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Dynamics of nitric oxide release in the cardiovascular system

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The endothelium plays a critical role in maintaining vascular tone by releasing nitric oxide (NO). Endothelium derived NO diffuses to smooth muscles, triggering their relaxation. The dynamic of NO production is a determining factor in signal transduction. The present studies were designed to elucidate dynamics of NO release from normal and dysfunctional endothelium. The nanosensors (diameter 100–300 nm) exhibiting a response time better than 100 μs and detection limit of 1.0×10^{-9} mol L^{-1} were used for *in vitro* monitoring of NO release from single endothelial cells from the iliac artery of normotensive (WKY) rats, hypertensive (SHR) rats, and normal and cholesterolemic rabbits. Also, the dynamics and distribution of NO in left ventricular wall of rabbit heart were measured. The rate of NO release was much higher (1200 ± 50 nmol L^{-1} s^{-1}) for WKY than for SHR (460 ± 10 nmol L^{-1} s^{-1}). Also, the peak NO concentration was about three times higher for WKY than SHR. Similar decrease in the dynamics of NO release was observed for cholesterolemic rabbits. The dynamics of NO release changed dramatically along the wall of rabbit aorta, being highest (0.86 ± 0.12 μmol L^{-1}) for the ascending aorta, and lowest for the iliac aorta (0.48 ± 0.15 μmol L^{-1}). The distribution of NO in the left ventricular wall of rabbit heart was not uniform and varied from 1.23 ± 0.20 μmol L^{-1} (center) to 0.90 ± 0.15 μmol L^{-1} (apex). Both, the maximal concentration and the dynamics of NO release can be useful diagnostic tools in estimating the level of endothelial dysfunction and cardiovascular system efficiency.

Nitric oxide generated by endothelium is a unique, ubiquitous messenger of cellular signals (Miyamoto *et al.*, 1998; Palmer *et al.*, 1987; Quyyumi *et al.*, 1995; Zeiher *et al.*, 1991). NO is not only involved in the regulation of blood pressure but also has been characterized as a neurotransmitter and plays an important role in the immune system. Its

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Abbreviations: H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; L-NAME, *N*-L-arginine methyl ester; L-NMMA, N^{ω}-nitro-L-arginine methyl ester; NOS, nitric oxide synthase.

chemical nature makes NO an excellent candidate for short-term and short-range signaling (Cohen *et al.*, 1997; Nathan *et al.*, 1994). NO is very lipophilic (therefore it diffuses readily through cellular membranes), it is synthesized rapidly on demand, and its short life (half-life 2–5 s) ensures a localized response. NO is synthesized from L-arginine and oxygen by the enzyme, nitric oxide synthase (NOS) (Moncada & Higgs, 1993). The oxidation of L-arginine is a five-electron-transfer reaction involving N-hydroxylation, formation of N^G-hydroxy L-arginine as an intermediary product, and stoichiometric formation of NO plus L-citrulline. The molecular mechanism of NO synthesis is complex and not completely understood. It involves the transfer of electrons between the various cofactors, which include FAD (flavine adenine dinucleotide), FMN (flavine mononucleotide) and NADPH (nicotinamide adenine dinucleotide phosphate), iron protoporphyrin IX heme, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B), and calmodulin. L-arginine and H_4B have been found to be the limiting factors of NO generation (Cosentino *et al.*, 1998; Huk *et al.*, 1997). L-Arginine analogues (including D-arginine) are not suitable as substrates for NOS. Furthermore, analogues with substitutions of one or both of the amino nitrogen groups in the guanidine group (N^G), for example N^G-monomethyl-L-arginine (L-NMMA) or *N*-methyl ester (L-NAME), competitively inhibit the formation of NO.

