Simultaneous Measurement of Nitric Oxide and Oxygen Dynamics During Myocardial Ischemia Reperfusion of Rat

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ABSTRACT

Myocardial infarction is one of the major causes of morbidity and mortality in industrialized countries despite the improvement in clinical management of the disease. Myocardial function is dependent on a constant supply of oxygen from coronary circulation. Therefore the accurate measurement of oxygen concentration in postischemic myocardium is crucial to the assessment of myocardial viability and function. Nitric oxide (NO) plays important roles in the cardiovascular system by mediating various physiological and pathophysiological processes. However, the role of endogenous NO in ischemia-reperfusion is still controversial. Besides, direct and real-time monitoring of NO is difficult because NO is present only at low concentrations in the body and has a short half-life Therefore, we utilized a sol-gel derived electrochemical NO and oxygen microsensor to monitor changes in NO and oxygen during myocardial ischemia-reperfusion. NO and oxygen sensors were built using a xerogel-modified platinum microsensor and coiled Ag/AgCl reference electrode. Hearts were placed on a Langendorff apparatus and treated by 30 min of ischemia and 60 min of reperfusion. In the ischemic myocardium, NO showed a time-course dependent change during the 30 min ischemic episode. And the restoration concentration of NO and oxygen was decreased below the pre-ischemic level after myocardial ischemia and early reperfusion. It seemed that endogenous NO formation was observed during ischemic episodes, which is a reaction against ischemia. And the oxygen recovery after ischemic episodes can be assessed by the extent of reoxygenation and the severity of ischemia.

Keywords: nitric oxide, oxygen, electrochemical sensor, myocardial ischemia-reperfusion, real-time monitoring

1 INTRODUCTION

Nitric oxide (NO) is an important signaling molecule that regulates a diverse range of physiological and cellular processes in many tissues [1]. Therefore, the accurate detection of physiological NO concentration is crucial to the understanding of NO signaling and its biological role. However, it is difficult to accurately measure NO levels in vivo because NO is present at nanomolar concentrations in the body and has a half-life of 2–6 s. Consequently, most methods used for NO detection are indirect including spectroscopic approaches such as the Griess assay for nitrite, detection of nitrate and nitrite with reductase enzymes, and detection of methemoglobin after NO reaction with oxyhemoglobin [2,3]. Unfortunately, these methods often fail to accurately reflect the dynamics of NO in vivo and in real time [4]. Direct measurement strategies are therefore necessary for examining biological process and diseases related to NO in biological conditions, in particular the action of endogenously produced NO.

Myocardial ischemia is characterized by insufficient oxygen and nutrient supply to the area at risk and leads to tissue infarction. Reperfusion therapy must be applied as soon as possible to attenuate the ischemic insult of acute myocardial infarction. Though reperfusion disturbs the process of ischemic cell death, it imposes injury in its early stage that results in further cell death [5]. It is well known that reperfusion injury is mainly induced by reactive oxygen species (ROS) [6]. Some studies have reported that the amount of oxygen delivered during reperfusion was an important determinant of postischemic cardiac dysfunction and ischemia-reperfusion (IR) injury [7]. Therefore, the accurate measurement of oxygen concentration in the post-ischemic myocardium is crucial for the assessment of myocardial viability and function.

In this study, we investigate changes in NO and O_2 dynamics during myocardial ischemia-reperfusion utilizing a sol–gel modified electrochemical NO and O_2 sensor. Therefore we attempted to clarify the correlation between endogenous NO release in ischemic myocardium and oxygen recovery in post-ischemic myocardium.

2 MATERIALS AND METHODS

2.1 Animals

Male Sprague–Dawley rats (Orient Bio., Inc., Seoul, Korea) weighing 250–350 g were kept under a 12 h light/12 h-dark cycle (lights on at 0600 h) at 24±0.5℃ in a central animal care facility. Food and water were provided ad libitum, but the animals were fasted for 1 day before the surgical procedure. All animal experiments were approved by the Committee of Animal Experiments in the College of Medicine, Kyung Hee University (KHUASP(SE)-10-023),

and were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Preparation of Sol-Gel Derived NO and O_2 Microsensors

The NO and O_2 microsensor (2-electrode configuration) consisted of a Pt working electrode and a Ag/AgCl reference electrode [8], respectively. Platinum wire (127 μm in diameter) was attached to a tungsten (W) rod with silver conducting epoxy, and inserted into a borosilicate glass capillary (1.5/0.86 mm o.d./i.d.; A-M Systems; Sequim, WA, USA). The end of the glass capillary was polished until the Pt tip was exposed. The ensuing Pt electrode was platinized in 3% chloroplatinic acid hexahydrate and 0.03% lead (II) acetate trihydrate (w/w in water) by cycling the potential from +0.6 to −0.35 V (vs. Ag/AgCl) at a scan rate of 20 mV/s using a CH Instruments 760B bipotentiostat (Austin, TX).

