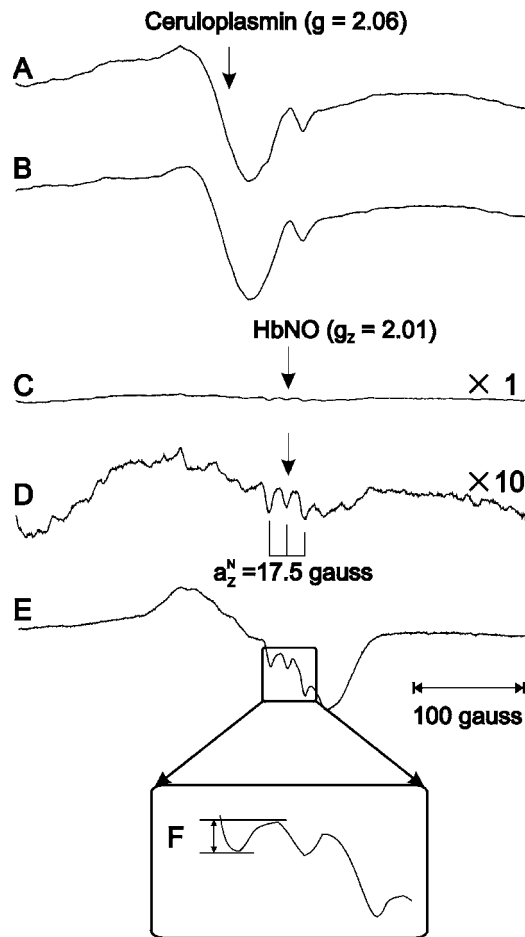
Fig. 2. Typical electron paramagnetic resonance (EPR) spectra of whole blood. Typical EPR spectra were obtained from whole blood of control (*A*) and L-NAME-treated rats (*B*). *C*: subtraction of *spectrum B* from *spectrum A*; *D*: *spectrum C* magnified by 10 times; *E*: nitric oxide (NO) added to whole blood (final concentration: 6.3 μM). Spectral conditions in *D* were the same as the typical EPR conditions described in MATERIALS AND METHODS except that power = 5 mW, sweep time = 15 min, and time constant = 0.3 s. *g* is the spectroscopic splitting factor of the hemoglobin-NO adduct (HbNO), and a_Z^N is the *Z*-factor hyperfine coupling constant.

The HbNO signal was obtained by subtracting the EPR spectrum of HbNO-depleted whole blood from that of each sample (14, 32). EPRMAIN computer software obtained from the National Institute of the Environmental Health Sciences (http://epr.niehs.nih.gov/pest.html) was used to accomplish this calculation.

Statistical Analysis

All data are expressed as means ± SE. Data were analyzed by a two-way ANOVA, followed by the Bonferroni test for comparisons between groups. $P < 0.05$ was accepted as statistically significant.

RESULTS

Signal Subtraction

Typical EPR spectra obtained from venous whole blood of untreated rats and L-NAME-treated NO-depleted rats are shown in Fig. 2, *A* and *B*, respectively. Most of the broad EPR signal in Fig. 2*A* consists of

serum ceruloplasmin ($g = 2.06$, where g is the spectroscopic splitting factor of HbNO). The EPR signal of HbNO consists of the components of the g_X, g_Y, and g_Z factors as shown in Fig. 3 (where *X*, *Y*, and *Z* indicate the direction of magnetic field and the *Z*-axis is parallel with it), and it overlaps with same field as ceruloplasmin. Therefore, the principal of the EPR signal subtraction method of HbNO is to bring the EPR signal of HbNO into prominence (14) by subtraction of the NO-depleted EPR signal from objective one. This manipulation is accomplished using special EPRMAIN software. The EPR signal of the *Z*-factor of HbNO gives a specific triplet EPR signal [$g_Z = 2.01$ and *Z*-factor hyperfine coupling constant (a_Z^N) = 17.5 gauss], and we used this region for qualification of HbNO and measured the signal height for quantitation of HbNO (Fig. 2*F*). Figure 2, *C* and *D*, shows the differential spectra obtained by subtracting the spectrum of Fig. 2*A* from that of Fig. 2*B* using EPRMAIN. Figure 2*D* shows the signal of Fig. 2*C* magnified by 10 times. It shows a weak but distinct triplet hyperfine structure ($g_Z = 2.01$) with a coupling constant of 17.5 gauss, and this EPR signal was assigned to HbNO (13, 19). Figure 2*E* shows a typical EPR signal of HbNO observed when NO (6.3 μM) was added to the venous blood.