In the mammalian system, there are two constitutive NOS isoforms, neuronal (nNOS) and endothelial (eNOS), and one inducible NOS isoform (iNOS) (Crane *et al.*, 1998; Stuehr, 1997). All three isoenzymes are homo-dimers. Each monomer has a molecular mass ranging from 130 000 to 150 000 Da containing four prosthetic groups: FAD, FMN, H_4B and heme. The turnover rate of NO production for each monomer of eNOS and nNOS is 12 and 55 molecules per second and strongly depends on the concentration of L-arginine and H_4B. Since NO is hydrophobic (solubility in water is only 1.82 mmol L^{-1}) and is somewhat lipophilic (K_{ow} about 6.5 at 37°C), NO freely diffuses rapidly through the hydrophobic environment of cell membranes just like O_2 and N_2. In the aqueous phase of the cytoplasm, the diffusion coefficient of NO is 3.6×10^{-5} cm^{-2} s^{-1}. Biosynthesized within the cell, NO may react with few types of molecules inside or outside the cell (after free diffusion through the cell membrane). The most rapid scavenger of NO is superoxide (Huk *et al.*, 1997) (O_2^-, $k = 6.7 \times 10^9$ mol L^{-1} s^{-1}); the peroxynitrite ($OONO^-$) formed in this reaction is quite stable, but when protonated ($pK_a = 6.8$), it usually quickly rearranges to H^+ and NO_3^- (Beckman & Koppenol, 1996; Meśaroš *et al.*, 1998). Reaction with O_2 is much slower and leads to the production of NO_2^-, followed by further oxidation to NO_3^-. NO may also react with a few metal ions (iron, copper or manganese), which are usually bound to proteins. The selective reactivity of NO with such proteins and its reaction with O_2^- and O_2 influences the dynamic of NO propagation and dominates the chemistry of NO in the biological systems. The short life of NO and its low concentration in biological systems make the measurement of this molecule a challenging analytical problem. Several indirect methods have been developed for NO detection. These methods include chemiluminescence, L-citrulline bioassay, and UV-visible spectroscopy. However, the only method that has been successfully applied for direct *in vivo* as well as *in vitro* measurement is electrochemical detection of NO (Malinski & Taha, 1992; Valance *et al.*, 1995). The advantages of direct electrochemical measurements include enhanced sensitivity of on-site measurements, as well as rapid response (at least 100 μs). 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 dynamics of NO release from endothelial cells of the cardiovascular system.

EXPERIMENTAL

Two different designs of porphyrinic sensors were used for NO measurements in a single cell or in the tissue (Malinski & Taha, 1992; Vergnani *et al.*, 2000). The NO nanosensor for single cell measurements was produced by threading a single carbon fiber with a diameter of 6 nm (Amoco Performance Products, Inc.) through the pulled end of an L-shaped glass capillary, with 3–5 mm length of the fibers left protruding. A copper wire was inserted into the opposite end of the glass capillary, which was sealed with conductive silver epoxy (AI Technology). The diameter of the tip of the carbon fiber was reduced to 100–300 nm by a gradual burning process using a propane microburner. Then the tip of the glass capillary was sealed with bee wax. A conductive polymeric film was deposited on the surface of the carbon fiber from a 0.25 mmol L^{-1} solution of nickel(II)-(3-methoxy-4-hydroxyphenyl) porphyrin in 0.1 mol L^{-1} NaOH under N_2, as previously described. After drying, the active tip of the sensor was immersed in 1% (wt/v) Nafion solution in alcohol (Aldrich Chemicals) and then allowed to dry again.

Measurement of NO amidst the dynamic of the *in vivo* conditions of cyclic breathing and heart beating is a challenging task (Pinsky *et al.*, 1997). In order to overcome these potential interferences and to record a reliable NO signal, a single fiber porphyrinic sensor was placed in an intravenous catheter. The catheter-protected porphyrinic NO sensor was constructed from a 24-gauge needle intravenous catheter/needle unit. The needle was roughened around the length of the shaft, truncated near the tip, and then polished flat so that it was 1 mm longer its 24 gauge protective catheter. A single carbon fiber (total length 55 mm, protruding 5 mm from the tip) was mounted inside the hollow truncated 24-gauge needle by conducting epoxy with the aid of a 0.10 mm long copper wire. After curing the exterior of the abraded and truncated needle, the needle was coated and allowed to dry 2 h (3×) with non-conductive epoxy. After curing for 24 h, the diameter and length of the protruding 5 mm carbon fiber was reduced to about 1 μm and 500 μm, respectively. The protruding 500 μm carbon fiber was made more sensitive and selective for NO using the process described for a single cell sensor.

