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V. BROVKOVYCH, S. PATTON, S. BROVKOVYCH, F. KIECHLE [1], I. HUK [2], T. MALIŃSKI *

IN SITU MEASUREMENT OF NITRIC OXIDE, SUPEROXIDE AND PEROXYNITRITE DURING ENDOTOXEMIA

Department of Chemistry, Institute of Biotechnology, Oakland University, Rochester, MI USA, [1] Department of Clinical Pathology, William Beaumont Hospital, Royal Oak, MI USA, [2] Department of Vascular Surgery, University of Vienna, Vienna, Austria

We report *in vivo* and *ex vivo* measurements of nitric oxide (electrochemical), *ex vivo in situ* measurements of superoxide (chemiluminescence) and peroxynitrite (chemiluminescence) and delineate the effect of endotoxemia on nitric oxide, superoxide and peroxynitrite release in aorta of rats. Nitric oxide release was measured in the aorta wall. An increase of nitric oxide concentration was observed immediately after administration of lipopolysaccharide (*Escherichia coli* serotype 0127: B8, 20 mg/kg), reaching a plateau ((50 nmol/L) after 180 ± 50 seconds; the plateau was followed by decreasing nitric oxide concentration and its subsequent gradual small increase after 45 minutes. Superoxide and peroxynitrite production increased dramatically during endotoxemia. Superoxide concentration increased from 10 ± 2 nmol/L to 28 ± 3 nmol/L at one hour, and reached a 50 nmol/L plateau at 4 hours. The pattern of peroxynitrite release paralleled the pattern of superoxide release during the time course of endotoxemia. Diametrical alteration of nitric oxide and superoxide concentration with subsequent production of peroxynitrite may be a major cause of endothelial cell injury during endotoxemia.

K e y w o r d s: *aorta, rat, lipopolysaccharide, endothelium, chemiluminescence, amperometry.*

INTRODUCTION

Nitric oxide (NO) generated by constitutive nitric oxide synthase (cNOS) is essential for homeostasis in the cardiovascular system, and also serves as a chemical messenger in the central and peripheral nervous system (1). Dysfunction of cNOS in the vascular endothelium has been implicated in the pathogenesis of ischemia/reperfusion injury, hypertension, and hypercholesterolemia (2).

The generation of NO is also important for nonspecific cytostatic host defense against tumor cells and pathogens. The signal to transcribe/translate

the gene sequence for inducible nitric oxide synthase (iNOS) can be transmitted by several endotoxins. In rodents, iNOS expression occurs mainly in macrophages, but also in several other cells types, including endothelial cells (3). Excessive, continuous NO-release from iNOS, over prolonged periods, under pathological conditions contributes significantly to circulatory failure, hypotension and septic shock (3). Based on direct measurement assessed by the accumulation of the NO decay products NO_2^- or NO_3^-, or the iNOS co-product L-citrulline, or the product of a NO activated enzyme cGMP, it has been assumed that the concentration of NO increases significantly during septic shock (4). This high NO concentration causes vasorelaxation, followed by cytostasis, and may initiate a cascade of events that results in cytotoxic effects.

Mammalian iNOS produces sufficient NO concentration ($> 1\,\mu mol/L$) to locally inhibit ribonucleotide reductase, the enzyme that converts ribonucleotides to the deoxyribonucleotides necessary for DNA synthesis; hence, the profound cytostatic effect of NO on the proliferation of rapidly dividing tumor cells or pathogens (5). In addition, DNA synthesis is a fundamental step in normal cell proliferation. Even NO concentration from mammalian cNOS can be high enough to inhibit local ribonucleotide reductase; thus halting the proliferation of cardiac myocytes and the smooth muscles around major arteries (6).

Cytotoxicity is often directly attributed to NO, which is often erroneously described in the biological literature as a highly reactive molecule. In reality, NO has all the desirable properties of a biological messenger molecule, it does not react with water and is only moderately reactive, making it a somewhat selective reactant (7). Also NO is small and neutral and only slightly more soluble in the lipid membrane than the aqueous cytosol, assuring its free diffusion across cell membranes just like dissolved oxygen (8). The only thing extreme about NO is its extremely fast reaction with superoxide (O_2^-, $K = 6.7 \times 10^9\ L \cdot mol^{-1} \cdot s^{-1}$) to form peroxynitrite ($OONO^-$), one of the fastest reactions in the liquid phase (9). $OONO^-$ is relatively stable; but, when protonated ($pK_a = 6.8$) the peroxynitrous acid (HOONO) formed usually rearranges to the relatively harmless waste products hydrogen cation and nitrate anion (10). However under certain pathological conditions, like ischemia/reperfusion or endotoxemia, sufficiently high concentration of HOONO may accumulate to assure its efficient transport to biomolecular targets several cell diameters away (10).