Preparation of Calibration Curve

Figure 3 shows the EPR spectra and calibration curve. The reaction mixture contained venous whole blood of untreated rats and the NO-saturated solution (final concentration: 0–152 μM). Each spectrum was obtained by subtracting the EPR spectrum of untreated whole blood from that of the NO-added sample, and the intensities of the EPR signals were determined by measuring the height of the left line of the five-coordinate α-nitrosyl heme-derived triplet signal (Fig. 2*F*). The intensity of the EPR signal linearly increased
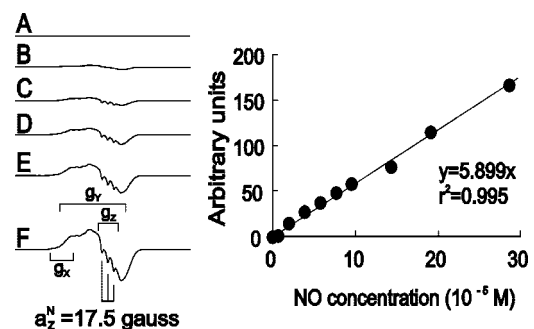

Fig. 3. Changes in HbNO signal intensity with increasing NO concentration. NO-saturated solution was added to venous blood of control rats. The final NO concentration was 0 μM (*A*), 6.3 μM (*B*), 19 μM (*C*), 38 μM (*D*), 76 μM (*E*), and 152 μM (*F*). *g* expresses the spectroscopic splitting factor of HbNO. Where g_X and g_Y are perpendicular factors, g_Z is a parallel factor against the magnetic field. Each spectrum was obtained by subtracting the EPR spectrum of untreated whole blood from that of NO-added samples. Spectral conditions were as follows: power, 5 mW; frequency, 9.045 GHz; field, 3,200 ± 250 gauss; mod width, 6.3 gauss; sweep time, 4 min; time constant, 0.1 s; amplitude, 250; and temperature, −196°C. Each point represents the average of 3 experiments.
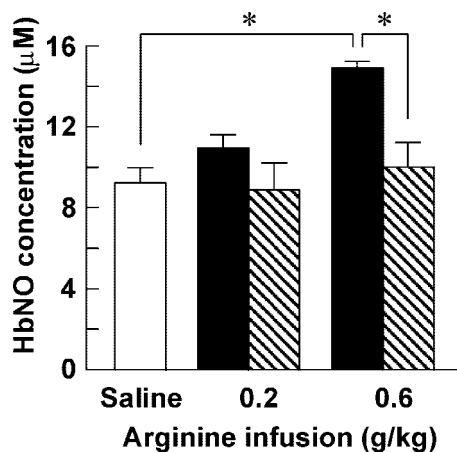
Fig. 4. Influence of L-arginine or D-arginine infusion on HbNO concentration. Saline (open bar), L-arginine (solid bars), or D-arginine (hatched bars) was infused at a rate of 0.1 ml/min for 10 min. The EPR signal of HbNO was defined by the subtraction of that of HbNO-depleted blood from that of each sample. HbNO concentration is expressed as means ± SE. Each group had 4 rats. *Statistically significant changes in HbNO concentration, $P < 0.05$ (Bonferroni test).
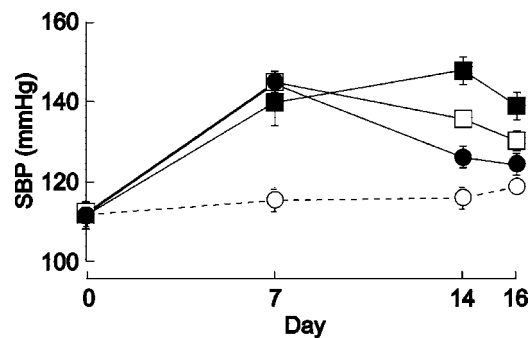

Fig. 5. Changes of systolic blood pressure (SBP). ○, Control; ■, L-NAME; □, low-dose temocapril; ●, high-dose temocapril. Values are expressed as means ± SE. Each group had 12 rats except for the control group ($n = 8$).

according to the NO concentration (32) with the correlation coefficient (r^2) = 0.995.

