Physiol. Res. 46: 251–255, 1997

New Strategy for Prolonging the Preservation Time of Hearts for Transplantation

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Received September 9, 1996
Accepted February 18, 1997

Summary

Our study concerned the findings that rat and rabbit heart transplants do not survive after six hours. They become dark, hard and fail to contract within 2 min after reperfusion and never regain their function. We tested the supplementation of solutions for heart transplant preservation with tetrahydrobiopterin (H4B) and L-arginine (L-ARG) to maintain the oxidative and reductive domains of the endocardial NO synthase. We decided to study the excised rabbit hearts preserved in Hank's balanced salt solution (HBSS) at 0 °C supplemented with different concentrations of H4B (0, 1, 5, 10 or 100 μM). At desired time intervals, successive pieces stored in the above solutions were warmed to rabbit body temperature in 4 ml of HBSS and maximally agonized by direct application of 20 μl of 200 μM bradykinin (or other agonist) onto the exposed endocardium. Nitric oxide bursts were monitored with a porphyrinic NO sensor lying on the exposed endocardium. Our goal was to find the lowest H4B concentration which would maximally agonize $NO^{\bullet}$ and prolong the time of heart preservation to more than 6 hours. Ten μM are a minimum H4B concentration which achieves maximum prolongation of heart preservation time up to 90 hours. This effect was based upon maximal potentiation of $NO^{\bullet}$ release and minimizing of superoxide production.

Key words
Tetrahydrobiopterin – Nitric oxide – Superoxide – Transplantation

Introduction

We recently reported (Pinsky *et al.* 1994) an unprecedented six-fold increase in the survival time (4 to 24 h) of excised rat hearts which can be safely preserved for successful transplantation. These stunning dose-dependent results were achieved by merely combining the clinical standard University of Wisconsin preservation solution (Jeevanandam *et al.* 1991) with optimal amounts of endogenous nitric oxide synthase (NOS) substrate, L-arginine (2 mM L-ARG), or with exogenous sources of $NO^{\bullet}$ – nitroglycerin (0.1 mg/ml, NG), sodium nitroprusside (0.01 mg/ml, SNP) or with 8-bromoguanosine 3',5' monophosphate (0.5 mM, 8Br-cGMP), a membrane permeant analog of the product of $NO^{\bullet}$ target enzyme guanylyl cyclase (Pinsky *et al.* 1994). As a negative control, we also observed that a competitive NOS antagonist N^{G}monomethyl-L-arginine (10 mM L-NMMA) was associated with poor preservation (Pinsky *et al.* 1994).

We found that rat heart transplants that did not survive, became dark, hard and failed to contract within 2 min after reperfusion and never regained their function (Pinsky *et al.* 1994). These observations combined with platelet aggregation studies, EPR studies, histochemical studies and with millisecond response time studied with an *in situ* porphyrinic NO microsensor (Malinski *et al.* 1994, 1996) compelled us to believe that highly reactive oxygen species (ROS) – most likely superoxide $O_2^{\bullet}$ – formed during the first few minutes of reperfusion quench the available cytoprotective $NO^{\bullet}$ leading to cell death and possible vasoconstriction and decreased blood flow to the reperfused transplant (Pinsky *et al.* 1994).

Nitric oxide ($NO^{\bullet}$), a gaseous free radical, is implicated in numerous physiological processes and diseases. $NO^{\bullet}$ causes relaxation of the vascular system, relaxation of bronchioles and acts as a non-adrenergic non-cholinergic neurotransmitter (Knowles and Moncada 1992). Alteration of $NO^{\bullet}$ production is involved in circulatory and respiratory disorders, diabetes, and cancer (Kiechle and Malinski 1993). Nitric oxide production can occur in many types of cells including endothelial cells, macrophages, neurones, and platelets. It has been suggested that the relatively short half-life (6-50 s) of $NO^{\bullet}$ in biological systems is due to its reaction with oxygen, although this decomposition appears to be relatively slow. Recently, the superoxide anion has been established as the main oxidant and a scavenger of nitric oxide (Dinerman *et al.* 1993, Mesaros *et al.* 1995). The reaction of $NO^{\bullet}$ with the superoxide is very fast, two different rate constants being reported: 3.7×10^7 $M^{-1} s^{-1}$ and 3.6×10^9 $M^{-1} s^{-1}$ (Hogg *et al.* 1992). The product of the reaction of $NO^{\bullet}$ with superoxide is peroxynitrite which has a relatively short half-life of 1–3 s and decomposes in water to form the biologically active radicals $NO_2^{\bullet}$ and $OH^{\bullet}$. The short half-life of $NO^{\bullet}$ and its loss due to reaction with superoxide anion makes difficult the accurate quantitative measurements of $NO^{\bullet}$. Recently, we published a design and application of a porphyrinic microsensor for the direct *in vitro* amperometric and voltammetric measurements of $NO^{\bullet}$ (Malinski *et al.* 1992).

