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Direct electrochemical measurement of nitric oxide in vascular endothelium

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Abstract

The endothelium plays a critical role in maintaining vascular tone by releasing vasoconstrictor and vasodilator substances. Endothelium-derived nitric oxide (NO) is a vasodilator rapidly inactivated by superoxide and by Fe(II) and Fe(III), all found in significant quantities in biological systems. Thus due to the short life of NO in tissue ($t_{1/2} = 3–6$ s), in situ quantification of NO is a challenging problem. We designed the present study to perform direct measurements of nitric oxide using the electrochemical porphyrinic sensor. The most significant advantages of this sensor is small size (0.5–8 μm), rapid response time (0.1–1 ms), and low detection limit (10^{-9} mol l^{-1}). The porphyrinic sensor was used for in vitro and in vivo measurements of NO in an isolated single cell or tissue. Effects of hypertension, endotoxemia, and ischemia/reperfusion on the release of NO and/or its interaction with superoxide (O_2^-) were delineated. In the single endothelial cell (rabbit endocardium), NO concentration was highest at the cell membrane (950 ± 50 nmol l^{-1}), decreasing exponentially with distance from cell, and becoming undetectable at distances beyond 50 μm. The endothelium of spontaneously hypertensive rats (SHR) released 35% less NO (580 ± 30 nmol l^{-1}) than that of normotensive rats (920 ± 50 nmol l^{-1}), due to the higher production of O_2^- in SHR rats. Endothelial NO synthase (eNOS) generated NO (140 ± 20 nmol l^{-1}) in lung during the acute phase (first 10–15 min) of endotoxemia, followed by production of NO by inducible NOS. High production of O_2^- was observed during the entire period of endotoxemia. Ischemia (lower limb of rabbit) caused a significant increase of NO peaking at 15 min and decreasing thereafter, also due to O_2^- production.

Keywords: Nitric oxide; Endothelium derived relaxation factor; Porphyrinic sensor; Amperometry

1. Introduction

The endothelium derived relaxation factor nitric oxide (NO) is known to be involved in a number of normal physiological processes as well as in pathological conditions. Recent comprehensive reviews provide a background for the understanding of the biochemical roles of the reactive NO molecule [1–4]. Nitric oxide production from L-arginine and oxygen is catalyzed by constitutive or inducible nitric oxide synthase. Vascular en-

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dothelial cells contain the calcium-dependent constitutive nitric oxide synthase (eNOS). In order to maintain normal blood pressure, eNOS produces relatively low concentrations of NO lasting for a few minutes. Synthesis of NO is stimulated by chemical agonists, including bradykinin and ATP, which stimulate Ca^{2+} flux through cell membranes [5]. However, physical agonists, such as shear stress or flow, can also stimulate NO release in the cardiovascular system by causing the release of internal Ca^{2+} stores. Turned on by Ca^{2+} flux, eNOS causes NO biosynthesis for about a minute; subsequently eNOS is turned off by phosphorylation of one of its serine residues. The kinetics of nitric oxide production in the brain by constitutive neuronal NOS (nNOS) is similar to that of NO produced by eNOS.

In contrast to eNOS, the inducible nitric oxide synthase (iNOS) is calcium-independent [6]. The stimulation of iNOS results from endotoxins and/or cytokines. The stimulated iNOS system produces large amounts of NO continuously for extended periods, up to several hours. NO derived from iNOS is bactericidal, and thus NO plays a beneficial role in host defense against bacterial and viral infections.

NO has been implicated in the pathogenesis of several diseases [7–11]; a deficiency of NO may play a role in some (hypertension, hyperglycemia, atherosclerosis, Parkinson's disease, Alzheimer's disease) and conversely, increased NO may participate in others [12–18] (arthritis, reperfusion injury, cancer). Thus from biochemical as well as medical perspectives, it is important to quantify the details of NO production in abnormal and normal tissues, including direct measurements. NO is stable in oxygen free and cell free solutions; however, NO reacts rapidly with cellular components in vitro or in vivo producing protein nitrosylation as well as reacting with hemoglobin and oxygen. In addition, in the presence of superoxide, NO is rapidly converted to peroxynitrite. Consequently, NO has a half-life of 2–6 s in vivo [4] and detection of NO in biological systems has thus proven technically difficult.