A silane solution was prepared by dissolving 18 μL of MTMOS and 4.5 μL of 17FTMS in 727.5 μL of ethanol. The solution was mixed with 160 μL of water, followed by 10 μL of HCl (0.5 M) for 1 h. The ensuing solution was deposited on the top of the Pt capillary electrode (0.44 $\mu L/mm^2$). The fluorinated xerogel-modified electrode was then allowed to cure for 1 day under ambient conditions.

2.3 Evaluation of NO and O_2 Microsensors

To evaluate the analytical performance of the NO and O_2 microsensors, amperometric measurements were performed using a Compactstat (Ivium Technology, Eindhoven, Netherlands). The sensing system was composed of the xerogel-modified Pt microsensor and the coiled Ag/AgCl reference electrode.

The NO standard solution (1.9 mM) was prepared by purging Krebs–Henseleit solution (K–H solution; 118 mM NaCl, 10.1 M glucose, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 0.5 mM Na–EDTA) with N_2 gas for 30 min to remove any oxygen, followed by purging with NO (99.5%) for 30 min. The solutions of NO and interfering species were freshly prepared every 2 days and stored at 4 ℃. All NO sensors were prepolarized for 30 min and tested in K–H solution under ambient conditions with constant stirring, and currents were recorded at an applied potential of +0.8 V (vs. Ag/AgCl).

The O_2 sensor performance was evaluated by suspending the sensor in a K-H solution differently tonometered with oxygen (5, 10, and 20% O_2, balance N_2). The solutions of oxygen were freshly prepared for every measurement. All sensors were pre-polarized for 30 min and tested in a K-H solution under ambient conditions, and currents were recorded at an applied potential of –0.4 V (vs. Ag/AgCl). Sensors were stored in a K-H solution at room temperature between measurements.

2.4 Induction of Ischemia and Reperfusion in Isolated Rat hearts

Rats were anesthetized via intraperitoneal injection of chloral hydrate (300 mg/kg) and thoracotomy was performed. Hearts were placed on a modified Langendorf apparatus via cannulation of the aorta. The hearts were perfused by gassed K-H buffer solution at a constant perfusion pressure of 70 mmHg. The perfusion buffer was filtered through a 5.0 μm microfilter (Millipore, Bedford, MA, USA) and bubbled with 95% O_2 and 5% CO_2 at 37℃ to achieve a pH of 7.4. The Langendorff system was maintained at 37℃ by a water circulation pump. A pressure transducer was connected to a probe inserted into the perfusion line immediately above the aortic cannula, enabling the measurement of aortic perfusion pressure.

The fluorinated xerogel-modified NO and O_2 sensor were inserted into the middle of the myocardium in the left ventricle, respectively. The current was stabilized at baseline during the 30 min before the induction of ischemia. Ischemia was initiated by stopping the perfusion. After the induction of ischemia, reperfusion was performed for 60 min.

3 RESULTS

The sensor performance of the resulting NO microelectrode was investigated in a K–H solution. The calibration and dynamic response curves for NO and interfering species (i.e., nitrite) are shown in Fig. 1. A sensor coated with fluorinated xerogel film did not exhibit serious interference from nitrite concentrations of up to 20 μM, which is a concentration much greater than the highest level present in physiological samples. In addition, the xerogelderived sensor responded to NO concentrations of up to 1000 nM with respect to sensitivity (−2.24 pA/nM), linearity (R^2 = 0.9960, 20–1000 nM NO range), detection limit (0.5 nM, based on S/N = 3), and response time ($t_{90\%}$ < 10 s).