In the presence of certain reactive centers, HOONO can undergo homolytic cleavage to form hydroxyl free radical (OH) and nitrogen dioxide free radical (NO_2), or heterolytic cleavage to form nitronium cation (NO_2^+) and hydroxide anion (OH^-) (10). Three of these moieties (OH, NO_2, NO_2^+) are among the most reactive and damaging species in biological systems each of which could

initiate a cascade of events resulting in severe tissue damage and the ultimate death of the host animal (11). These three extremely reactive HOONO cleavage products are most likely the source of the observed cytotoxicity erroneously attributed to NO.

Indirect measurements of nitric oxide based on NO_2^-/NO_3^-, L-citrulline, or cGMP concentration may provide a very misleading picture of NO release and its real (active) concentration in the tissue during endotoxemia. This paper describes direct *in situ* measurements of the change of net NO production (the portion which does not react with O_2^-) during endotoxemia. In these experiments, continuous *in vivo* measurement of NO concentration were correlated with intermittent measurements of O_2^- and $OONO^-$ concentration.

MATERIAL AND METHODS

Animals

Normotensive Wistar Kyoto (WKY) rats with body weight 300 ± 20 grams and systolic blood pressure of 115 ± 5 mmHg were obtained from Harlen Sprague Dawley, Inc. For *in vivo* measurements, rats were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, intubated and ventilated with room air using a Harvard small animal ventilator set at a tidal volume of 2.5 mL and a breathing rate of 100 breaths/minute. A porphyrinic sensor was implanted in a wall of the aorta and its exact localization was confirmed by postmortem section.

Preparation of aorta for in vitro measurements

Rats were decapitated, and the aorta was removed and placed in (4°C, pH 7.40) modified Hanks' balanced salt solution (HBSS, Sigma Chemical Co., St. Louis, MO) of the following composition (in millimoles per liter): NaCl 137, Tris-HCl 10, $MgCl_2$, 1, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, KH_2PO_4 0.44, Na_2 HPO_4 0.33, and L-arginine 0.1. The aorta was cleared of adhering tissue under a dissection microscope and cut into small segments (2—3 mm long) under a dissection microscope (Wild M3G, Wild AG.)

Preparation of NO sensors for in vitro and in vivo measurements

NO was measured with a porphyrinic microsensor that was free of interference (at physiologic conditions) from all reagents used in these experiments and all known readily-oxidizable secretory products that may be found in mammalian blood to at least two orders of magnitude greater than their expected concentrations. Moreover, the NO sensors were not effected by ambient light (12) or blood pressure fluctuations, and had an acceptably small thermal coefficient (a 1.7—1.9% increase in signal/°C) (13). Also, the NO sensors were remarkably free of piezoelectric noise generated by contracting muscles or the cardiac conducting system (14).

The porphyrinic sensors for *in vitro* and *in vivo* measurements were prepared by methodologies described in detail previously (3, 15, 16). Briefly, the needle from a intravenous cathether unit

(24 gauge, 25 mm long, Angiocath®, Becton-Dickinson) was roughened along the shaft, then truncated and polished flat so that it was 3 mm shorter than its 24-gauge, 25-mm-long catheter. A single carbon fiber (6 μm in diameter, protruding 3 mm, Amoco) was mounted inside the hollow truncated 24 gauge needle with the aid of a 0.1 mm diameter bare copper wire coated with conducting epoxy. After curing, the exterior of the truncated needle was coated with non-conductive epoxy (2-TON®, Devcon) and allowed to cure again.

The protruding 3-mm carbon fiber tip was made more sensitive to NO and less sensitive to possible interferences by the cyclic voltammetric deposition (from −0.20 to 1.00 V and back at 0.10 V/sec for 10 cycles) of a highly conductive polymeric porphyrin from a solution of 0.50 mmol/L nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin (TMHPPNi, synthesized as previously described (21)) in 0.1 mol/L NaOH, under nitrogen. Dip coating the dried polymerized TMHPPNi coated carbon fiber tip (three times for 5 s) in 1% Nafion in alcohol (Aldrich), after drying, produced a thin anionic film that repelled or retarded charged species while allowing small neutral and hydrophobic NO access to the underlying catalytic surface.

