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Analysis of Blood Flow-dependent Blood Nitric Oxide Level and Half-life of Nitric Oxide in Vivo

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Abstract

Endothelial release of nitric oxide (NO) contributes to the regulation of vascular tone by inducing vascular relaxation. To estimate the blood flow-dependent nitric oxide level and half-life (T1/2) of nitric oxide in vivo state, we investigated the change of aortic NO currents during the change of aortic blood flow rate using NO-selective electrode system and electromagnetic flowmeter in the aorta of anesthetized rats. Resting mean aortic blood flow rate was 49.6 ± 5.6 ml/min in the anesthetized rats. NO currents in the aorta were increased by the elevation of blood pressure and/or blood flow rate. When the aortic blood flow was occluded by the clamping, aortic NO currents were decreased. The difference of NO concentration between resting state and occluded state was 1.34 ± 0.26 μM (n=7). This NO concentration was estimated as blood flow-dependent nitric oxide concentration in the rats. Also, while the aortic blood flow was occluded, NO currents were decreased with exponential pattern with 12.84 ± 2.15 seconds of time constant and 7.70 ± 1.07 seconds of half-life. To summarize, this study suggested that blood flow-dependent NO concentration and half-life of nitric oxide were about 1.3 μM and 7.7 seconds, respectively, in the aorta of anesthetized rats. The nitric oxide-selective electrode system is useful for the direct and continuous measurement of NO in vivo state.

Key words : Nitric Oxide, Blood Flow, NO-selective electrode, Half-life, NO decay rate

Introduction

Endothelial synthesis and release of vasoactive mediators has been widely recognized as one of the main mechanisms involved in the regulation of vascular tone[1]. Endothelial cells have been reported to synthesize nitric oxide (NO) continuously without stimulation by agonist[2]. Direct evidence for basal generation of NO came from bioassay cascade experiments, which revealed the continuous formation and release of NO in effluents collected from perfused and/or superfused vascular preparations[3-7]. Although the intrinsic stimulus for basal generation of nitric oxide (NO) was not appreciated in the early 1980s, later studies revealed that the shear stress or tangential shear force generated by flowing blood against the endothelial cell surface triggered the generation of NO in the endothelial cells[2,3]. However, there is little

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information about the level of blood flow-dependent nitric oxide in vivo state because there have been no appropriate tools with which to investigate dynamic change of nitric oxide in vivo.

NO was a very reactive substance and was easily converted to other molecules. It was known that half-life of NO in vitro using cultured endothelial cells or bioassay cascade experiments was about 2 ~ 7 sec[4-6]. However, there is possibility that half-life of NO in vivo state is different with that in vitro condition, maybe due to several kinds of physiological factors. Direct in vivo assessment of changes in aortic NO activity during changes in aortic blood flow was not performed yet.

In the present study, using NO-selective microelectrode, we evaluated a continuous and direct assessment of changes in aortic NO levels in vivo during the change of blood flow, and we also estimated basal NO level and decay rate of NO in anesthetized rats.

Materials and Methods

Animals

Male Sprague-Dawley rats (weight 250-350g) were obtained from Chemical Research Institute (Taejon, KOREA). The rats were kept in individual cages in a room in which lighting was controlled (12 hours on / 12 hours off), temperature was maintained at 23° C to 24° C. The rats were given food and tap water ad libitum and fasted for 12 hours before the experiment.

Experimental Procedure

The animals were anesthetized with urethane 1 g/kg, intraperitoneally. Polyethylene catheters were inserted into the left carotid artery to measure blood pressure and into the right jugular vein for infusions. The abdominal aorta was exposed by a midline abdominal incision. Then, the abdominal aorta was separated gently from the inferior vena cava. Aortic blood flow rate was measured with an electromagntic blood flow meter (Narco-Biosystem, USA). Aortic blood flow rate was measured with a 1-mm flow probe placed around the abdominal aorta connected to an electromagnetic flowmeter. In the blood flow experiments, a mechanical occluder was placed around the aorta located above the renal arteries to allow for changes in aortic blood flow. Blood pressure, mean blood flow rate (MBF), and pulsatile blood flow rate (PBF) were continuously recorded throughout the experiment. PBF and MBF was obtained as flow rate and expressed as ml/min. Respiration resistance (RR) was measured with impedence pneumograph coupler (Narco-Biosystem, USA). Pneumograph signals were obtained by measuring the impedance between two electrode applied to the thorax in the rats.

