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V. BROVKOVYCH, L.W. DOBRUCKI, S. BROVKOVYCH, I. DOBRUCKI,
C.A. Do NASCIMENTO, A. BUREWICZ, T. MALINSKI

NITRIC OXIDE RELEASE FROM NORMAL AND DYSFUNCTIONAL ENDOTHELIUM

Center for Biomedical Research, Oakland University, Rochester, MI 48309, USA

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 (O_2^-) found in significant quantities. The porphyrinic sensor (0.5—8 μm diameter) and chemiluminescence methods were used to measure NO and O_2^- respectively. Effects of hypertension, low density lipoprotein (LDL), and heart preservation on the release of NO and O_2^- were delineated. In the single endothelial cell (rat aorta) NO concentration was the highest in the cell membrane decreasing exponentially with distance from cell, and becoming undetectable beyond 50 μm and 25 μm for normotensive (WKY) and hypertensive (SHR) rats respectively. The endothelium of SHR released 40% less NO (300 ± 25 nmol L^{-1}) than that of normotensive rats (500 ± 20 nmol L^{-1}), due to the higher production of O_2^- in SHR rats. An exponentially decreasing NO production (from 1.20 ± 0.15 to 0.10 ± 0.05 μmol L^{-1}) and concomitant increase of O_2^- generation (from 10 ± 0.3 to 300 ± 25 nmol L^{-1}) were observed in left ventricle of stored (eight hours) rabbit heart. Native and oxidized low density lipoproteins (nLDL and oxLDL) inhibited NO generation and increased O_2^- production. The local depletion of the L-arginine substrate may disarrange the nitric oxide synthase, leading to production of O_2^- from oxygen.

Key words: *Nitric oxide, superoxide, endothelium, vascular system, free radicals*

INTRODUCTION

Vascular endothelial cells contain the calcium dependent constitutive nitric oxide synthase (eNOS). The endothelium plays a critical role in maintaining vascular tone by releasing nitric oxide (NO) (1). Synthesis of NO is stimulated by chemical agonists (bradykinin, ATP and acetylcholine) and physical agonists (shear stress, flow and pressure). Ca^{2+} influx turns on NO biosynthesis by eNOS for about a minute; subsequently eNOS is turned off by phosphorylation of its serine residue (2). Compared with other messengers

(neurotransmitters and hormones), nitric oxide is an inferior chemical messenger. NO is extremely non-specific and cannot be stored and released on demand. Its high rate of isotopic diffusion ($D = 2.6 \times 10^{-5} cm^2 s^{-1}$) requires the generation of high amounts of NO to attain a given concentration at the target cell.

The activity of nitric oxide at any target site depends on its local concentration, which is determined by a combination of its rate of production and non-enzymatic destruction (3). The rate of production of NO can be limited by the local concentration of eNOS, Ca^{2+}, NADPH, tetrahydrobiopterin, oxygen and L-arginine. Therefore an oscillatory NO output can be observed, even when the intracellular level of Ca^{2+} is steady. The level of eNOS can be affected by that of Fe^{2+}, which is needed for its synthesis, while Fe^{3+} inhibits NOS synthesis. The enzymatic production of NO is down regulated by NO, which binds to eNOS.

The NO concentration is not homogeneous in the endothelial cell. Propagation of neutral NO in the hydrophobic cell membrane is much faster (permeability for NO is 26.9 cm s^{-1}) than propagation in an equivalent layer of aqueous cytoplasm. The cell membrane does not present a significant barrier to the diffusion of NO, and is not rate determining for NO propagation.

NO has been implicated in the pathogenesis of several diseases. The deficiency of NO on a target may play a role in hypertension, hyperglycemia, atherosclerosis, Parkinson's disease, Alzheimer's disease, and controversially, increased NO concentration may participate in rheumatoid arthritis, reperfusion injury, stroke and cancer (4). Thus from a pharmacological as well as medical aspect, it is important to quantify the details of NO production, its diffusion and propagation in abnormal and normal cells and tissues. 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 ($k = 6 \times 10^9 M^{-1} s^{-1}$) (5, 6). Consequently NO has a half-life of 2—5 s *in vivo* and detection of NO in biological systems has thus proven technically difficult. To study NO dynamics and its rate as a messenger one cannot use the build up of products (nitrate and nitrite), nor can one use NO swept out into the gas phase, to be determined by chemiluminescence. Also EPR cannot be used, because this technique does not detect unbound NO in the biological milieu. The only technique currently available that can be used for the studies of the release of NO and its propagation is an electrochemical assay of NO by a NO-selective electrode (7, 8). Using an electrochemical porphyrinic sensor (0.5—8 μm in diameter, response time of 0.1—1 ms) with a detection limit of 10^{-9} mol/L this study describes comparative *in vitro* measurements of NO release by eNOS in normal and dysfunctional endothelium.