A single carbon fiber sensor is flexible and can be bent and placed directly on the surface of a microvessel without catheter protection for *in vivo* or *in vitro* measurements. An opened aortic ring was immersed in physiologic solution and positioned under a dissecting microscope. A porphyrinic sensor was lowered with the help of a stereotactic micromanipulator until the surface of the observed blood vessel was reached. This was indicated by a small (picoampere) and short (millisecond) piezoelectric signal. Two auxiliary electrodes (platinum, and silver/silver chloride, SSCE) were positioned in a physiologic solution near the opened aortic strip.

To implant the porphyrinic NO sensor in the wall of the aorta, smooth muscle tissue was pierced with a standard 24-gauge angiocatheter needle (clad with its catheter with 4×50 mm ventilation holes near the tip). The catheter/needle unit was advanced to a desired place in the aorta wall. The position of the catheter was secured, and the placement needle was removed and replaced by a truncated needle with mounted porphyrinic NO sensor. A platinum wire counter electrode and SSCE were placed in contact with adjacent tissue.

Two techniques for measuring NO: differential pulse voltammetry (DPV) and chronoamperometery (CA) were performed with a PAR model 273 voltammetric analyzer interfaced with an IBM 80486 computer with data acquisition and control software. DPV was used to measure the basal NO concentration. Briefly, in the DPV method, current vs potential curves were generated in the potential range between 0.45 and 0.75 V vs SSCE. The DPV peak current at the peak potential characteristic for NO oxidation (0.65 V) was found to be directly proportional to the local NO concentration in the immediate vicinity of the sensor. CA, fixed at the peak potential for the oxidation od NO vs SSCE, was used for fast (resolution time 0.1 to 1 ms) and continuous measurement of the changes of NO concentration from its basal level with time.

The volume sampled is approximately equal to the volume of the sensor ($10^{-10} - 10^{-12}$ L). Therefore, the concentration of NO measured was a local or surface concentration (not a bulk or global concentration). The porphyrinic microsensor had a response time of 0.1 milliseconds at micromolar NO concentrations and 10 milliseconds at the detection limit of 1 nmol/L. Linear calibration curves were constructed for each sensor from 2×10^{-9} to 2×10^{-5} mol/L NO, before and after *in vivo* or *in vitro* measurements, with aliquots of saturated NO prepared as described (17).

Superoxide measurements

O_2^- was measured *in vitro* in rabbit aorta rings. The concentration of O_2^- was determined by a chemiluminescence method (18). O_2^- produced chemiluminescence of lucigenin

(bis-N-methylacridinium nitrate), which was detected with a scintillation counter (Beckman 6000 LS, with a single photon monitor). Each tissue sample (0.8—1.5 milligrams) was placed in 2 mL of Hanks Balanced Salt Solution (HBSS) adjusted to pH 7.4 at 25°C, then enough lucigenin was added to make its concentration of 0.25 mmol/L. Basal O_2^- concentration produced by the tissue was measured after a two minute incubation in HBSS. The sum of the O_2^- produced by disarranged cNOS and other sources (basal) was measured in a similar manner, except that the two minute incubation period was followed by injection of 20 μL of 1 mmol/L A23187 calcium ionophore (a receptor independent cNOS agonist). Photon counts were calibrated as O_2^- concentration by construction of standard curves based on photons emitted by O_2^- stoichiometrically generated by treatment xanthine with xanthine oxidase. The measured concentration of O_2^- (unlike the sensor monitored NO) represents an average concentration in the volume of the sample and is reported per 1 milligram of wet tissue.

Peroxynitrite measurement

Concentration of $OONO^-$ was determined directly by another chemiluminescence method in freshly excised tissue as described previously (19). $OONO^-$ produced chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-phathalazinedione), which was detected with scintillation counter (Beckman 6000 LS, with a single photon monitor). Each tissue sample (8—10 milligrams) was placed in 0.5 ml of Hanks Balanced Salt Solution (HBSS) adjusted to pH 7.4 at 25°C, then enough calcium ionophore (A23187) solution was added to make its concentration of 20 μmol/L. After 3 s 1.0 ml alkaline sodium bicarbonate luminol solution was added to achieve a final concentration of luminol (400 μmol/L), bicarbonate (50 mmol/L) and pH 10.0. Photon counts were recorded immediately after luminol addition. The sum of $OONO^-$-dependent photons produced by calcium ionophore was calculated after the substraction of photons count, obtained in the same condition without tissue.