Materials and Methods

Animals

Using protocols submitted to, and approved by the Oakland University Animal Care and Use Committee, hearts were rapidly excised from 2.5–3 kg male New Zealand white rabbits following pentobarbital anaesthesia, then cut into 20 or more small pieces and stored in HBSS (Hank's balanced salt solution) supplemented with the desired amount of L-ARG and H4B (tetrahydrobiopterin).

NO microsensor fabrication

The NO microsensor was produced by threading an array of 10 to 20 carbon fibres (Amoco, TX) through the pulled end of an L-shaped glass capillary with 5 mm of the fibres left protruding. The tip of the glass capillary was sealed with bee's wax. Then a copper lead was inserted into the opposite end of the glass capillary and sealed with conductive silver epoxy (A.I. Technology, NJ). A conductive polymeric porphyrinic film was then deposited on the surface of the carbon fibers from a solution of nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin under nitrogen as previously described (Malinski *et al.* 1992). Finally the sensor's active tip was immersed in 1 % Nafion solution (Sigma, MO), then allowed to dry.

Experimental setup and NO measurement

At desired intervals of time, successive pieces of heart were warmed to rabbit body temperature in 4 ml of HBSS and then maximally agonized by direct application of 20 μl of 200 μM bradykinin (maximal stimulation of the NO synthase) onto the exposed endocardium. Nitric oxide bursts were monitored with an porphyrinic NO sensor applied on the exposed endocardium; a platinum wire counter electrode and saturated calomel reference electrode were also immersed and placed in contact with adjacent tissue. Chronoamperometric recording was performed at the peak potential for the oxidation of $NO^{\bullet}$ using a PAR Model 273 voltammetric analyzer interfaced with an IBM AT-80486 computer with custom data acquisition and control software.

Measurement of the superoxide anion

$O_2^{\bullet}$ was measured at the same time intervals as that of $NO^{\bullet}$. The successive pieces of heart were warmed to physiological temperature in 4 ml of HBSS, taking care not to damage the endothelium, then gently transferred to glass scintillation vial containing 0.25 mM lucigenin and HBSS (final volume of 2 ml). Counts were obtained at 2-min intervals at room temperature after injection of the agonist (10 μl of 200 μM bradykinin) using a liquid scintillation counter (LS 6000; Beckman, CA). Calibration of the scintillation counter for $O_2^{\bullet}$ was done by the xanthine/xanthine oxidase reaction (Ohara *et al.* 1993).

Chemicals

Bradykinin, lucigenin (bis-*N*-methyl-acridinium nitrate), xanthine, xanthine oxidase and chemical components of the modified Hank's balanced salt solution (HBSS) were obtained from Sigma Chemicals Co., MO. H4B was obtained from Dr. B. Schircks Laboratory, Jonas, Switzerland.

Results and Discussion

Figure 1 presents typical amperometric curves obtained for *in situ*, *ex vivo* measurements of $NO^{\bullet}$ in the absence of H4B in the endocardium. In the heart stored for one hour, an initial rapid increase of $NO^{\bullet}$ concentration was observed within one to two seconds after bradykinin injection, with a rate of 415 nM/s reaching the maximum of 1.47 μM in 5 s (Fig. 1a). In hearts stored for 24 h, the rate of $NO^{\bullet}$ concentration increase was slower by a factor of about four (91 nM/s), and the maximum reached after 5 s was only 0.32 μM (Fig. 1b). An $NO^{\bullet}$ decay rate of 644 nM/s was observed in hearts stored for 24 h as compared to 110 nM/s in hearts stored for 1 h. The

decrease of NO• concentration as well as the kinetics changed with the duration of tissue storage. The maximum NO• concentration decreased by a factor of more than five between 1 h and 24 h of storage and the rate of NO• decay also increased more than five times. Therefore the amount of NO• available as a vasodilator decreased by the same order of magnitude in the stored tissue.