MATERIALS AND METHODS

Nitric oxide and superoxide release in a stored heart

Male New Zealand white rabbits (2.5—3.0 kg, Kuiper Inc) were anesthetized with xylazine-hydrochloride (5 mg kg^{-1}) and ketamine-hydrochloride (50 mg kg^{-1}). After median sternotomy the heart was excised, perfused with cold physiological saline solution and placed into University of Wisconsin preservation solution (UW solution). Hearts were preserved up to eight hours. Nitric oxide and superoxide were measured in the myocardial wall (left ventricle).

Nitric oxide and superoxide release in aorta of SHR and WKY rats

The experiments were performed on aortas taken from male SHR and WKY rats (45—60 days old). Rats were anesthetized with mixture of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). The aorta was excised and immediately placed into cold HBSS. The isolated aorta was cleaned of adherent connective tissue under a dissection microscope.

Nitric oxide and superoxide release in endothelial cells

Human umbilical endothelial cells were obtained from American Type Culture Collection (cat. # CRL—1730). Cells were seeded in collagen coated flasks and monitored until 75% of the cell clumps adhere (0.5—1 hour). Non-adhering cells were poured off and the adhering cells were incubated within minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 and 95% air. The medium was changed every two days. After 4—6 days, the primary cultures formed a confluent monolayer. Cell monolayer from stock flask was dissociated by exposure for 2—3 minutes at 24°C to 0.05% trypsin in 0.15 mol L^{-1} NaCl, 0.01 mol/L sodium phosphate and 0.02% EDTA. When the cells rounded up, they were resuspended in MEM supplemented with 10% FBS and seeded at the final concentration of 2×10^4 cells/35 mm dish. 4 or 6 days later, when the cultures became confluent (4—5×10^5 cells/35 mm dish) the medium was replaced with MEM containing 5% human lipoprotein-depleted serum (LPDS). The cells were further incubated at 37°C for 12—14 hours and then treated for 1/2 hour with different concentrations of nLDL or oxLDL with or without pretreatment with 2 mM L-arginine. Nitric oxide release was measured from single endothelial cell. Superoxide was measured in cells treated with heparin and suspended in buffer solution.

nLDL preparation

Venous blood plasma from healthy normolipidemic volunteers was immediately separated by centrifugation at 3000 g for 10 min. at 4°C. LDL (d = 1.020—1.063 g ml^{-1}) was separated from freshly drawn plasma by preparative potassium bromide ultracentrifugation. The purified LDL was dialyzed for 96 hours against phosphate buffer solution (PBS) containing 0.3 mmol L^{-1} EDTA, at 4°C, then stored at 4°C in the dark and used within one week. Cholesterol content was determined according to the method of Kattermann (9).

oxLDL preparation

A 10 mg sample of native LDL was extensively dialyzed against Tris/NaCl buffer (50 mmol L^{-1} Tris in 0.15 mol L^{-1} NaCl, pH 8.0) to remove the EDTA. Tris-NaCl buffer was added to the

dialyzed native LDL to adjust the protein concentration to 30 mg ml^{-1}. 1 ml aliquot of 20 μmol L^{-1} $CuSO_4$ was added to 1 ml of dialyzed native LDL. Oxidation was performed at 37° C over 24 hours. The ox-LDL was then exhaustively dialyzed at 4° C with 4 L Tris buffer, filtered using a 0.22 μm filter, and stored under a nitrogen atmosphere at 4° C. Oxidation was monitored by measurements of thiobarbituric acid-reactive substances (TBARS). TBARS concentration was determined as malondialdehyde (MDA) equivalents using a MDA standard curve.

Nitric oxide measurement

Nitric oxide was measured with a porphyrinic sensor according to the procedure published previously (7, 8). A three-electrode system was used for the nitric oxide measurements, consisting of the porphyrinic sensor working electrode, standard calomel reference electrode, and a platinum wire counter electrode. An EG&G PAR Model 283 Potentiostat/Galvanostat in amperometric mode at constant potential of 0.65 V was used for the measurements. Cell or tissue was placed in fresh Hank's balanced salt solution (HBSS), containing 137 mM NaCl, 8 mM KCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 0.44 mM K_2HPO_4, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Tris-HCl, and 0.1 mM L-arginine. Working electrode was placed with the help of a computer controlled micromanipulator (resolution ±1μm) on the surface of the tissue. During cell culture experiments, working electrode was placed on surface of cells monolayer. A solution of calcium ionophore A23187 (final concentration 10 μmol L^{-1}) was injected to reach maximum nitric oxide release. The nitric oxide concentration was determined from the measured current by means of a calibration curve with nitric oxide standard (10).

Superoxide measurement

Superoxide release was measured by a chemiluminescence method (11). Superoxide produced chemiluminescence of lucigenin (bis-N-methylacridinium nitrate), which was detected by a scintillation counter (Beckman 6000LS, with a single photon monitor). Tissue samples (0.8 to 1.5 mg) or cells culture mixture were placed in 2 ml of HBSS, then enough lucigenin was added to make its concentration 0.25 mM. Basal superoxide concentration produced by sample was measured after 2 minutes incubation in HBSS. After that 20−μl of 1 mM A23187 was injected to reach maximum superoxide release (basal plus calcium ionophore stimulated O_2^- release). Photon counts were calibrated as superoxide concentration by construction of standard curves based on photons emitted by superoxide stoichiometrically generated by treatment of xantine with xantine oxidase.