RESULTS

Fig. 1a shows a typical amperometric curve (current calibrated as NO concentration versus time) measured *in vivo* during endotoxemia with a porphyrinic sensor placed in the wall of the aorta. During administration of 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 $L^{-1} \cdot s^{-1}$. The concentration of NO reached a peak of 150 ± 20 nmol/L after 180 ± 15 seconds, which persisted for 12 ± 2 minutes and then decayed at a rate of -0.20 nmol $\cdot L^{-1} \cdot s^{-1}$. After 40 minutes NO concentration started to rise again, but at much slower rate. The rate of increase of NO concentration was 0.020 ± 0.005 nmol $\cdot L^{-1} \cdot s^{-1}$ significantly slower, than for first NO peak. A plateau of release was established after 70 ± 10 minutes from LPS administration and after 90 ± 8 minutes a small decay of NO concentration was observed.

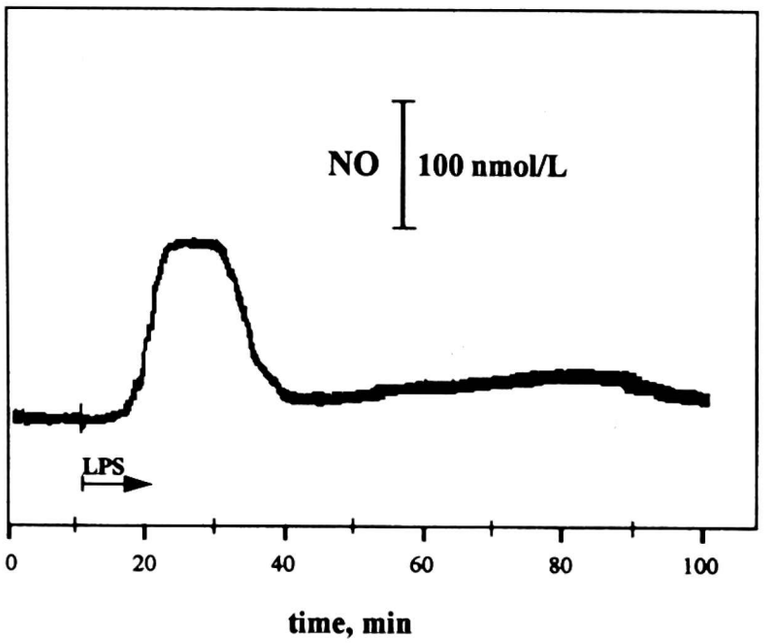

Fig. 1. In vivo measurement of nitric oxide (NO) release in rat aorta during the first 100 minutes after administration of LPS (20 mg/kg).

Changes of endothelial NO concentration released from freshly excised aorta after stimulation with the receptor independent (agonist calcium inophore A23187, 10 μmol/L) were measured *in vitro* at different time after LPS administration. A single-fiber porphyrinic sensor placed close to the surface (5—7 μm from the source) of endothelial cells in opened segment of the aorta. The amperometric curves showing the change of endothelial NO concentration with time recorded in the absence and presence of membrane permeable superoxide dismutase (PEG-SOD 100 U/ml) are depicted in *Fig. 2*. We used this indirect approach to estimate production of O_2^- at the time of calcium stimulated NO release from endothelium during time of progressing endotoxemia.