Several indirect methods have been developed for NO detection [19]. These methods include chemiluminescence, L-citrulline bioassay, UV–visible spectroscopy (Griess reagent, hemoglobin) and ESR spectroscopy. However, the only method which has been successfully applied for direct in vivo as well as in vitro measurement is electrochemical detection of NO [20,21]. The advantages of direct electrochemical measurements include enhanced sensitivity of on site measurements as well as rapid response. As a result, less NO is lost prior to measurement. Using the electrochemical porphyrinic sensor, this study describes comparative in vitro and in vivo measurements of the kinetics of NO release by both eNOS and iNOS in normal and dysfunctional endothelium.

2. Materials and methods

NO was measured using a catheter-protected porphyrinic microsensor prepared in a previously described manner [22]. Measurements were performed with a PAR model 273 voltammetric analyzer interfaced with an IBM 80486 computer software; the amplified and recorded analytical signal was sampled at 4 KHz. Amperometry, fixed at the potential for the oxidation of NO from its basal level with time. Measurements used the three electrode system: a catheter-protected porphyrinic microsensor working electrode, a platinum wire counter electrode and a reference silver/silver chloride electrode. The porphyrinic microsensor had a response time of 0.1 ms at micromolar NO concentration and 10 ms at the detection limit of 1 nmol l^{-1}. Linear calibration curves were constructed for each sensor from 5×10^{-9} to 3×10^{-6} mol l^{-1} NO before and after in vivo or in vitro measurements, with aliquots of saturated NO prepared as described previously [23].

Measurement of NO in the beating heart is a challenging task. In order to overcome potential interferences and to record a reliable NO signal, we modified the porphyrinic sensor of our earlier in vivo design [22] in two ways. First, the active sensor tip was shortened to 3–5 μm from the previously described 50–60 μm. In addition, the truncated needle from which the active sensor tip emerges was cut 5–6 μm shorter than its protec-

tive catheter, so that the tip of the sensor was completely recessed within the ventilated catheter tip, rather than protruding from an unventilated catheter tip as previously described [24].

New Zealand White rabbits (4 kg), normotensive Wistar–Kyoto rats (300 g) or spontaneously hypertensive rats (250 g) were anesthetized (50 mg kg^{-1} Ketamine and 5 mg kg^{-1} Xylazine for rabbits; 100 mg kg^{-1} Ketamine and 10 mg kg^{-1} Xylazine for rats), incubated and ventilated with room air using a Harvard small animal ventilator (tidal volume of 25 ml and rate of 70 breaths min^{-1} for rabbits; tidal volume 2.5 ml and rate of 100 breaths min^{-1} for rats). After median sternotomy was performed, NO concentration was measured as follows: to implant the porphyrinic NO sensor, tissue was pierced with a standard 0.8-mm-diameter angiocatheter needle (clad with its catheter with two ventilation holes near the tip). The catheter/needle unit was advanced to a desired place in the tissue. The position of the catheter was secured, and the placement needle was removed and quickly replaced with a porphyrinic NO sensor mounted in a truncated needle. The exact localization of the active tip of the sensor was confirmed by postmortem section of the tissue.

2.1. Measurement of NO release from isolated cells

Endocardial cells were mechanically removed from the surface of the left ventricle. The cells were plated on round cover slips maintained in HEPES-buffered saline (Sigma) at 37° for later experiments. To measure NO concentrations, an L-shaped thermally sharpened porphyrinic microsensor (diameter, 2–3 μm; length 5–7 μm) was placed on the membrane of a single cell using a motorized computer controlled micromanipulator [25]. When the tip of the sensor touched the cell membrane, a transient small electrical noise was observed, assumed to indicate zero distance from the cell. From this point the sensor was moved away from the surface in 0.2-μm increments controlled by computer. Injection of NO agonists employed a nano- or femto-injector. Injection of large volumes of agonist with a micro-injector caused a 'jet effect' accompanied by release of NO due to shear stress.