In vivo nitric oxide measurements

All NO measurements were done using the NO-501 Nitric Oxide Monitoring Device (Inter-Medical Ltd, Japan). The NO-selective microelectrode is made of platinum/iridium alloy and is 200 micrometers in diameter. NO-electrodes were allowed to equilibrate for at least 30 min in phosphate-buffered saline (PBS) before use. To measure aortic NO levels in the abdominal aorta, the NO-selective microelectrode was inserted into the aortic lumen, and only in the small area of the aortic upper wall and the impaled NO electrode was tied with 10-0 mononylon filament for the maintenance of the aortic blood flow. The reference electrode was placed on the surface of the mesenteric adipose tissue.

We calibrated the device by generating a standard curve using the NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP) in PBS according to the manufacturers instructions. It was assumed that a 1 mM of SNAP will generate an NO concentration of 1.3 μM and that there is a linear relationship between the amount of SNAP and the amount of NO produced over the range we were measuring. A linear fit was obtained for each standard curve and used to calculate for each standard curve and used to calculate the NO concentrations for each experiment. Nitric oxide electrodes were precalibrated in vitro by adding known doses of the NO donor compound SNAP to a

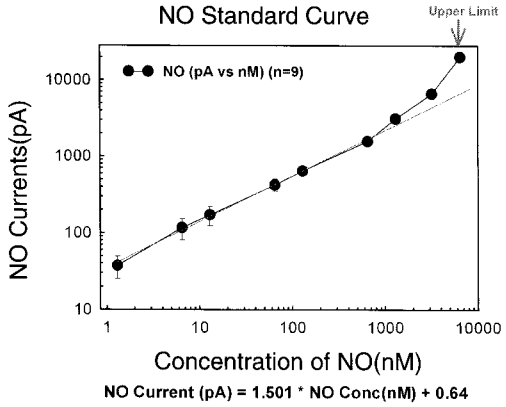

Figure 1 In vitro calibration curve of the NO currents (n=9). Nitric oxide electrodes were precalibrated in vitro by adding known doses of the NO donor compound SNAP to a cuvette in which they were immersed. The NO calibration curve was generated from the 9 different experiments. Using the calibration curve, NO current-concentration conversion formula was represented below the figure. Linear line was regression line of calibration curve. Upper limit for the NO currents using the NO monitor (Inter-Medical Co, Japan) was 20,000 pA (Arrow).

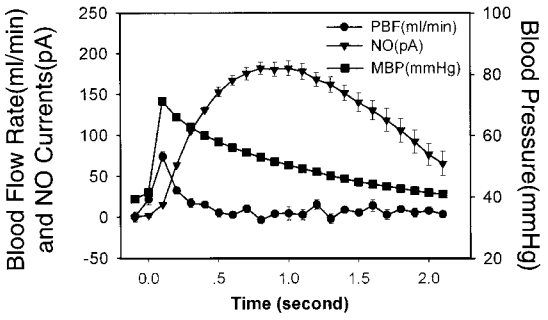

Figure 2 The change of aortic blood flow and nitric oxide currents by the single pulse of blood pressure in the state that the voluntary respiration was stopped by the treatment of a high-dose of acetylcholine (10 mg/kg) in the anesthetized rats. Pulsatile aortic blood flow (PBF, ml/min) and nitric oxide current (NO, pA) were measured with electromagnetic flowmeter (Narco-biosystem, USA) and NO monitor (Inter-Medical Co, Japan) in the aorta, respectively. Mean blood pressure (MBP, mmHg) was measured in the carotid artery. Data was means ± S.E. from 7 different sets of experiments.

cuvette in which they were immersed. Figure 1 illustrates the calibration curve generated from the 9 electrodes used in this study. Using the calibration curve, we made the NO current-concentration conversion formula as follows; NO currents (pA) = 1.501 * NO concentration (nM) + 0.636.