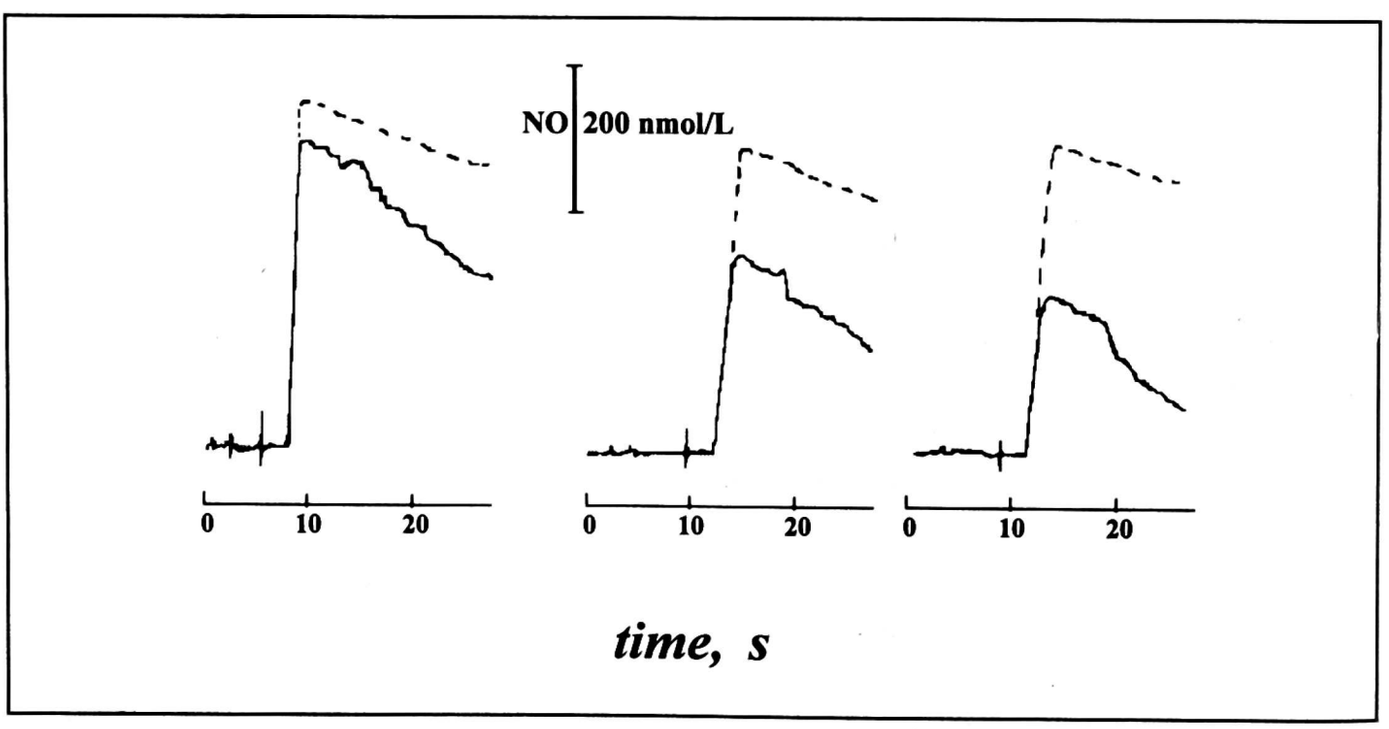

Fig. 2. In vitro measurements of nitric oxide (NO) from endothelial cells of aorta before administration of LPS (a); 40 minutes (b), and 5 hours (c) after administration of LPS. NO was stimulated with calcium ionophore (A23187, 10 μmol/L) in the absence (solid line) and presence of PEG-SOD (100 U/ml, dashed line).

The peak release in pre-endotoxemia tissue was 425 ± 10 nmol/L and increased slightly (460 ± 20 nmol/L) in the presence of PEG-SOD. In the tissue collected at 40 minutes of endotoxemia the NO concentration was 280 ± 15 nmol/L (37% lower than before endotoxemia) and increased in the presence of PEG-SOD, but did not reach the level observed before LPS administration. A further significant decrease of NO concentration to 200 ± 10 nmol/L with concomitant increase in the presence of PEG-SOD was observed in tissue collected after 5 hours of endotoxemia. A plot showing A23187 stimulated NO release in aorta endothelium (in the presence and absence of PEG-SOD) during endotoxemia is shown in *Fig. 3*. A dramatic decrease of endothelial NO production (as measured *in vitro*) was observed during first 2 hours of endotoxemia. Most of this loss was recovered when A23187 stimulated NO release was in the presence of PEG-SOD. These data suggest that A23187 not only stimulates NO release from endothelial cells, but also activates the release of superoxide.