2.2. Intermittent ex vivo superoxide measurement

Superoxide was measured intermittently by a chemiluminescence method [26] in freshly excised parenchyma from the lung, before or during endotoxemia. Superoxide produced chemiluminescence of lucigenin (bis-*N*-methylacridinium nitrate, Sigma, St. Louis, MO), detected using a scintillation counter (Beckman 6000LS, with a single photon monitor). Each tissue sample (0.8–1.5 mg) was placed in 2.0 ml of HBSS (Sigma, St. Louis, MO) adjusted to pH 7.4 at 25°C, then enough lucigenin added to make its concentration 0.25 mmol l^{-1}. Basal O_2^- concentration produced by the tissue was measured after a 2-min incubation in HBSS.

The sum of the O_2^- produced by the cNOS plus other sources (basal) was measured in a similar manner, except that the 2-min incubation period was followed by injection of 20 μl of an ethanolic solution of calcium ionophore A23187 (1 mmol l^{-1}, Sigma, St. Louis, MO), a receptor independent cNOS agonist. Photons were counted during the first 20 s after addition of calcium ionophore A23187. Photon counts were calibrated as O_2^- concentration by constructing standard curves based on photons emitted by the reaction of xanthine and xanthine oxidase (both from Sigma, St. Louis, MO).

3. Results

3.1. In vitro measurements of NO in a single cell

Fig. 1a shows an amperogram (current vs. time) of NO released from a single isolated endothelial cell (endocardium). A porphyrinic sensor was placed 10 ± 2 μm from the cell surface. NO release was stimulated with a calcium ionophore (A23187, 8 μmol l^{-1}). About 0.9–1.2 s after injection of the calcium ionophore, a rapid in-

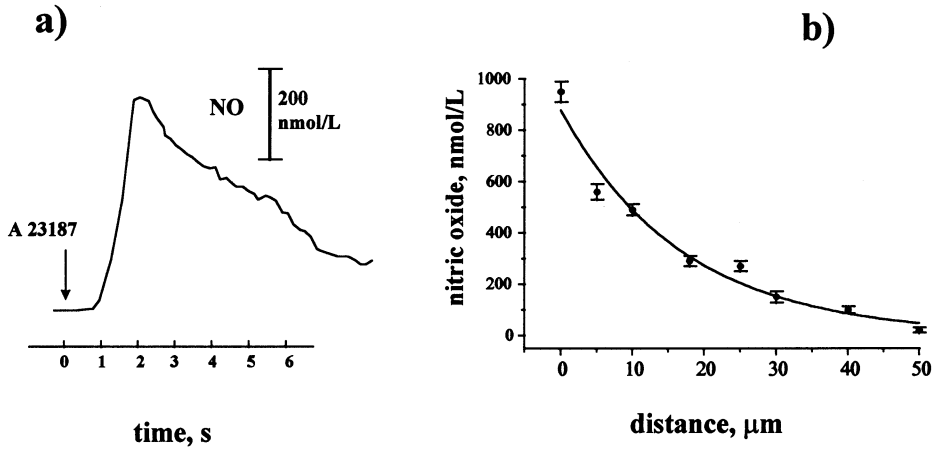

Fig. 1. Typical amperogram of NO recorded with porphyrinic sensor placed 10 ± 2 μm from the surface of an isolated single endothelial cell (endocardium of rabbit) (a); Exponential decrease of NO concentration with increasing distance from the membrane of single endothelial cell. (b); NO release was stimulated with calcium ionophore A23187, 8 μmol l^{-1}