Data Analysis

The time constants and half-life of NO were determined from the fitted exponential equation. All values are means ± SEM. All data was compared by using Student s paired t test, with significance defined as $P < 0.05$.

Results

Relation between blood pressure and/or blood flow and nitric oxide production

We investigated the change of aortic blood flow and nitric oxide currents by the single blood pressure in the acetylcholine (10 mg/kg)-treated anesthetized rats. As shown as Figure 2, aortic blood flows were immediately and transiently increased and then rapidly decreased to near zero levels during a single pulse of blood pressure. However, aortic nitric oxide pulse of blood pressure. However, aortic nitric oxide currents were also immediately response to the blood pressure pulse. After nitric oxide currents were reached at maximum about 1 second of blood pressure pulse, its currents were more slowly decreased than that of blood flow rate.

The change of nitric oxide levels during the change of blood flow

Effects of acute blood flow change on the aortic nitric oxide currents were examined in the anesthetized rats. The typical result was shown in Figure 3. Resting NO output signals in the aorta were slightly affected by the respiratory movements (Figure 3). Table 1 summarized the mean values obtained in 7 rats. When the aortic blood flow rate was almost stopped by the aortic clamping for 30 seconds, systemic blood pressure in the left carotid artery was slightly increased, however, aortic nitric oxide current was gradually decreased. Inversely, when the aortic blood flow rate was recovered by the release of the clamp, systemic blood pressure was slightly decreased and the aortic nitric oxide current was rapidly

Table 1 The changes of hemodynamic parameters and aortic NO currents during the change of aortic blood flow in the anesthetized rats.

	Resting	Flow stop	Flow recovery
Mean arterial pressure (mmHg)	89.7 ± 2.3	96.6 ± 2.1*	87.0 ± 3.1
Mean aortic blood flow rate (ml/min)	49.6 ± 5.6	3.8 ± 2.9**	61.9 ± 6.2*
Change of NO Currents (pA)		Δ 2006 ± 393	Δ 2162 ± 433
Estimated NO (μM)		Δ 1.34 ± 0.26	Δ 1.44 ± 0.29

Values are means ± SE from 7 different sets of experiments. * $P < 0.05$, ** $P < 0.01$ versus resting

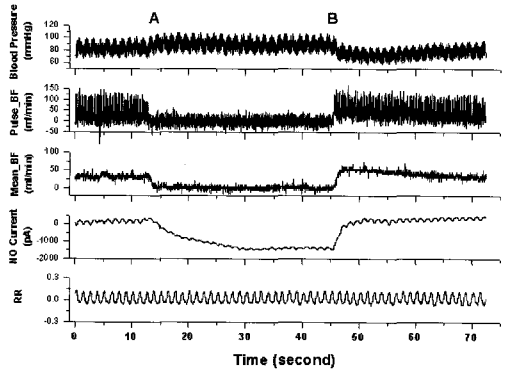

Figure 3 Example of the hemodynamic responses to the change of aortic blood flow in the anesthetized rats. A arterial clamp was placed around the aorta located above the renal arteries to allow for the stop (A) and recovery (B) in the aortic blood flow. Blood pressure (BP, mmHg), pulsatile blood flow (Pulse_BF, ml/min), mean blood flow (Mean_BF, ml/min), nitric oxide currents (NO, pA), and respiration resistance (RR) were continuously recorded throughout the experiment.