Chronoamperometry, fixed at the potential for the oxidation of NO (630 mV *vs* SCE), was used for fast and continuous measurement of the changes of NO concentration. A three-electrode system; porphyrinic sensor working electrode, a platinum wire counter electrode, and references electrode (SCE), was applied. PAR model 273 voltammetric analyzer with an IBM 80486 computer with Lab-view software was sampled at 4 KHz, amplified, and recorded the analytical signal.

Each NO sensor's performance was characterized under static as well as dynamic (flow) conditions before and after *in vivo* or *in vitro* measurements. The calibrations were performed under laminar flow similar to that of the blood flow in the vessel of interest. A dextran (70 000 av. mol. mass) solution (pH 7.4, phosphate buffer) with the dynamic viscosity of blood (2.5–0.1 cP), was used for the calibration under flow conditions.

Endothelial cells were mechanically removed from the surface of the iliac artery of normotensive rats (WKY), hypertensive rats (SHR), rabbits with normal cholesterol level and cholesterolemic rabbits. Groups of seven laboratory adult animals of each of these species were used to perform the experiments. The cells were plated on round cover slips maintained in Hepes-buffered solution (Sigma) at 37°C for later experiments. To measure NO concentrations, an L-shaped artery porphyrinic microsensor (diameter 0.2–0.3 μm, length 5–7 μm) was placed on the membrane of a single cell using a motorized computer controlled micromanipulator. When the tip of the sensor touched the cell membrane, a transient small electrical noise was observed, assumed to indicate zero dis-

tance from the cell. From this point, the sensor was moved away from the surface in 0.2 μm increments controlled by computer.

New-Zealand White rabbits (2 kg), were anesthetized (50 mg kg^{-1} Ketamine and 5 mg kg^{-1} Xylazine), incubated, and ventilated with room air using a Harvard small animal ventilator (tidal volume of 25 ml and rate of 60 breaths min^{-1}). After median sternotomy was performed, the NO concentration was measured as follows: to implant the porphyrinic NO sensor, the 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 on a truncated needle. The exact localization of the active tip of the sensor was confirmed by postmortem dissection of the tissue.

DATA ANALYSIS

Results are expressed as mean ± S.E.M. In each set of experiments, n = 7 is the number of animals studied. Statistical analysis was performed by ANOVA followed by the Student–Newman–Keuls test, means are considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

A single fiber (tip diameter 0.3 μm) porphyrinic sensor was placed in close proximity (5 ± 2 μm) to the endothelial cell membrane. Nitric oxide release from the endothelial cell was stimulated with the receptor-independent agonist, calcium ionophore (A23187, 1 μmol L^{-1}). After injection of the calcium ionophore, a rapid increase in NO concentration was observed (Fig. 1a). The maximum NO concentration, the rate of NO release, and the rate of decay varied significantly between normal and dysfunctional endothelium. For normotensive rats, the release of NO was observed at 400 ± 20 ms after injection of calcium ionophore. The rate of NO release was 1200 ± 50 nmol L^{-1} s^{-1} with a peak concentration of 430 ± 15 nmol L^{-1} reached after 600 ± 20 ms. The rate of NO decay was 140 ± 15 nmol L^{-1} s^{-1}. The observed change in NO concentration with respect to time was significantly different for hypertensive rats (Fig. 1b). First, the time needed to stimulate NO release after injection of A23187 was longer (550 ± 30 ms). Also, the kinetics of NO release was much slower. The rate of NO increase was 460 ± 10 nmol L^{-1} s^{-1} and a nitric oxide peak of the concentration 140 ± 15 nmol L^{-1} was reached after 900 ms.