Arginine Infusion and HbNO Formation

To confirm that the HbNO level reflects the enzymatic formation of NO, we infused L- or D-arginine (0.2 or 0.6 g/kg) into rats and determined the HbNO levels in blood. When saline was infused, SBP was unchanged and the HbNO concentration was 9.23 ± 0.80 μM (Fig. 4). Although L-arginine did not alter SBP, the HbNO concentration increased in a dose-dependent manner. In contrast, D-arginine altered neither the HbNO concentration nor SBP even at the maximum dose.

L-NAME-Induced Hypertension and HbNO Concentration

Body weight increased continuously in all groups during the experimental period (Table. 1). The increase was slightly attenuated in the L-NAME-treated groups.

When the experiment was started, basal SBP was ~110 mmHg in all groups (Fig. 5). In the control (*group 1*), SBP and HR did not alter throughout the experiment. Administration of L-NAME induced hypertension and bradycardia, consistent with previous findings (26, 58, 59). Temocapril treatment dose dependently lowered SBP in the L-NAME-treated rats (*groups 3* and *4*; Fig. 5). After the washout period (*days 14* and *15*), both SBP and HR in the L-NAME-induced hypertensive rats were slightly improved. Figure 6 shows representative EPR spectra and HbNO concentration for each group. In the control group, the HbNO concentration was 10.12 ± 2.03 μM, which was similar to that for normal animals (9.23 ± 0.80 μM). Treatment with L-NAME alone significantly reduced the blood HbNO concentration (4.39 ± 0.65 μM), and temocapril restored the L-NAME-induced HbNO reduction in a dose-dependent manner (2 mg·kg⁻¹·day⁻¹, 5.71 ± 0.53 μM; 20 mg·kg⁻¹·day⁻¹, 7.02 ± 0.82 μM). When we administered temocapril (20 mg·kg⁻¹·day⁻¹) to untreated rats ($n = 6$) for 9 days, the HbNO concentration slightly but not significantly increased (data not shown).

DISCUSSION

NO is one of the most important physiological factors in the cardiovascular, immune, and nervous systems, and abnormal NO dynamics may cause diseases.

Table 1. *Heart rate and body weight in control and treated groups*

	Day 0	Day 7	Day 14	Day 16
Control group				
Body weight, g	373 ± 8	405 ± 4	423 ± 6	423 ± 6
Heart rate, beats/min	345 ± 6	344 ± 6	341 ± 8	337 ± 5
L-NAME group				
Body weight, g	389 ± 4	404 ± 5	428 ± 6	422 ± 6
Heart rate, beats/min	356 ± 7	294 ± 3	306 ± 6	315 ± 7
Low-dose temocapril group				
Body weight, g	379 ± 5	396 ± 4	409 ± 4	407 ± 4
Heart rate, beats/min	350 ± 7	302 ± 5	305 ± 6	314 ± 6
High-dose temocapril group				
Body weight, g	372 ± 5	391 ± 4	405 ± 4	397 ± 3
Heart rate, beats/min	347 ± 7	311 ± 3	311 ± 6	315 ± 5