Drugs and chemicals

Calcium ionophore A23187, polyethylene glycol-superoxide dismutase (PEG-SOD), L-arginine and the chemical components of the modified HBSS were obtained from Sigma[R].

Statistical analysis

Statistical evaluation was done using ANOVA followed by Student-Newman-Keuls test. Values are expressed as means±SEM, with $p<0.05$ considered statistically significant.

RESULTS

NO release in a single cell

Figure 1 shows an amperogram (current vs. time) of NO released from a single isolated endothelial cell (rats aorta). A porphyrinic sensor was placed on the surface of endothelial cell. NO release was stimulated with calcium ionophore (A23187, 10 μmol L^{-1}). About 0.8−1.1 s after injection of the calcium ionophore, a rapid increase of NO concentration was observed with a rate of NO increase 192±9 $nmolL^{-1}s^{-1}$ (n = 5). After about 3.6 s, the maximal peak concentration (500±20 $nmolL^{-1}$) was observed. The duration of plateau of the peak was about one second after which NO steadily decreased with an average NO decrease of 133±6 nmol $L^{-1}s^{-1}$. Peak NO concentration was higher (500±20 nmol L^{-1}) in WKY than in SHR rats (300±25 $nmolL^{-1}$).

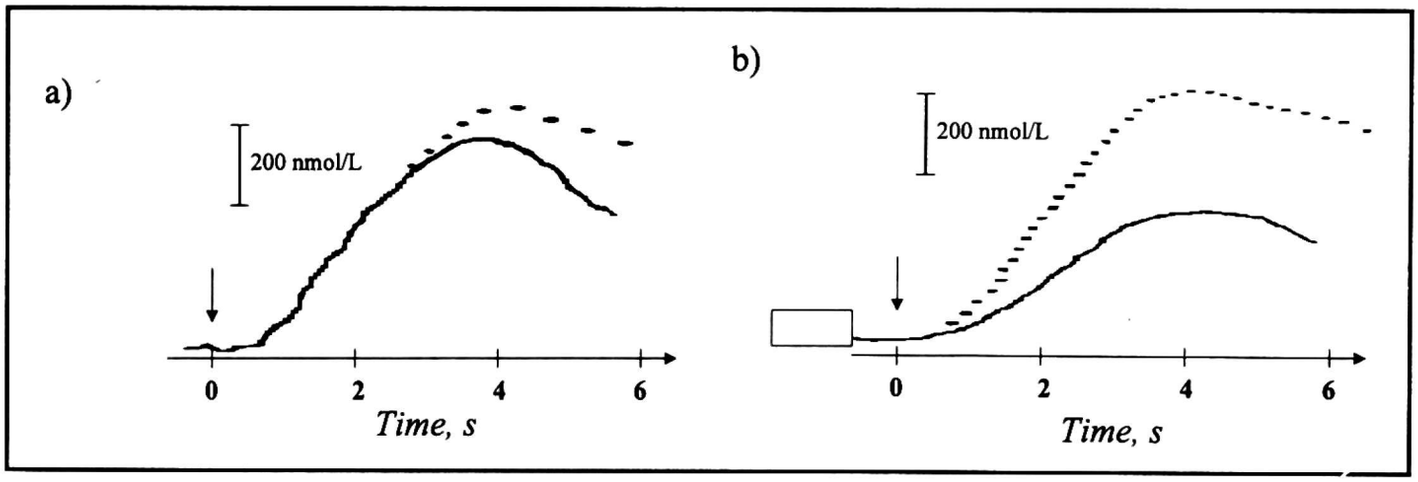

Fig. 1. Amperogram showing nitric xide release from single endothelial cell (aorta) of (a) WKY rat and (b) SHR rat in the presence (dashed line) and absence (solid line) of PEG-SOD. NO release was stimulated by calcium ionophore A23187 (10 $\mu MolL^{-1}$).

Amperometric curves showing the change of NO concentration with time were also recorded in the presence of membrane permeable superoxide dismutase with attached polyethanol glycol 400 (PEG-SOD activity 100 U ml^{-1}). Since PEG-SOD rapidly dismutase superoxide (O_2^-), we use this indirect approach to estimate production of O_2^- at the time of NO release (*Fig. 1*). 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 10%. However, for SHR rats this increase of NO peak concentration was much higher (80%) in the presence of PEG-SOD than for WKY rats. This finding indicates that a significant concentration of O_2^- is generated within the heart of hypertensive rats and that superoxide consumed a portion of NO in a fast chemical reaction. The process of NO consumption by O_2^- affects the distance of NO diffusion when it is released from a single endothelial cell (*Fig. 2*). The height of the peak of NO release from a single cell