A similar trend for the change in the dynamics of NO generation was observed for endothelium obtained from a rabbit aorta at normal and elevated levels of cholesterol (Fig. 1c, d). The rate of NO release was 1400 ± 80 nmol L^{-1} s^{-1} for normal rabbits and 480 ± 30 nmol L^{-1} s^{-1} for cholesterolemic rabbits. Also, maximum NO concentration was much higher for normal rabbits (480 ± 15 nmol L^{-1}) than for cholesterolemic rabbits (260 ± 15 nmol L^{-1}). It is interesting to note that the rate of NO decay differs significantly between normal and dysfunctional endothelium. NO decreased with a rate 155 ± 10 and 80 ± 5.0 nmol L^{-1} s^{-1} WKY and SHR, respectively. The half lifetime of NO decay was 1.8 ± 0.1 s for WKY, 1.0 ± 0.2 s for SHR and 2.1 ± 0.1 s, 1.0 ± 0.1 s for normal and cholesterolemic rabbits, respectively. This slow dynamics of NO release from dysfunctional endothelium has a severe implication on the propagation of NO and the signal transduction into smooth muscles (Van der Loo *et al.*, 2000; Vergnani *et al.*, 2000).

The propagation of NO in the cardiovascular system is based on diffusion, and the diffusion is based on a gradient of NO concentration between a generator (the surface of endothelium) and a target (vascular wall). The low gradient of NO in dysfunctional endothelium de-

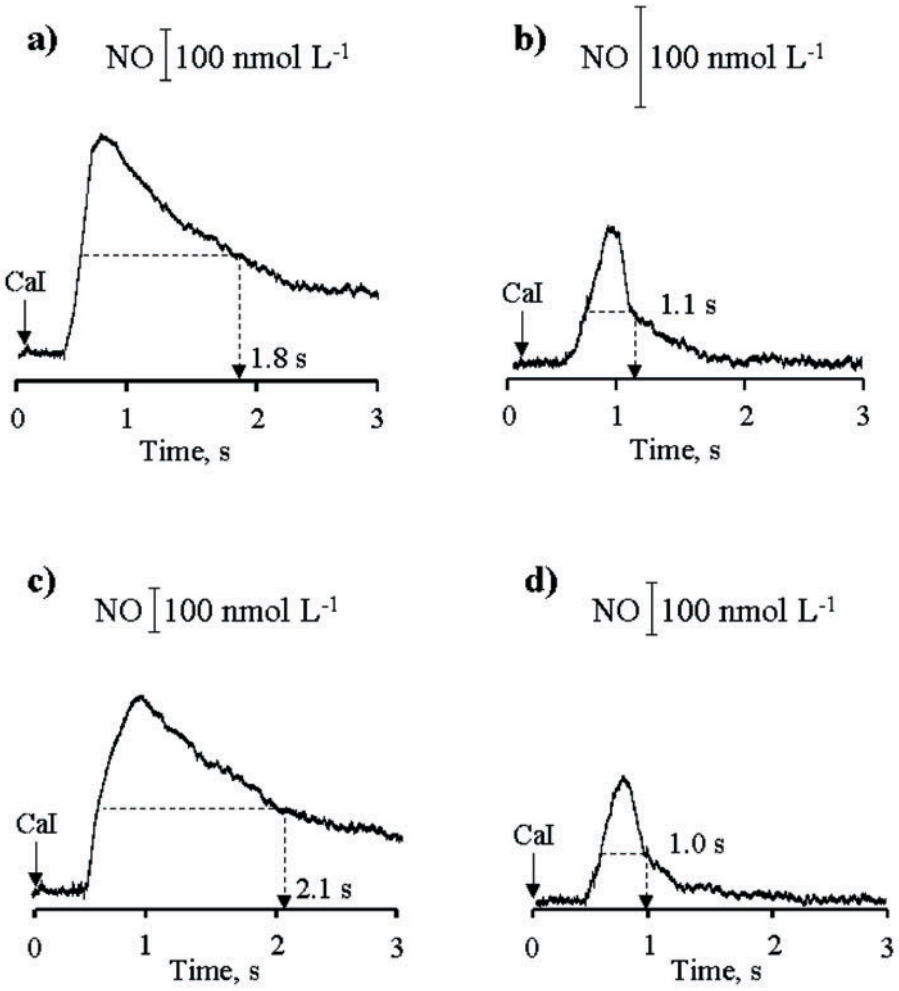

Figure 1. Nitric oxide release measured *in vitro* from a single endothelial cell of the iliac artery of a) normotensive rat (WKY); b) hypertensive rat (SHR); c) rabbit (normal cholesterol level); and d) cholesterolemic rabbit.