crease of NO concentration was observed, with a rate of NO increase of 500 ± 20 nmol l^{-1} s^{-1} ($n = 7$). After about 1 s, the maximal peak concentration of 520 ± 25 nmol l^{-1} was observed. The duration of plateau of the peak is about 0.5 s, after which NO steadily decreases, with an average NO decrease of 100 nmol l^{-1} s^{-1}. The height of the peak of NO released from a single cell depended on the distance of the sensor from the cell membrane. The highest NO concentration was observed on the cell membrane (950 ± 40 nmol l^{-1}), with concentration decreasing exponentially with the distance from the cell membrane. At about 50 μm from the cell membrane, the NO concentration was 20 ± 10 nmol l^{-1} (Fig. 1b), and at a distance greater than 50 μm from the cell membrane, NO was not detectable by the porphyrinic sensor.

3.2. In vitro measurements of NO in tissue

NO concentration was measured in vitro in the heart endocardium of WKY and SHR rats using a porphyrinic sensor placed near the cell surface (8 ± 2 μm). Amperometric curves showing the change of NO concentration with time were recorded in the absence and presence of membrane superoxide dismutase with attached polyethylene glycol 400 (PEG-SOD activity 100 U ml^{-1}) (Fig. 2). Since PEG-SOD rapidly decomposes superoxide (O_2^-), we used this indirect approach to estimate production of O_2^- at the time of NO release. After addition of calcium ionophore, a rapid increase of NO concentration was observed. Peak NO concentration was higher for WKY than SHR rats (910 ± 40 and 580 ± 35 nmol l^{-1}, respectively, $n = 6$, $P < 0.005$). In the presence of PEG-SOD, an increase of peak NO concentration was observed for both WKY and SHR rats. In the WKY strain, SOD treatment increased the peak NO release by 8%. However, for SHR rats the increase of NO peak concentration was much higher (83%) in the presence of PEG-SOD than for WKY rats. This finding indicates that a significant concentration of O_2^- is generated in the heart of hypertensive rats, and this superoxide consumed a portion of NO in a fast chemical reaction.

3.3. In vivo measurements of NO in lung

Fig. 3 shows the amperometric curve measured in vivo during endotoxemia with a porphyrinic sensor placed in the rat lung parenchyma. During administration of lipopolysaccharide (LPS), an increase of NO production from its basal concentration was observed. The average rate of NO concentration increase was 0.80 ± 0.05 nmol l^{-1}

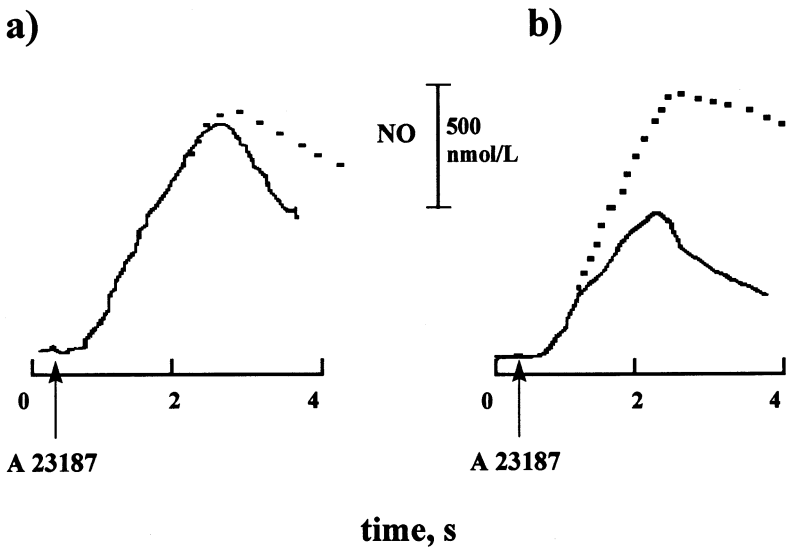

Fig. 2. Typical amperogram showing changes of NO concentration near the surface of the endocardium (left ventricle) of the heart of (a) a WKY rat, and (b) an SHR rat. NO was stimulated with calcium ionophore (A23187, 8 μmol l^{-1}) and its concentration was measured in the presence (dotted line) and absence (solid line) of PEG-SOD.