depends on the distance of the sensor from the cell membrane. The highest NO concentration was observed on the cell membrane (500 ± 20 nmolL^{-1}), with concentration decreasing exponentially with the distance from the cell membrane. The NO concentration decreased to 15 ± 5 nmolL^{-1} for WKY cells and at a distance 50 μm from the cell membrane, NO concentration was not detectable by the porphyrinic sensor. The distance of the diffusion of NO from endothelial cells is even shorter for SHR cells. The initial concentration at the surface of cell membrane is 300 ± 25 nmolL^{-1} and also decreased exponentially when NO was released from endothelial cells of SHR rats. The decrease is rapid and at the distance 20 μm from the membrane of endothelial cell the NO concentration is only 20 nmolL^{-1} and at a distance higher than 30 μm from the surface of endothelial SHR cells, NO cannot be detected. An initial concentration of NO on the cell membrane of WKY and SHR cells depends clearly on the concentration of superoxide, which can be released simultaneously in endothelial cells. The concentration of superoxide was measured during nitric oxide release, stimulated by calcium ionophore (*Fig. 2c*). Superoxide concentration is significantly different for WKY and SHR cells. For WKY cells O_2^- concentration of 22 ± 4 nmolL^{-1} was observed, for SHR cells the concentration of superoxide was 73 ± 7 nmolL^{-1}. Also the basal concentration of superoxide (the concentration before the stimulation with calcium ionophore) is slightly higher for SHR cells than for WKY cells. For SHR cells the basal concentration of superoxide was 15 ± 3 nmolL^{-1} while for WKY cells the basal concentration of superoxide was 12 ± 2 nmolL^{-1}.