Dashed lines indicate half time of NO decay. NO release was stimulated with calcium ionophore A23187, 1 μmol L^{-1}.

creases the rate of NO diffusion, resulting in increasing the time for NO to reach the target, as well as decreasing the final concentration of NO on the target. Therefore, the efficiency of NO signaling in hypertension and in cholesterolemia is severely impaired. The observed kinetics of NO release and its propagation for these dysfunctional endothelial cells may be attributed to the high production of superoxide from different sources, including the nitric oxide synthase (Gryglewski *et al.*, 1986; Huk *et al.*, 1997).

Further specific studies for the concentration of NO released by normal endothelial cells indicated that the concentration is not uniform and depends on the location of the cells within the aorta. The highest NO concentration (0.86 ± 0.12 μmol L^{-1}) was found in the ascending aorta and lowest in iliac aorta (0.48 ± 0.15 μmol L^{-1}) (Fig. 2). These are maximum concentrations of NO measured close to the membrane surface after stimulation with a receptor independent agonist. Under physiological conditions, the normal endothelium operates usually below 50% of this maximum NO concentration.

Although, the porphyrinic sensor has been used for both *in vitro* and *in vivo* measurements in prior published studies, several characteristics of the sensor were tested to ensure

that recordings in the beating heart would reflect authentic NO concentration. The experiments, defining the analytical response of the sensor to potential piezoelectric or *in vivo* electric current interferences showed that the measured NO signal was in most cases at least 30 to 100 times larger and temporarily shifted from the conservatively estimated background noises. Anesthetized rabbits were used to measure local dynamics of the NO concentration in the apical left ventricular endocardium. Rapid changes in cardiac NO concentration related to the cardiac cycle were observed (Fig. 3a). In the rabbit's heart (myocardium), each cardiac cycle (period about 340 ± 20 ms) begins and ends with an inter-cycle NO concentration of 0.10 ± 0.02 μmol L^{-1}. During early systole, NO concentration increases to a semi-plateau of 0.24 ± 0.03 μmol L^{-1}. Early diastolic filling is accompanied by a rapid NO increase with a peak diastolic 0.63 ± 0.04 μmol L^{-1}. After this peak, there is a sharp decay of the inter-cycle NO concentration. To demonstrate the relationship between the ECG and the instantaneous NO concentration, simultaneous recording of each of these was performed (Fig. 3a). Endothelial cells within the heart are subject to mechanical deformation during filling and beating (Pinsky *et al.*, 1997). The mechanical deformations open mechanical Ca^{2+} channels

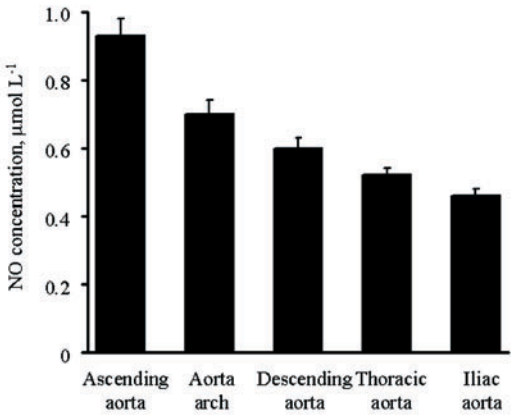

Figure 2. A maximal NO concentration measured *in vitro* in endothelial cells of aorta.

NO release was stimulated with calcium ionophore (A23187, 1 μmol L^{-1}).