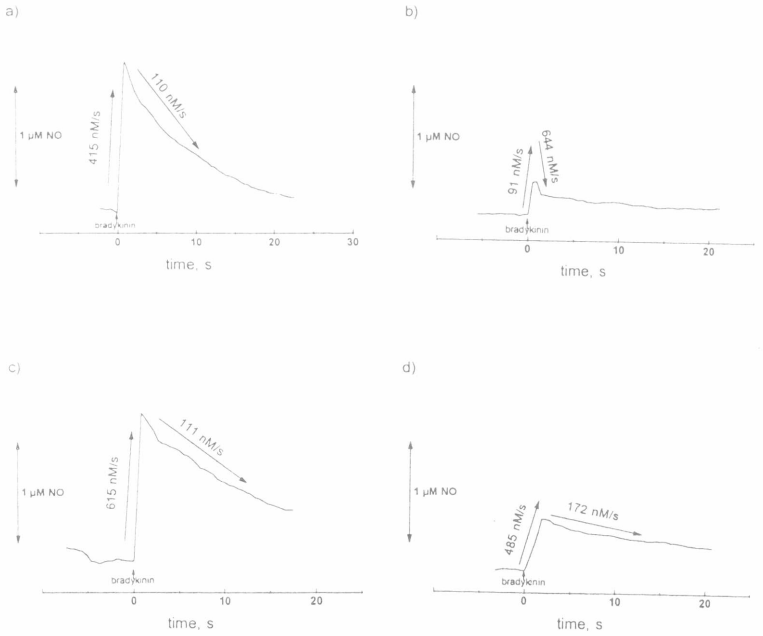

Fig. 1. *Nitric oxide release in rabbit heart stored 1 h (a) and 24 h (b) in HBSS alone and 1 h (c) and 24 h (d) in HBSS with 10 µM of H4B.*

Experiments performed in stored heart in the presence of 10 μM H4B (Fig. 1c,d) showed differences in the maximum NO• concentration as well as in the kinetics of NO• decay (compare with Fig. 1a,b). Again, 1-2 s after the injection of bradykinin an initial rapid increase of NO• was observed in both cases reaching a maximum 1.50 μM in 5 s with a rate of 615 nM/s (Fig. 1c) in hearts stored for one hour with 10 μM H4B. In hearts stored for 24 h (Fig. 1d), the rate of concentration increase was about same as in hearts stored for 1 h only (485 nM/s) with a maximum peak 0.52 μM NO•. An NO• decay rate of 111 nM/s was observed in hearts stored for 1 h as compared to 172 nM/s for hearts stored for 24 h. The NO• concentration decreased but the kinetics did not change with storage time in the tissue preserved with 10 μM of H4B.

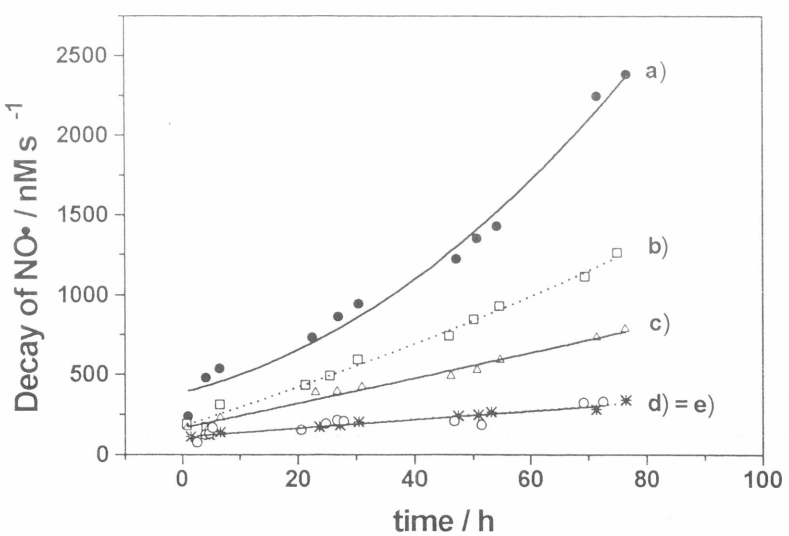

Fig. 2. *Time dependences of NO• decay for different concentration of H4B in HBSS (a = 0 µM; b = 1 µM; c = 5 µM; d = 10 µM; e = 100 µM).*

Figure 2 presents the time dependence of NO• decay at different concentrations of H4B (0, 1, 5, 10 and 100 μM). The NO• decay is markedly dependent on H4B concentration. The optimal H4B concentration for preservation of heart tissue is about 10 μM (Fig. 2) with a minimal increase of NO• decay with the time of storage. The 100 μM concentration of tetrahydrobiopterin has a similar effect on the decay of NO• as 10 μM H4B, but a higher concentration has a negative influence on the tissue.

Because a minimal value of 250 nM/s of NO• release rate is the important value for successful transplantation of heart, we tested the time-dependent NO• release rate for four different solutions: a) HBSS alone; b) University of Wisconsin preservation solution; c) University of Wisconsin preservation solution + 2 mM L-arginine; d) HBSS + 10 μM H4B. The NO• release rate for HBSS solution alone decreased very rapidly and the minimal value was reached after about 4 h of heart storage (Fig. 3a). The curve was approximately the same as for the University of Wisconsin preservation solution (Fig. 3b). After adding L-arginine (2 mM) to University of Wisconsin solution (as a substrate of endogenous NO synthase) the maximal time of stored heart for successful transplantation was prolonged to 23 h, as can be seen from Figure 3c. Our newly proposed solution (HBSS + 10 μM H4B) allowed to increase the storage time of heart to more than three days (about 90 h) (Fig. 3d).