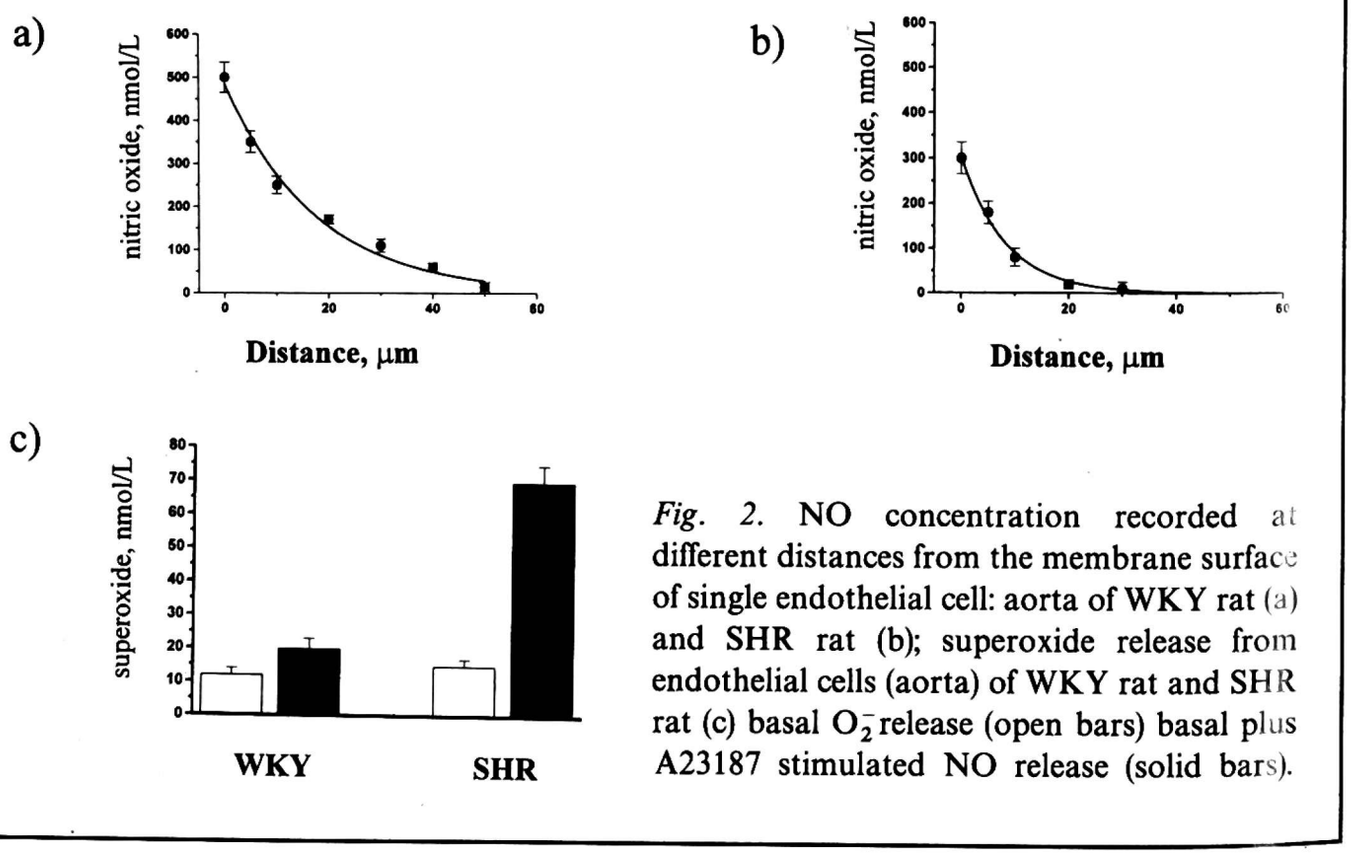

Fig. 2. NO concentration recorded at different distances from the membrane surface of single endothelial cell: aorta of WKY rat (a) and SHR rat (b); superoxide release from endothelial cells (aorta) of WKY rat and SHR rat (c) basal O_2^- release (open bars) basal plus A23187 stimulated NO release (solid bars).

Nitric oxide distribution in the heart

Figure 3 shows the distribution of nitric oxide in the left ventricle (myocardium) of rabbit heart. NO concentration was measured *in vitro* after stimulation with calcium ionophore. The concentration of NO on the top of the left ventricle was about 0.60 ± 0.05 $\mu molL^{-1}$ and steadily increased reaching maximum, at the distance of about 40% from the top of the left ventricle. This maximum NO concentration was 1.10 ± 0.06 $\mu molL^{-1}$. After the peak and small plateau, a linear decrease of NO was observed reaching a level of 0.65 ± 0.08 $\mu molL^{-1}$ in the apex. This data indicate that NO concentration when measured in the heart or in any other segment of the cardiovascular system has to be measured within very well defined area.

Maximum of NO and O_2^- release (after stimulation with calcium ionophore) was measured in the left ventricle (myocardium; 40% of distance from the top) of heart preserved up to eight hours in University of Wisconsin preservation solution (*Fig. 4*). Nitric oxide concentration decreased exponentially, while superoxide concomitantly increased with time.

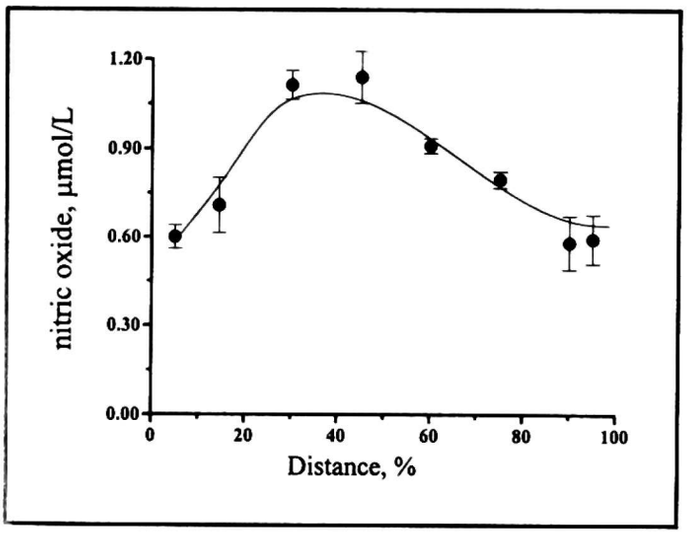

Fig. 3. NO distribution (vertical distribution expressed as % distance from the top of left ventricle) in the left ventricle of rabbit myocardium.

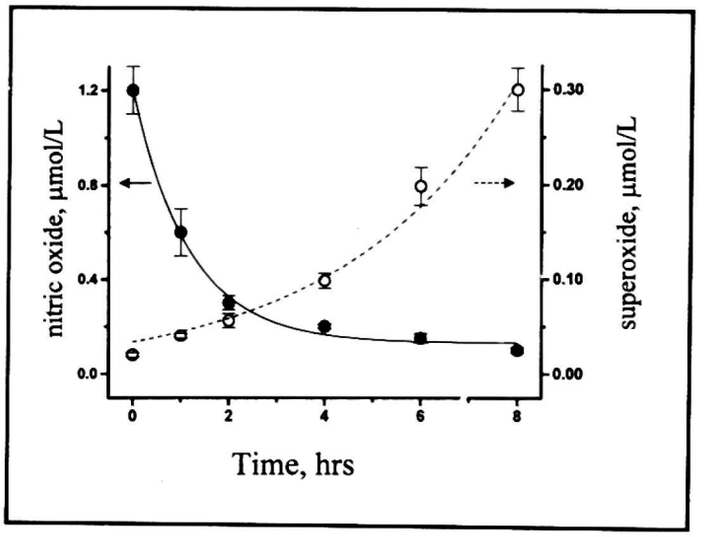

Fig. 4. NO (solid line) and superoxide (dashed line) release (stimulated by A23187) from the heart of myocardium (left ventricle) after storage of the rabbit heart in University of Wisconsin preservation solution

NO concentration decreased by 74% (from original 1.10 ± 0.06 $\mu molL^{-1}$) after two hours of preservation and by 91% after eight hours of preservation. Superoxide increased from 20 ± 2 $nmolL^{-1}$ to 50 ± 8 $nmolL^{-1}$ and 300 ± 20 $nmolL^{-1}$ after two and after eight hours of preservation respectively. NO concentration decreased with increasing concentration of lipoproteins. Endothelial cells (human umbilical endothelial cells) were exposed to the increasing concentration of nLDL for 30 min. A steady decrease of NO concentration with an average rate 3.25 ± 0.15 nmol mg^{-1} with increasing

concentration was observed up to 60 mg/dl of nLDL (*Fig. 5*). Beyond 60 mg/dl of nLDL the NO level reached a low NO production plateau (about 20% of the control).