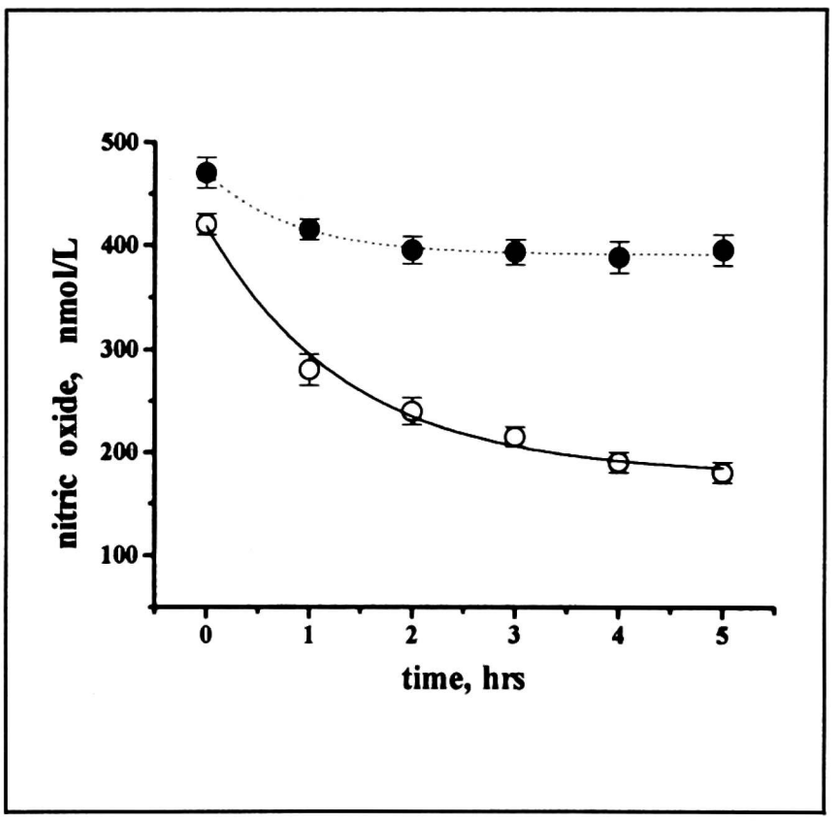

Fig. 3. Concentration of nitric oxide (NO) release from the aorta plotted versus the time of endotoxemia. NO stimulated with calcium ionophore (A23187, 10 μmol/L) in the absence (solid line) and presence of PEG-SOD (100 U/ml, dashed line).

It may be that the rising calcium-independent O_2^- production during endotoxemia was responsible for the decreasing net production of NO concentration by cNOS observed in *Fig. 2 and 3*. This possibility was substantiated by the use of a chemiluminescence method to make direct *in vitro* measurement of O_2^- concentration in freshly excised tissue. Also, a chemiluminescence method was used to measure concentration of $OONO^-$, the product of rapid reaction of O_2^- with NO.

Superoxide generation

The mean basal O_2^- concentration (measured before stimulation of NO release with A23187) in the tissue was 12 ± 1 nmol $\cdot$ L^{-1} before endotoxemia. This concentration increased to 18 ± 2 and to 25 nmol $\cdot$ L^{-1} after 45 minutes and 5 hours of endotoxemia, respectively. The total O_2^- concentration (basal plus concentration observed during stimulation of NO release by A23187) also substantially increased during endotoxemia (*Fig. 4*). Before endotoxemia, the

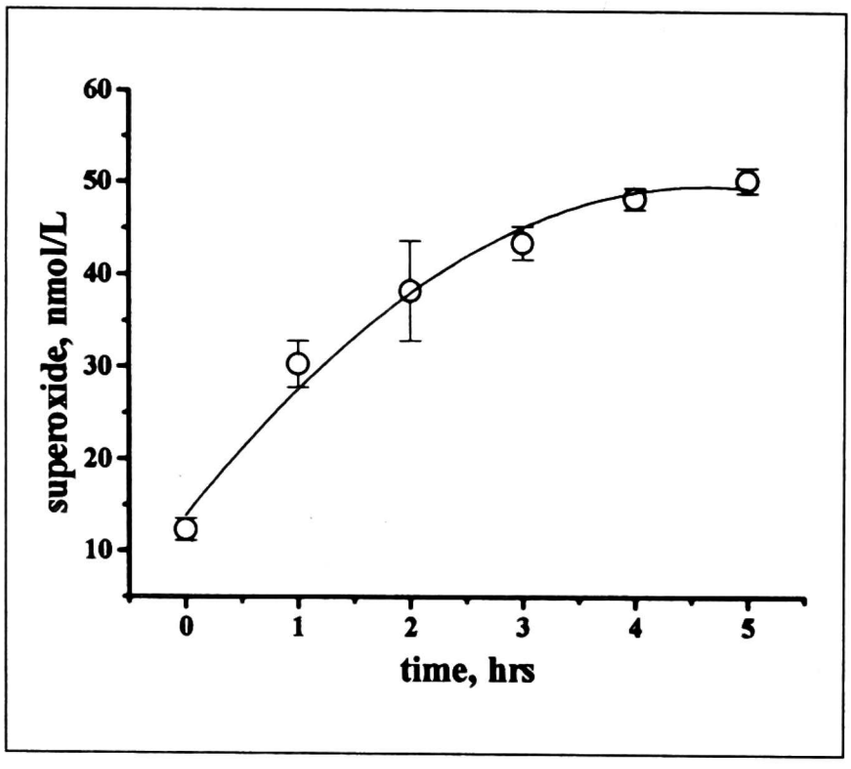

Fig. 4. In vitro chemiluminescence measurements of superoxide concentration in the tissue of aorta during endotoxemia. Data were obtained in the absence (open circles) and presence (solid circles) of A23187 (10 µmol/L).