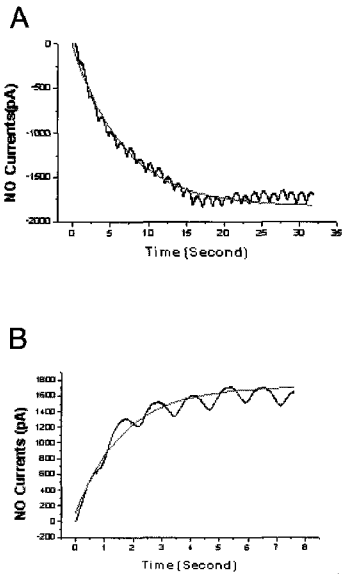

Figure 4 Typical changes of aortic nitric oxide currents and fitted curve during the stop (A) and recovery (B) of aortic blood flow in the aorta of anesthetized rats.

increased. Aortic nitric oxide currents were decreased by the reduction of aortic blood flow (Δ2006 ± 393 pA). This change of NO output current was estimated as Δ1.3 ± 0.3 μM of NO concentration with in vitro calibration curves for each electrode. The change of nitric oxide currents during the reduction of aortic blood flow was similar with those during the recovery of aortic blood flow in the anesthetized rats (Δ2006 ± 393pA vs Δ2163 ± 433 pA, n=7, NS).

Nitric Oxide Decay Rates

To evaluate the NO decay time constant and half-life during the stop of aortic blood flow, the changes of nitric oxide currents were fitted using first order exponential decay formula. Also, NO recovery rate and NO recovery time constant during the restoration of aortic blood flow were estimated. Typical data was shown in Figure 4. These data summarized in Table 2. As shown in Table 2, NO decay time constant was greater than NO recovery time constant (12.8 ± 2.2 seconds vs 1.1 ± 0.3 seconds, $P < 0.01$, respectively). NO half-life was estimated as 7.7 ± 1.1 seconds in the aorta of anesthetized rats.

Table 2 Estimation of NO decay and recovery rate during the change of aortic blood flow in the aorta of rats.

	NO Decay / NO Recovery
Time Constant (τ, sec)	12.8 ± 2.2 ,1.1 ± 0.3*
Half-life ($T^{1/2}$, sec)	7.7 ± 1.1

Values are means ± SE from 7 different sets of experiments. * $P < 0.05$ versus decay rate

Discussion

Because NO decays within seconds, dynamic assessment of changes in NO level is particularly important. In the present study, we firstly demonstrated that acute alteration in aortic blood flow resulted in the correlated change of NO level in the aorta of anesthetized rats. When the aortic blood flow was stopped, we also estimated NO decay time constant and half-life in the aorta of anesthetized rats.

The shear stress exerted by the streaming blood on the endothelial cell lining of the luminal surface of the vascular wall causes a permanent activation of these endothelial cells, resulting in the release of an endothelium-derived relaxing factor, of which NO is the active principle[8]. This released NO results in a relaxation of the adjacent smooth muscle and in a dilation of the vessel in response to an augmentation of flow through that vessel. Several important early studies contributed to the development of this concept that flow-dependent vasodilatation is endothelium dependent[9-13]. It now appears that shear forces trigger the mechanotransducer[14], thereby leading to the calcium-independent activation of endothelial NO synthase and increase local production of NO[15].

To minimize respiratory movement artifacts in NO electrode output signal recordings, NO electrode and impaled tissue have to be kept in a fixed position[19]. Because the resting nitric oxide currents in the impaled NO electrode to the aorta were oscillated by the muscle movement of respiration. Therefore, in the present study, rats were pretreated with high doses of acetylcholine intravenously to stop transiently the spontaneous respiration. In the transiently stop state of respiration, it has been confirmed that pulsatile blood flow in response to blood pressure stimulates nitric oxide production in the aorta of anesthetized rats (Figure 2). Even though blood flow in response to blood pressure reached the resting level, nitric oxide current was increased during approximately 1 second. This result may be due to the triggering of the cascade signal for nitric oxide synthesis by the blood flow in the endothelial cells. In the present study, blood pressure was measured in the left carotid artery, the upper level of arterial clamping site (abdominal aorta). Arterial clamp of abdominal aorta increased systemic blood pressure in the anesthetized rats. This phenomenon was due to volume overloading to blood vessels. Therefore blood pressure was recovered to original level after the release of arterial clamp (Figure 3)