The initial decrease of NO production was accompanied by a steady increase of superoxide concentration up to about 40 mg/dl of nLDL. In the range of nLDL concentration between 40 and 100 mg/dl a dramatic increase of superoxide concentration was observed.

The same set of experiments was done exposing endothelial cells for half-hour to increasing concentration of oxLDL. Ox-LDL caused a sharper decrease in NO concentration compared to nLDL. NO decreased about 80% (compared to control) at oxLDL concentration of 20 mg/dl. Simultaneously, a 7-fold increase of superoxide production was observed. Finally, at oxLDL concentration of 40 mg/dl the superoxide level reached a plateau at 128 ± 5 $nmolL^{-1}$ (control 10 ± 2 $nmolL^{-1}$).

L-arginine pre-treatment resulted in a significant increase of NO production in nLDL as well as in oxLDL treated cells. In both cases, nLDL and oxLDL concentration of 80 mg/dl the NO level was two times higher than before L-arginine supplementation (*Fig. 6*).

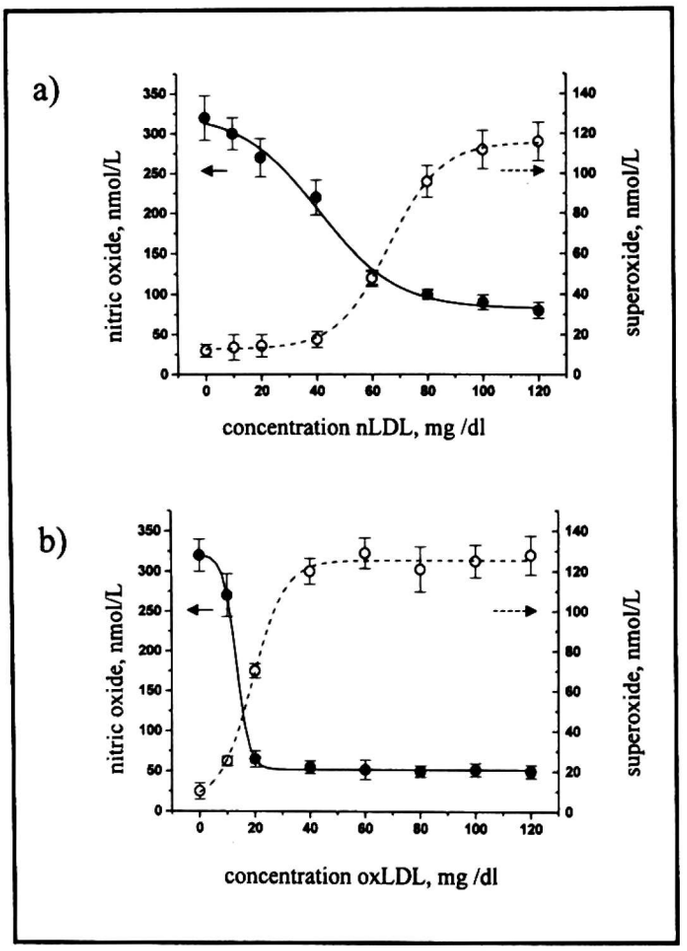

Fig. 5. A23187 stimulated nitric oxide (solid line) and superoxide (dashed line) release from endothelial cell (human umbilical endothelial cells) in the presence of nLDL (a) and oxLDL (b).