Values are expressed as means ± SE; $n = 8$ rats in the control group and 12 rats in all other groups. L-NAME, N^ω-nitro-L-arginine methyl ester.
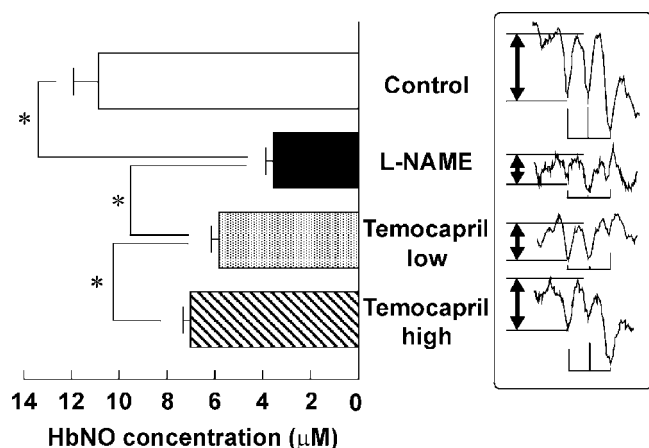
Fig. 6. Effect of chronic L-NAME treatment in combination with temocapril on HbNO concentration. *Left*: HbNO concentration expressed as means ± SE. Each group had 12 rats except for the control group ($n = 8$). *Statistically significant changes in HbNO concentration, $P < 0.05$ (Bonferroni test). *Right*: typical spectra in each group.

Therefore, an accurate method is required for the measurement of systemic NO dynamics under physiological and pathophysiological conditions. To date, the plasma concentration and urinary excretion of NO_x and cGMP have been employed as indexes of systemic NO levels in vivo (35, 62). However, NO_x and cGMP are regulated by factors other than NO. The NO_x level is affected not only by endogenous NO production but also by dietary NO_x and metabolism (8, 20, 53). The formation of cGMP is also altered by carbon monoxide and atrial natriuretic factor via the activation of guanylate cyclase (27, 64, 66, 71). Therefore, a specific and reliable method of detection is necessary to understand the physiological and pathophysiological role of NO.

Recently, it has been reported that endothelium-derived NO diffuses into blood, permeates the erythrocyte membrane, and then binds to Hb to form relatively stable HbNO in erythrocytes (48). In the present study, we tried to develop a method to detect HbNO as an index of blood NO by the EPR technique. The EPR method is more specific for NO compared with other methods, such as the Griess and cGMP methods, and offers a characteristic EPR spectrum of HbNO (22, 30).

Previous studies have already shown that LPS and NO donor treatment increase the HbNO concentration in blood, and clear HbNO spectra have been successfully obtained using the EPR method without any modifications (10, 28, 31, 67, 69). However, we and others failed to detect the distinct EPR signal of HbNO in the blood of normal humans or animals.

There are several difficulties in detecting the distinct EPR signal for HbNO in whole blood. Other EPR signals, such as for ceruloplasmin ($g = 2.06$) and semiquinone radical ($g = 2.00$), overlap the magnetic field of HbNO ($g_Z = 2.01$) (5, 7, 10, 13, 19, 42). Because the basal HbNO level is quite low (μM range) (11, 25), and its EPR signal overlaps with the strong EPR signal of ceruloplasmin (Fig. 2A), the EPR spectrum of HbNO cannot be observed in most cases. To solve these prob-

lems, we introduced and modified an EPR signal subtraction method (14, 32).

In the previous reports, prepared Hb was directly injected into animal body to trap endogenous NO ($Hb^{2+} + NO \rightarrow HbNO$) (32). However, this method is inappropriate for general use in NO detection in living systems due to the prooxidative activity of Hb^{2+}. In addition, although Glover et al. (14) first reported the definitive EPR spectra of HbNO in the systemic circulation of humans receiving hydroxyurea, they did not determined the absolute value.

Therefore, in this study, we adopted a whole blood as an NO trap agent and tried to quantitate the HbNO concentration with the EPR subtraction method.

First of all, we tried to produce NO-depleted venous blood. L-NAME is a nonselective NO synthase (NOS) inhibitor (12, 17, 50) commonly used both in vivo (9, 38) and in vitro (33, 49), and chronic L-NAME (0.5–1 g/l)-treated rats have been widely used as a model of NO deficiency (6, 26, 56). To make NO-depleted rats, we administered a high dose of L-NAME for 1 wk. When we subtracted the EPR signal of blood of L-NAME (2 g/l)-treated rats from that of untreated rats, the apparent EPR signal of HbNO was observed (Fig. 2, *C* and *D*), whereas no HbNO EPR signal appeared when we subtracted the signal of blood of L-NAME (2 g/l)-treated rats from that of L-NAME (1 g/l)-treated rats (data not shown). Although we cannot confirm the completely depletion of NO in the L-NAME (2 g/l)-treated rats, these results suggest that 1-wk L-NAME treatment (2 g/l) is sufficient for the preparation of HbNO-depleted blood, and hence this treatment was employed to the prepare NO-depleted rats in the present study.