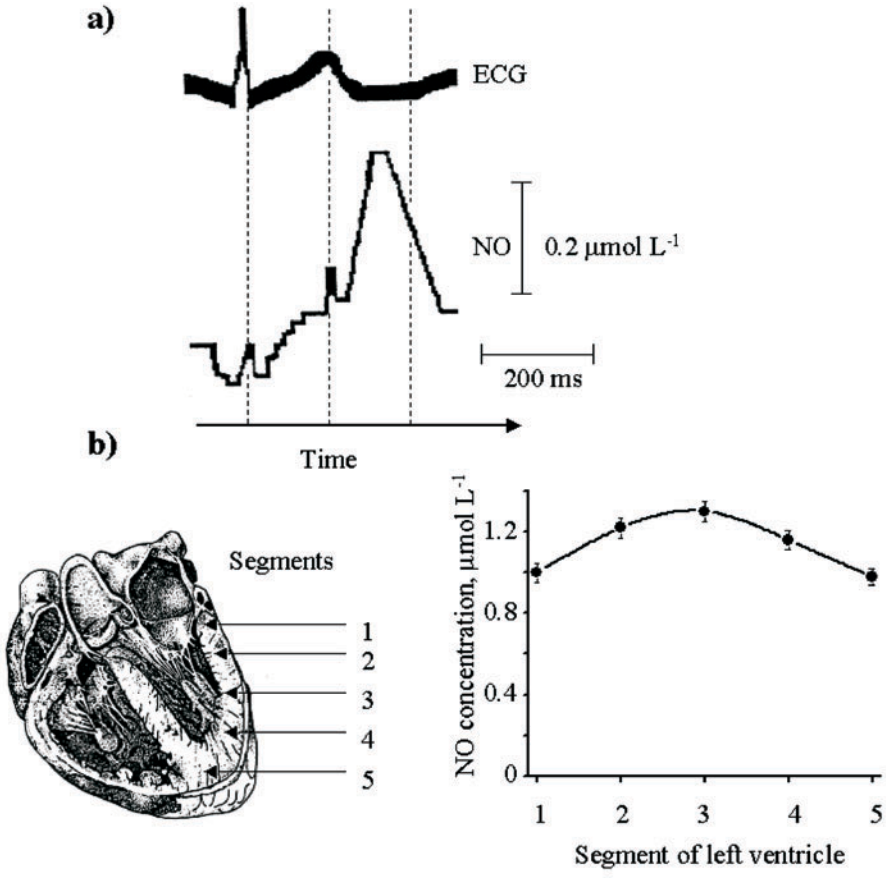

Figure 3. A dynamics of NO release measured *in vivo* during a single beat of rabbit heart (a); distribution of NO concentration measured *in vitro* in the wall of left ventricle of the heart (rabbit) (b).

The arrows indicate a location of the sensors. NO release was stimulated with calcium ionophore A23187, 1 μmol L^{-1}.

and stimulate the NO release. This NO affects mechanical properties of cardiac myocytes *via* increasing cGMP to facilitate relaxation and to mediate an acetylcholine-stimulated decrease in contractibility. NO can be also produced by myocytes only after stimulation with chemical agonists but not *via* the mechanical stimulation (Kanai *et al.*, 1997). The high concentration of NO released during each beat of the heart also prevents the aggregation and adhesion of platelets.

We expected that the distribution and dynamics of NO release would vary significantly in the different segments of the wall of the left ventricle. For this reason, a mapping study of NO release in the wall of left ventricle was performed. The sensors were placed in the median of the ventricular wall and NO release was measured after stimulation with calcium ionophore. The maximum NO concentrations, which can be produced in the different regions of the left ventricular wall, are depicted in Fig. 3b. The highest NO concentration (1.23 ± 0.20 μmol L^{-1}) is produced in the center of ventricular wall and the lowest is in the apex (0.90 ± 0.15 μmol L^{-1}). Direct measurement of the dynamics of NO release in a beating heart may help to explain certain aspects of the beat-to-beat regulation of cardiac performance and also provide insight into the pathophysiology of diseases associated with increased myocardial distention, such as valvular heart disease or heart failure.

In summary, the presented data demonstrated that the dynamics of nitric oxide release differs significantly in the different subunits of the cardiovascular system. The data also suggests that under pathological conditions this dynamics is much slower than that observed under normal conditions. The concentration of NO produced by eNOS is an important indicator of the efficiency of the cardiovascular system. However, the dynamics of NO release directly affect its transmission and signaling capability. Therefore, both NO concentration as well as the dynamics of its release may be considered as diagnostic tools to estimate a level of endothelial dysfunction and an efficiency of the cardiovascular system.

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