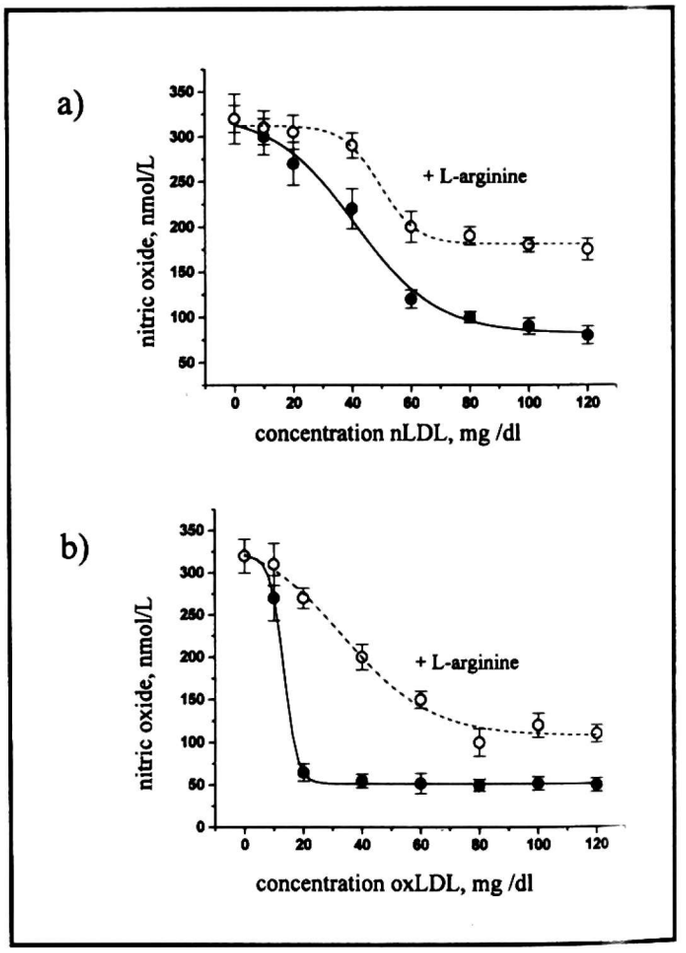

Fig. 6. A21387 stimulated nitric oxide release from endothelial cells (human umbilical endothelial cells) incubated with L-arginine (dashed line) and without incubation (solid line) in the presence of nLDL (a) and oxLDL (b)

DISCUSION

By using the porphyrinic microsensor, a method suitable for measurements of small difference in NO concentration in a time-frame of a millisecond, the kinetic NO release was investigated in normal and dysfunctional endothelium. This study provides direct evidence that in dysfunctional endothelium NO production decreases with a parallel increase in O_2^- generation.

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 is 6.3), the membrane is a storage reservoir for NO (3). 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, a significant dilution occurs. Thus, *in situ* measurement of NO released from a single isolated cell, from a group of tissue culture cells or 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. 2*, NO decreased exponentially with distance from endothelial cell, therefore, even under static conditions of cell culture medium it would be impossible to detect NO at the distance beyond 50 μm and 30 μm for normal and dysfunctional endothelium (rats aorta). NO release depends primarily on the location of endothelium being the highest in the left ventricular endocardium. However, in myocardium distribution of NO concentration is not homogeneous and may var significantly being highest at a distance 50—60% from the apex.

It has been reported based on spectroscopic measurements, that endothelium of hypertensive rats produced more NO_2^-, NO_3^- than endothelium of normotensive rats (12). However, these reports were in contradiction to data obtained on smooth muscle relaxation, hindered in hypertensive rats (13). This means that the endothelium of hypertensive rats should produce less nitric oxide. Electrochemical measurements reported here (*Fig. 1*) clearly show that the net concentration of NO produced by endothelium of SHR rats is lower than that produced by 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 normotensive rats. However, the endothelium of SHR rats also generated a significant amount of superoxide which rapidly reacts ($k = 6 \times 10^9$ $Lmol^{-1}s^{-1}$) with NO to produce the unstable product peroxynitrite ($OONO^-$). Therefore, the net NO concentration as detected by the porphyrinic sensor is much lower in SHR rats as compared to WKY rats. In the presence of membrane permeable PEG-SOD, an efficient conversion of superoxide

occurred, followed by the large increase of net NO concentration in SHR rats finally exceeding that observed for WKY rats in the absence of PEG-SOD (*Fig. 2c*). Peroxynitrite when protonated ($pK_a = 6.8$) to HOONO usually undergoes isomerization ($t_{1/2} < 1s$) to form hydrogen cation and nitrate anion (*Fig. 7*). However, at high concentration of NO, and O_2^-, large concentrations