As expected, the EPR signal of whole blood of chronic L-NAME-treated rats is identical to that of untreated rats except for the HbNO signal in the field of $3,200 \pm 250$ gauss (Fig. 2, *A* and *B*). Therefore, we subtracted the EPR signal of L-NAME-induced NO-depleted rats as a background to obtain a pure HbNO signal. HbNO can exist as a mixture of five-coordinate and six-coordinate hemes dependent on the environmental oxygen tension (54, 73). The differential signal showed a resolved triplet hyperfine structure ($g_Z = 2.01$) with a coupling constant of 17.5 gauss, which was assigned to a five-coordinate α-nitrosyl heme (Fig. 2, *C* and *D*). The signal intensity of HbNO increased with the NO concentration (Fig. 3), which means that NO specifically binds to Hb in red blood cells and forms HbNO (32).

L-Arginine is a physiological precursor of NO production. NO is synthesized from the terminal guanidine group of L-arginine by NOS (45, 52). To determine whether the EPR signal of HbNO is derived from L-arginine-dependent NO or not, we infused arginine into rats via the femoral vein and measured the EPR signal of HbNO. L-Arginine infusion (0.2 or 0.6 g/kg) significantly increased the HbNO concentration, but SBP was not affected by L-arginine at any dose. When D-arginine was infused, neither an increase in HbNO concentration nor a change of SBP was observed at the same dose as L-arginine. These results demonstrated

that the EPR signal for HbNO reflects the endogenous changes in NO dynamics.

It has been reported that endothelium-derived vasodilation is impaired in hypertensive animals and patients compared with normotensive subjects (36, 44, 47, 63). This endothelial dysfunction may result in the progression of vascular structural change, cardiac hypertrophy, and renal failure, which may be associated with a decreased bioavailability of NO (16). ACEIs have been shown to significantly improve the endothelial dysfunction and structural changes of some organs in experimental and clinical studies (2, 21, 36, 37, 57, 65). It seems conceivable that the improvement of endothelial function caused by ACEIs may result from the restoration of blood NO levels. Therefore, we used chronic L-NAME-treated rats as an animal model of endothelial dysfunction and examined the effect of temocapril (24, 43) on the level of NO in blood using the HbNO signal subtraction method.

The oral administration of L-NAME (1 g/l) for 2 wk induced a time-dependent hypertension, and the HbNO concentration was reduced to ~40% of the control (Fig. 6). However, the administration of temocapril dose dependently improved the HbNO concentration and SBP (Figs. 5 and 6). Because the increase in the HbNO level was accompanied by a reduction in SBP (Fig. 5), the SBP-lowering effect of temocapril may be, at least in part, associated with an improvement in systemic NO levels (60). Although the exact mechanism behind the effect of temocapril in improving NO dynamics is still unclear, we confirmed using our EPR HbNO signal subtraction method that temocapril reversed the decrease of blood NO in L-NAME-treated rats.

In the present study, we succeeded in measuring the systemic HbNO levels as an index of NO by the EPR HbNO signal subtraction method. Our method is specific for NO and does not require any pretreatment, and hence it seems simple and relevant as a method for measurement of the NO concentration in blood. As the balance between production and quenching of NO determines the level of bioactive NO, the determination of blood HbNO levels by our method may be useful in investigating a potential role of NO in physiological and pathophysiological functions. We recognize that there are some limitations with our method, and we will attempt to correct for these in successive experiments. The detection limit of HbNO was in the micromolar range (Fig. 3) with the present equipment. Further improvements will be required to apply this technique in clinical use.

DISCLOSURES

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