s^{-1} ($n = 6$). The concentration of NO reached a peak of 140 ± 20 nmol l^{-1} after 180 ± 20 s, persisting for 12 ± 3 min before decaying at a rate of 0.20 ± 0.01 nmol l^{-1} s^{-1}. After 45 min, the NO concentration started to rise again, but at a much slower rate. The rate of increase of NO concentration was 0.020 ± 0.005 nmol l^{-1} s^{-1}, significantly slower than for the first NO release. A plateau of NO release was established 80 ± 15 min after LPS administration; after 95 ± 7 min, a small decay of NO concentration was observed.

UV–visible spectroscopy (Griess reagent [25]) was used to determine a change of nitrite/nitrate concentration in blood during endotoxemia. Both NO_2^- and NO_3^- are end products of NO oxidation in biological systems. Fig. 3b shows clearly that the concentration of NO_2^- and NO_3^- significantly increased from basal 1.0 ± 0.3 to 6.4 ± 0.6 μmol l^{-1} after 6 h of endotoxemia. The most dramatic rate of increase was observed after 2 h of endotoxemia. The concentration of superoxide (measured in vitro during stimulation with A23187 using the chemiluminescence method) [26] also increased significantly during endotoxemia (Fig. 3c). During the first hour of endotoxemia, the O_2^- concentration increased threefold relative to the control. A plateau established after 4 h of endotoxemia showed three times the concentration of O_2^- compared to the control.

3.4. In vivo measurement of NO in the femoral artery

Fig. 4a shows an amperometric curve recorded in vivo during I/R with a porphyrinic sensor placed in the wall of the femoral artery in the lower limb of a rabbit. Shortly (340 ± 30 s) after the femoral artery was clamped, a rapid increase of NO concentration from its basal concentration of 52 ± 15 nmol l^{-1} ($P < 0.005$) was observed, with an average NO concentration increase of 0.2 nmol l^{-1} s^{-1}. The concentration of NO reached a peak of 140 ± 20 nmol l^{-1} ($n = 5$) after 15 min of ischemia, persisting for 2–3 min before decay at a rate of 0.15 ± 0.01 nmol l^{-1} s^{-1}. After ~ 50 min, the NO concentration decreased to its basal level, and after another 30 ± 5 min, the NO concentration dropped significantly below normal preischemic basal concentration. During reperfusion, a rapid decrease of NO concentration below 10 nmol l^{-1}