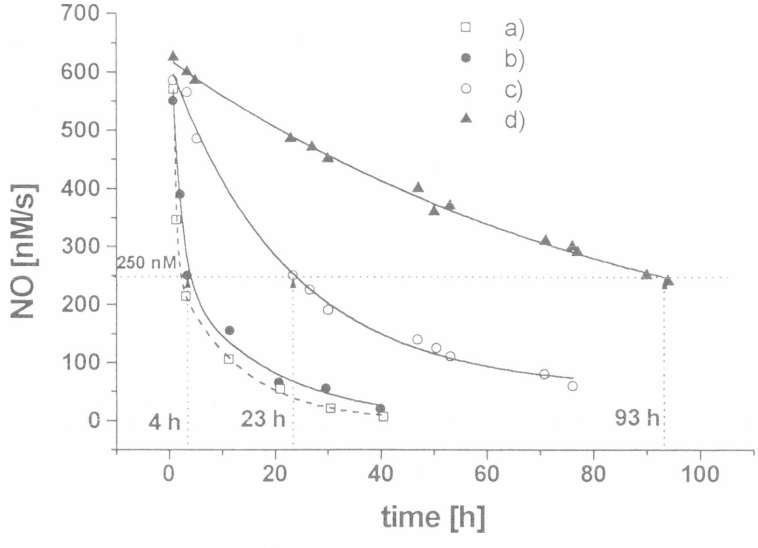

Fig. 3. *NO• release rate for four different preservation solutions: a) HBSS alone (broken line); b) University of Wisconsin preservation solution (solid line); c) University of Wisconsin preservation solution + 2 mM L-arginine; d) HBSS + 10 μM H4B.*

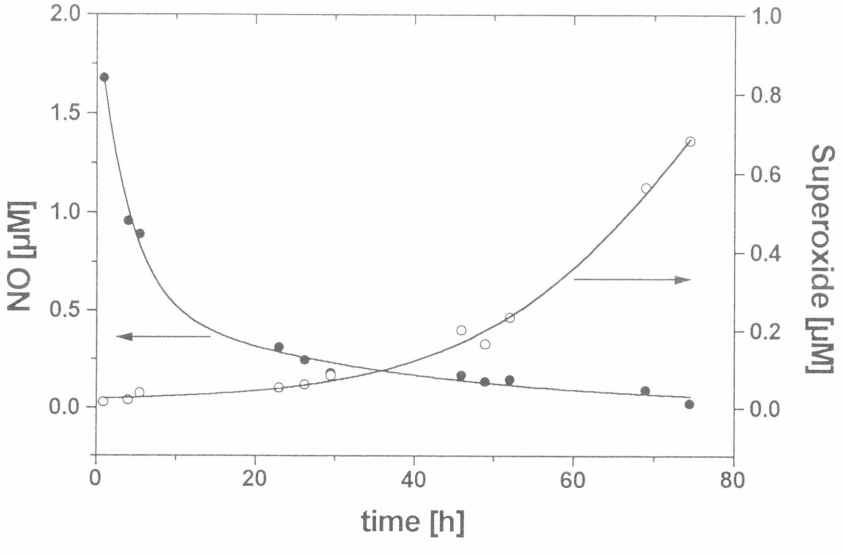

Fig. 4. *Time-dependent release of NO• and superoxide from the stored heart.*

The same results were obtained when measuring $O_2^{\bullet}$ as can be seen from Fig. 4, which represents time-dependent release of $NO^{\bullet}$ and superoxide from the stored heart. These data indicate that the nitric oxide synthesis pathway deteriorates in the stored tissue and/or $NO^{\bullet}$ is consumed in chemical reaction(s), i.e. with the superoxide anion (Mesaros *et al.* 1995).

Endothelial NOS (eNOS) generates $NO^{\bullet}$ as a membrane bound "monomer" when sufficient concentrations of O_2, L-arginine and NADPH as well as stochiometric amounts of the labile cofactor H4B are readily available (Busconi and Michel 1993). We expect that the oxidase and reductase domains of the eNOS may undergo a conformational change relative to each other (after the loss of labile H4B) to form such an eNOS conformation that cannot make $NO^{\bullet}$ but can still receive electrons from NADPH and can donate them to the substrate O_2 to form $O_2^{\bullet}$.

The presented data suggest that the release of superoxide follows the release of nitric oxide. Preservation of the $NO^{\bullet}$ synthesis pathway along with inhibition of the superoxide production may be an important strategy for improving cardiac preservation before transplantation.

Therefore, our goal was to find the lowest concentration of H4B (10 μM) which maximally agonized $NO^{\bullet}$ a reactive oxygen species, remained high and stayed extinguished for at least three days (the time needed to transport a preserved heart by express mail to anywhere in the world).

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