Endothelial cells in an un-stimulated state spontaneously releases endothelium-derived relaxing factor (EDRF) which was identified as nitric oxide[16]. It is likely that the tonic, basal release of EDRF plays a major role in the modulation of blood pressure and systemic vascular resistance[17,18]. When EDRF was bioassayed from perfused vascular segments, it became obvious that it was released by the endothelium even in the absence of vasodilator agents. Direct evidence for basal generation of NO came from bioassay cascade experiments, which revealed the continuous formation and release of NO in effluents collected from perfused and/or superfused vascular preparations[6,7]. Also, the basal release of NO has been reported to be stimulated or augmented by shear stress[2]. However, these previous experiments were

performed with *in vitro* condition isolated from their physiological environment so that can not evaluate the amount the basal nitric oxide. In the present study, we demonstrated that the difference of NO currents before and after the change of blood flow was estimated as blood flow-dependent nitric oxide levels. However, limitations of this experiment existed; NO standardization curve was made in the *in vitro* condition such as phosphate-buffer solution, and the possibility of change of basal NO production rate during the change of blood flow rate.

It is known that half-life of NO *in vitro* using cultured endothelial cells or bioassay cascade experiments was about 2 ~ 7 sec[4-6]. It was reported that the half-life of NO in oxygenated Kreb s buffer was approximately 6.4 seconds[4]. In the present study, a possible NO decay rate was estimated by using of the fitting curve of the nitric oxide currents which is measured during the stop of aortic blood flow in the aorta of anesthetized rats. Interestingly, estimated half-life of NO in the present study (7.7 seconds) was similar with the results of previous *in vitro* experiments. Also, the recovery rate of NO during the recovery of blood flow was faster than the decay rate of NO in the aorta of anesthetized rats. The possibility of a fast NO recovery rate may be due to the stimulation of NO production by the increase of blood flow rate as well as due to already made NO above level of clamped site. Therefore, these data can not explain the exact NO production rate using only our data in vivo state.

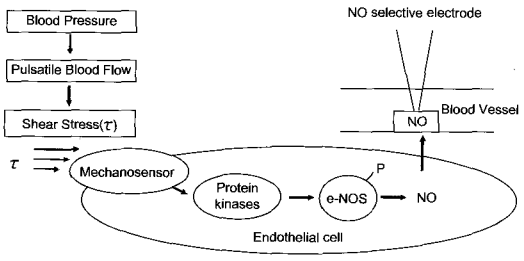

Figure 5 Schematic diagram for blood flow-induced nitric oxide production in the blood vessels. Shear stress induced by blood flow can induce the phosphorylation of endothelial nitric oxide synthase (eNOS) through the activation of several kinds of protein kinases. Intracellular molecular responses to shear stress are transduced by the candidate mechanosensor in the endothelial cell of blood vessels. Real time change of nitric oxide (NO) can be measured with nitric oxide-selective microelectrode which is impaled in the blood vessel.

Shear stress can modulate endothelial cell functions by sequentially activating the mechanosensors, intracellular signaling pathways, specific transcription factors, and the expression of genes and proteins[20]. The activation of mechanosensors by shear stress leads to the triggering of phosphorylation cascades of signaling molecules (Figure 5). Shear-induced production of NO involves Ca^{2+}/calmodulin-independent mechanisms, including phosphorylation of eNOS at several sites and its interaction with other proteins, including caveolin and heat shock protein-90. There have been conflicting results as to which protein kinases protein kinase A, protein kinase B (Akt), other Ser/Thr protein kinases, or tyrosine kinases are responsible for shear-dependent eNOS regulation21. However, the precise mechanism of signaling events in endothelial cells in the response to blood flow and /or shear stress remains to be elucidated.

In summary, we demonstrated that blood flow-dependent NO concentration and half-life of nitric oxide in vivo state of anesthetized rats using NO-selective electrode system. The nitric oxide-selective electrode system is useful for the direct and continuous measurement of NO from the aorta of rats.

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