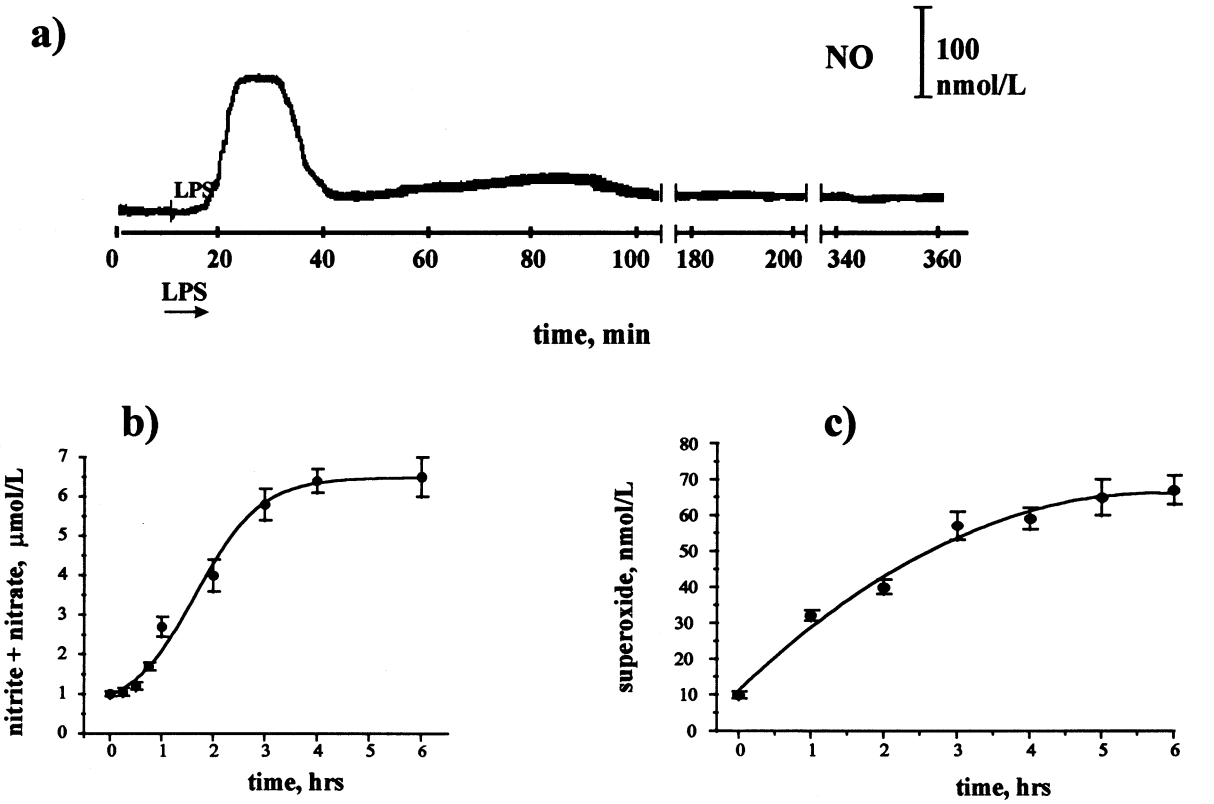

Fig. 3. (a) In vivo measurement of nitric oxide in the rat aorta during 6 h after administration of LPS (20 mg kg^{-1}); (b) UV–visible spectroscopic measurement of NO (as sum of NO_2^- and NO_3^- concentration) in blood during 6 h after administration of LPS (20 mg kg^{-1}); (c) In vitro chemiluminescence measurements of superoxide concentration in the lung tissue during endotoxemia; superoxide was measured during nitric oxide release stimulated by calcium ionophore (A21387, 8 μmol l^{-1}).

was noticed. After 30 min of reperfusion, a very slow increase of NO concentration to ~15 nmol l^{-1} was observed.

The L-arginine analog (L-NMMA) was used to inhibit NO release by endothelial NOS during the ischemia/reperfusion (I/R) experiment. This experiment was performed to verify that the signal recorded was really NO signal. Infusion of L-NMMA (20 mg kg^{-1}) before femoral artery occlusion caused significant changes of the kinetics of NO release for the duration of the I/R (Fig. 4b). The NO concentration decreased 10 min after administration of L-NMMA by 65% (from 52 ± 15 nmol l^{-1} to 18 ± 5 nmol l^{-1}). During the first 20 min of ischemia, the NO peak concentration was 40 ± 8 nmol l^{-1}, more than three times lower than in the absence of L-NMMA.

4. Discussion

From an analytical standpoint, the detection of NO at the site of highest concentration, the surface of the endothelial cell membrane, is the most convenient and accurate method for measurement of endogenous NO. Due to the hydrophobic properties of NO (partition coefficient between nonaqueous/aqueous phase = 6.3), the membrane is a storage reservoir for NO [27]. Therefore, a small volume membrane develops a relatively high steady-state concentration of NO within a short period of time after activation of NO-synthase. During the diffusion of NO through the aqueous phase, significant dilution occurs. Thus, in situ measurement of NO released from a single isolated cell, from a group of tissue culture cells, or