total O_2^- concentration was 12 ± 3 nmol $\cdot$ L^{-1}. After 5 hours of endotoxemia, the concentration of O_2^- was 49 ± 2 nmol $\cdot$ L^{-1} in A23187 stimulated aortic tissue. As a negative control 10 µmol/L doses of known inhibitor of cNOS after 5 minute incubation was found to reduce calcium-dependent O_2^- release from aortic tissue. L-NMMA inhibited O_2 by 75% (data not shown). In pre-ischemic tissue no significant inhibition of basal O_2^- by L-NMMA was observed.

Production of peroxynitrite

The concentration of peroxynitrite (measured *in vitro* during stimulation with A23187) increased linearly during endotoxemia and followed the pattern of O_2^- increase. This increase was especially noticeable during first two hours. During the first hour of endotoxemia $OONO^-$ concentration increased two times compared to the control. A plateau established after three hours of endotoxemia showed three times higher concentration of $OONO^-$ than in the control.

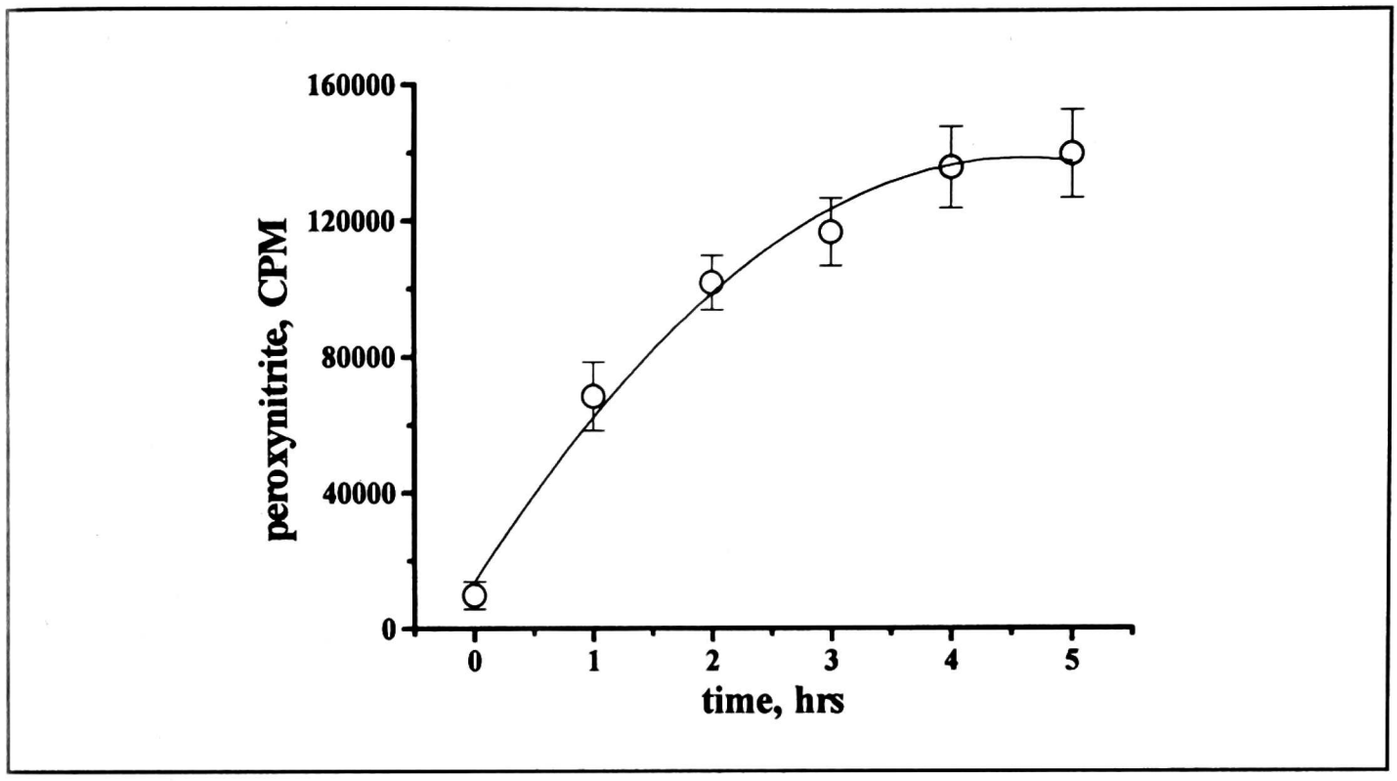

Fig. 5. In vitro chemiluminescence measurements of peroxynitrite concentration in the tissue of aorta during endotoxemia. Data were obtained in the absence (open circles) and presence (solid circles) of A23187 (10 μmol/).

DISCUSSION

As evident from data obtained by direct and continuous *in vitro* measurement of NO, a few minutes after administration of LPS, a rapid increase of NO concentration in the aorta was observed. This pattern of NO release is similar to that observed after stimulation of endothelial cell with typical NO release agonist i.e. bradykinin or acetylcholine. NO production during this period catalyzed by eNOS and can be inhibited by inhibition of calcium flux. It appears that some phenomenon connected with LPS elevates transient intracellular calcium gradients and turns on the membrane bound eNOS to start transforming L-arginine and O_2 into citrulline, NO and H_2O (3).

Conformation that cNOS rather than other sources produced most of the O_2^- after addition od LPS come from experiments showing O_2^- release after treatment with known NO agonist, as well as inhibition after incubation with known cNOS inhibitor. The rapid accumulation of O_2^- concentration in the presence of calcium ionophore suggest that production of O_2^- can be also inhibited (like NO production) by certain L-arginine derivatives. L-NMMA was a potent inhibitor of the production of O_2^- from freshly excised aorta.

The rationale underlying these studies is that local depletion of L-arginine concentration, during endotoxemia reduces NO concentration in endothelium by concomitant increase of O_2^- production followed by high production of