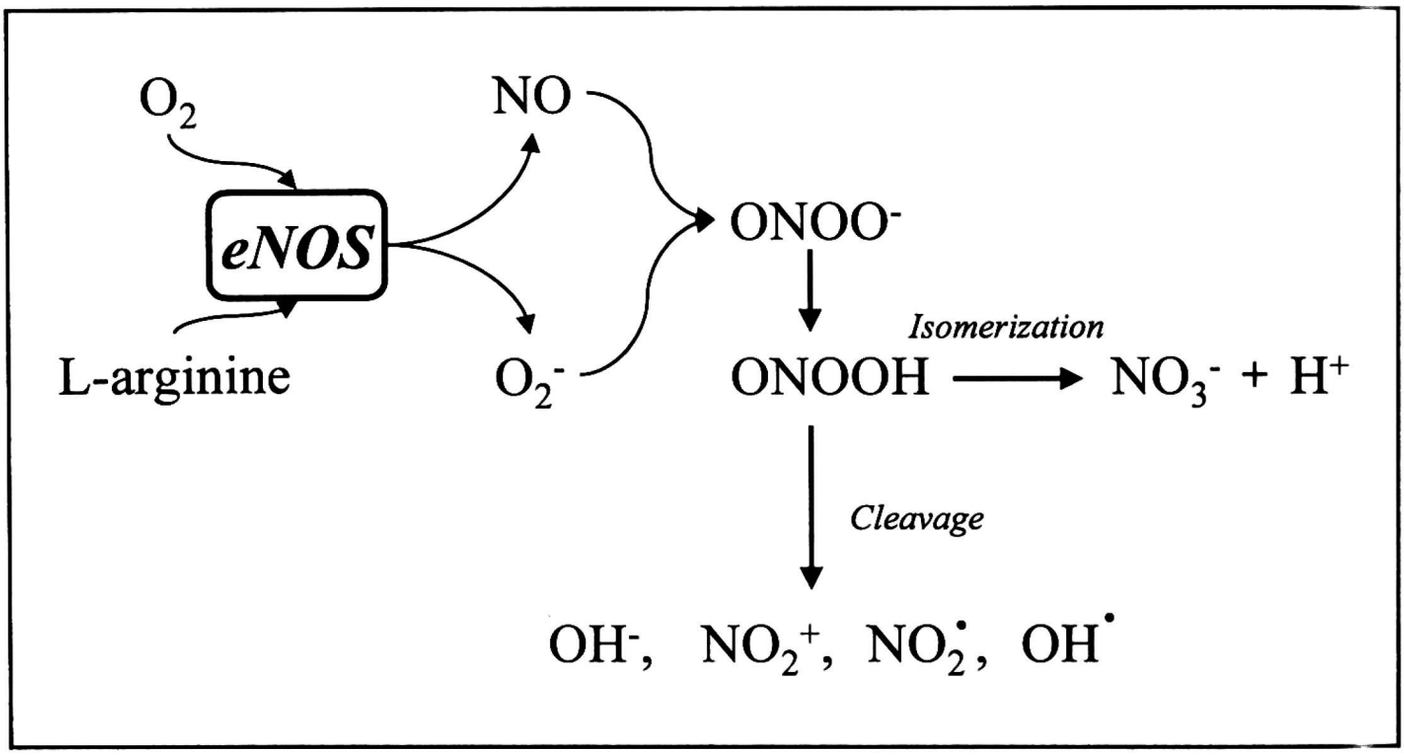

Fig. 7. A pathway for generation of highly oxidizing species by eNOS

of HOONO can be formed. Under these conditions HOONO may undergo cleavage to a hydroxyl free radical (OH*), nitrogen dioxide free radical (NO_2^*) or nitronium cation (NO_2^+) and hydroxide ion (OH^-). Three of these cleavage products (OH*, NO_2^* and NO_2^+) are among the most reactive and damaging species in the biological systems (14), and maybe major contributors to the severe damage of endothelial and the cardiovascular system occuring in hypertension. A similar increase of superoxide production by dysfunctional endothelial was also observed in the presence of nLDL and oxLDL (*Fig. 6*). Other studies of NO production in cells incubated with nLDL and oxLDL demonstrated that high level of nLDL cause a decrease in NO production, but were carried out by using indirect methods based on the determination of NO metabolic products (15). Furthermore the precise LDL concentration in which an early loss of NO production can be observed has not yet been established. Our findings indicate that the endothelial NO production is already altered at low nLDL concentration and also demonstrate that physiological concentrations of nLDL perturbed the endothelial cell metabolism and lead to the increase of O_2^- production. The positive effect of L-arginine supplementation on NO production mainly suggests that L-arginine transport

maybe impaired in lipoprotein-treated cells. Therefore, the decrease of NO and increase of O_2^- production by dysfunctional endothelium maybe correlated with inadequate L-arginine/eNOS coupling. Under normal physiological conditions endothelial NOS activity is triggered by the calmodulin binding which occurs in response to any phenomenon that elevates intracellular calcium ion concentration (6). Any process hindering the transport of L-arginine to the cytoplast or consuming a large amount of this amino acid, or reducing the quantity of L-arginine transported through the cell membrane may cause the depletion of this limiting substrate just as the derangement of other enzymes occurs at deficient substrate concentration. The depletion of arginine may eventually lead to derangement of oxidase/reductase domains of eNOS (6). It has been already documented that NOS, when turned on in L-arginine — poor environment, is still able to receive electrons from NADPH and donate them to its other substrate oxygen, resulting in one electron reduction to form O_2^- (6). eNOS in the endothelial cells seem to be the most active source of O_2^- production. This finding shaded the role of superoxide in the impaired NO production and suggest that the amount of L-arginine may play a key role in NO generation and acts complimentary with the increased production of O_2^- perturbing endothelial function in this way. The relatively high levels of O_2^- produced by endothelial cells incubated within increasing concentrations of nLDL and oxLDL were decreased when the cells were supplemented with L-arginine. These results indicate that the local depletion of L-arginine may cause the derangement of eNOS leading to an overproduction of superoxide which can react very rapidly with NO to form $ONOO^-$.

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Author's address: T. Malinski, Center for Biomedical Research Oakland University, Rochester MI 48309, USA