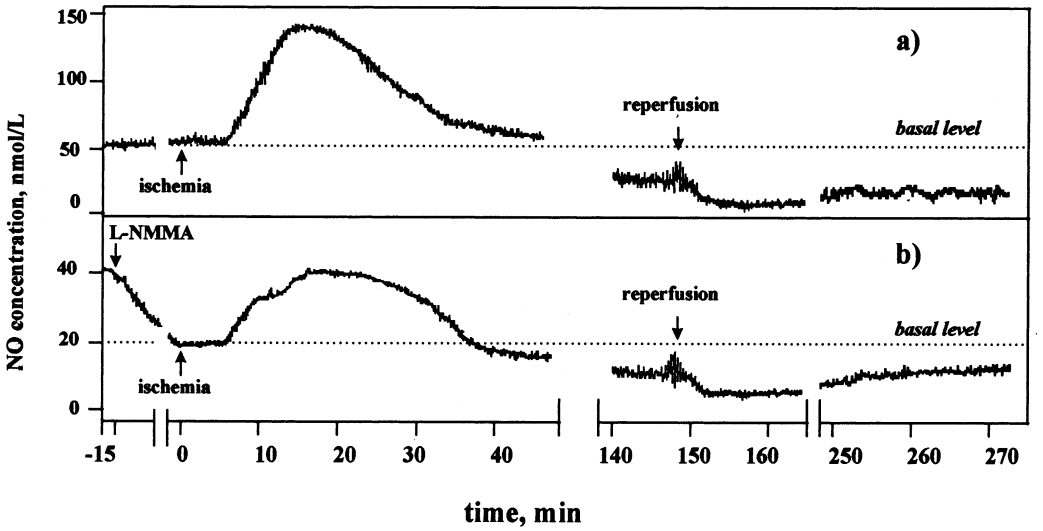

Fig. 4. (a) In vivo measurements of NO release in rabbit limb during ischemia/reperfusion; (b) L-NMMA administered before ischemia.

from an isolated artery, requires the positioning of the electrochemical porphyrinic sensor on the membrane surface of the endothelium or in close proximity. As can be seen in Fig. 1b, NO decreased exponentially with distance from the endothelial cell; therefore, even under static conditions of cell culture medium, it would be impossible to detect NO by the sensor at a distance greater than 50 μm. The diameter of the sensor (smaller than, or comparable to the diameter of the endothelial cell) allows its placement on or close to the membrane surface. These features of electrochemical detection with the sensor offer significant practical advantages over UV–visible spectroscopy, ESR spectroscopy, or biochemical assays in real time detection of NO.

An additional feature of electrochemical detection is the direct measurement of net NO concentration, and not a total concentration of NO produced by the endothelium, which includes its oxidation products NO_2^-, NO_3^-, or $ONOO^-$. The net NO concentration measured by the sensor is that concentration detectable after about a millisecond time, the response time of the sensor. This concentration of NO has real physiologic meaning since the NO can rapidly diffuse to smooth muscle cells and cause their relaxation.

A typical example of this unique application of the electrochemical sensor is a detection of NO release from normotensive and hypertensive rats. It has been reported based on spectroscopic measurements, that endothelium of hypertensive rats produced more NO_2^-/NO_3^- than endothelium of normotensive rats [28]. However these reports were in contradiction to data obtained on smooth muscle relaxation, hindered in hypertensive rats [29]. This means that the endothelium of hypertensive rats should produce less NO. Electrochemical measurements reported here (Fig. 2) clearly show that net concentration of NO produced by endothelium of SHR rats is lower than that produced by the endothelium of normotensive WKY rats. Our results correlate well with previously reported smooth muscle relaxation data. Total production of NO by the endothelium of hypertensive rats is slightly higher than in normotensive rats. However, the endothelium of SHR rats also generated significant amounts of superoxide which rapidly reacts ($k = 6 \times 10^9$ l mol^{-1} s^{-1}) with NO to produce the stable product peroxynitrite ($OONO^-$). Therefore, the net NO concentration as detected by the porphyrinic sensor is much lower in SHR rats as compared with WKY rats. In the presence of