peroxynitrite. Under normal physiologic condition, the concentration of L-arginine in the endothelial cells is high enough (10^{-4} to 10^{-3} mol/L) to provide a sufficiently high concentration gradient to facilitate fast transport of this molecule to the active site of the cNOS enzymes.

However, under conditions of extensive NO production (first by cNOS after addition of LPS and after 30—45 minutes by iNOS), local depletion of L-arginine concentration is likely to occur. It has been demonstrated that cNOS enzymes can be deranged in the absence of sufficient concentration of L-arginine or tetrahydrobiopterin (20, 21). Deranged cNOS can catalyze both a one electron reduction of O_2 to O_2^- and a five electron oxidation of L-arginine to NO. O_2^- reacts very rapidly with NO to form $OONO^-$, which protonated ($pK_a = 6.8$) normally rapidly rearranges to form the stable product nitrate (10). Therefore, the net concentration of NO (which can effectively diffuse to a target muscle cell and is not scavenged) measured by porphyrinic sensor depends not only on the accumulation of NO production from cNOS but also on the concentration of O_2^-.

Direct measurement of O_2^- concentration in tissue using chemiluminescence method provides strong indication that superoxide is produced in high concentration during endotoxemia and this production is inversely proportional to net NO production and directly related to $OOONO^-$ accumulation. The onset of increasing production of O_2^- (above its basal level) was observed about 30 minutes after the administration of LPS. After 5 hours of endotoxemia O_2^- production (presumably by cNOS) increased significantly in comparison to its basal level before endotoxemia.

As the OONO concentration increases, as maximal O_2^- accumulations react with freshly synthesized NO during endotoxemia, local HOONO concentration may become sufficient enough to assure its efficient transport to reactive sites as far as several cell diameters away. In the vicinity of certain reactive centers, HOONO may undergo homolytic cleavage to produce a hydroxyl free radical or nitrogen dioxide free radical (NO_2); or heterolytic cleavage to produce a nitronium cation (NO_2^+) or hydroxide anion (OH^-) (10). Three of these cleavage products ($OH^\cdot$, NO_2 radicals and NO_2^+) are among the most reactive and damaging species in biological systems, and may be major contributors to endothelium damage and initiation of cascade of events leading to organ failure in endotoxemia (11). High production of superoxide followed by generation and cleavage of peroxynitrite maybe a major cause of endothelial dysfunction during endotoxemia.

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Author's address: T. Malinski, Department of Chemistry, Oakland University, Rochester, MI 48309—4401, USA.