membrane permeable PEG-SOD, an efficient conversion of O_2^- occurred, followed by a large increase of net NO concentration in SHR rats, finally exceeding that observed for WKY rats in the absence of PEG-SOD (Fig. 3b). Peroxynitrite when protonated ($pK_a = 6.8$) to HOONO usually undergoes isomerization ($t_{1/2} < 1$ s) to form hydrogen cation and nitrate anion. However, at high concentrations of NO and O_2^-, large concentrations of HOONO can be formed. Under these conditions HOONO may undergo cleavage to a hydroxyl free radical ($OH^{\bullet}$) a nitrogen dioxide free radical ($NO_2^{\bullet}$) or nitronium cation (NO_2^+) and hydroxide anion (OH^-). Three of these cleavage products ($OH^{\bullet}$, $NO_2^{\bullet}$ radicals and NO_2^+) are among the most reactive and damaging species in biological systems [15,18], and may be major contributors to the severe damage of endothelium and the cardiovascular system occurring in hypertension. A similar increase in O_2^- production by dysfunctional endothelium was also observed during endotoxemia and hypoxia (Fig. 3).

Induction of iNOS by bacterial toxins directly or through cytokines leads to generation of NO in arterial walls. An administration of toxin (LPS) also generated NO by calcium-dependent eNOS in the endothelium during the endotoxemia (Fig. 3). The high production of NO by the endothelium is observed only in the early acute phase of endotoxemia (first 25–40 min), however, it has a profound effect on the late chronic phase of endotoxemia when iNOS becomes the main generator of NO. The porphyrinic NO sensor measured only free NO; that is the net NO concentration not consumed in the extremely fast chemical reaction with O_2^- and other redox centers in the tissue. This free NO, measured intermittently, remained approximately the same during the second chronic phase of endotoxemia, in spite of high NO production by iNOS (after 45 min from LPS infusion), measured indirectly by assaying the accumulation of the NO decay products NO_2^-/NO_3^- in blood plasma by the Griess method. These data clearly indicate that the net concentration of NO is only two times higher during the late chronic phase of endotoxemia than the observed pre-endotoxemic basal concentration. This net NO concentration is much lower than the total NO produced during this period, but still sufficient to account for the coincident chronic hypotension observed. However, low net NO concentration cannot be accountable for a sudden death of animals after about 6 h of endotoxemia. Direct electrochemical measurements suggest that the dysfunction of endothelial cells eventually leading to the death of the animal is triggered by $O_2^-/OONO^-$ rather than by NO itself in endotoxemia.

Selectivity of the porphyrinic sensor is mainly based on very high current generated by NO. Several molecules found in the in vivo system can also be oxidized at the operational potential of the sensor. These include tyrosine, cysteine, serotonin, dopamine, and norepinephrine. However, the response of the porphyrinic sensor due to oxidation of these molecules is at least two orders of magnitude lower (at equimolar concentration) than for NO. In order to verify that a sensor response is actually due to oxidation of NO, the measurements must be routinely repeated in the presence of NOS inhibitors. L-Arginine analogs such as L-NMMA inhibit nitric oxide release by blocking active sites of NOS. A maximum inhibition of NO by L-NMMA is about 70–75%. The percentage of NO inhibition by L-NMMA was found to be on the level of 70% as measured directly during ischemia/reperfusion in rabbit (Fig. 4b).

5. Conclusions

The electrochemical method offers a unique opportunity for direct monitoring of kinetics and dynamics of nitric oxide release in a complex biological environment. The porphyrinic sensor measures trace concentrations of NO at the precise sensor location with a rapid response time. This observed concentration is a net NO concentration of physiological relevance, since NO can diffuse through tissues and cause smooth muscle relaxation. By contrast, the total NO concentration as measured by most spectroscopic methods or bioassays is an average concentration over a longer period, and includes NO consumed in rapid chemical reactions.

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