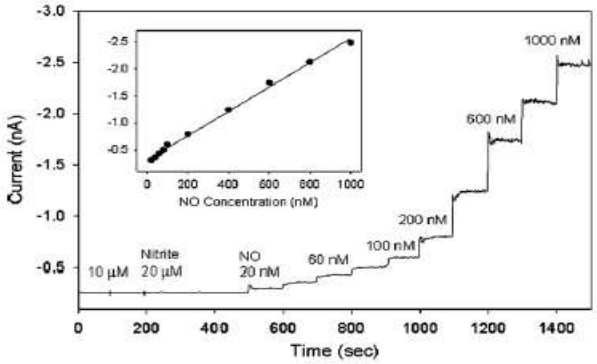

Figure 1: Dynamic response to NO and interfering species, nitrite. Inset: calibration curve of the NO microsensor modified with the fluorinated xerogel gas-permeable membrane.

The sensor performance of the resulting oxygen microelectrode was also investigated in a K-H solution. The dynamic responses at different oxygen levels ranging from 37 to 149 mmHg (corresponding to 5 to 20% O_2, balance N_2) are shown in Fig. 2. The fluorinated xerogel-modified microsensor exhibited a linear response up to 149 mmHg O_2 with respect to sensitivity (266.3 ± 0.61 nA/mmHg), linearity (R^2 = 0.9999, 37–149 mmHg O_2 range), and detection limit (1 mmHg, based on S/N = 3).

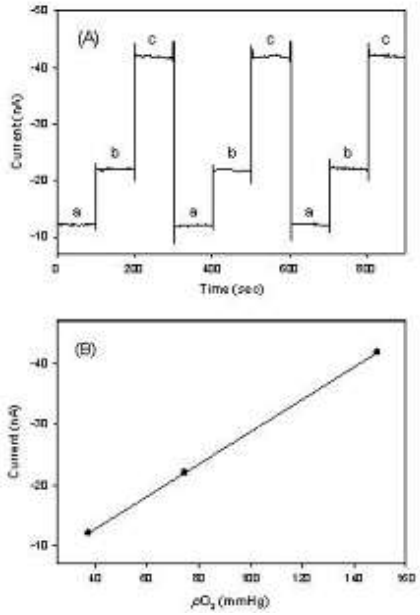

Figure 2 : Dynamic response (A) and calibration curve (B) of the oxygen microsensor modified with the fluorinated xerogel gas-permeable membrane. The partial pressure of oxygen in the Krebs-Henseleit solution was varied between (a) 37, (b) 74, and (c) 149 mmHg.

To investigate the changes of endogenous NO release and pO_2 during myocardial ischemia reperfusion, we performed real time monitoring of NO and oxygen pressure in isolated Langendorff-perfused rat hearts. During controlled perfusion for 100 min, there were no changes in the current by the sol–gel derived NO sensor and left ventricular pressure (LVP) in either group. However, as soon as the ischemia was initiated, pressure and pO_2 rapidly declined to near zero levels. The ischemic plateau was maintained during the ischemic period. The NO level initially decreased and gradually increased during the ischemic period. After 40 min of ischemia followed by the 30 min of reperfusion, NO eventually decreased below the pre-ischemic level. When reperfusion was initiated, the changes in pO_2 the pO_2 did not begin to increase until 2714 ± 1252 sec. After 60 min of reperfusion, the degree of restoration level of pO_2 was 3.40 ± 4.82 % ($p < 0.001$, n = 3, respectively).

For efficient analysis, we proposed 11 parameters for NO dynamics and 9 parameters for pO_2 dynamics during the ischemic and reperfusion episodes.

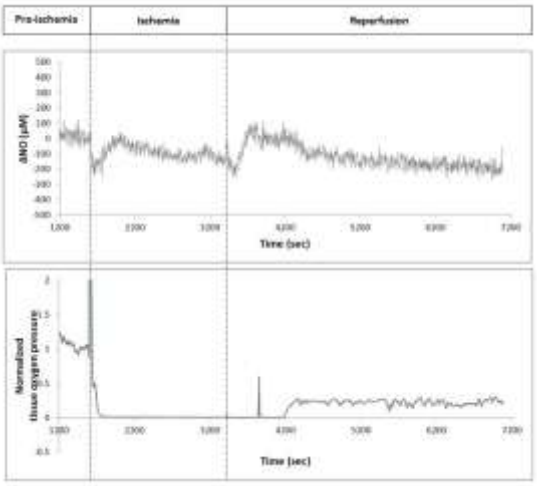

Figure 3 : Representative real-time measurement of nitric oxide and oxygen during myocardial ischemia-reperfusion of Langendorff-perfused rat hearts

4 DISCUSSION

Acute myocardial infarction is the most common cause of morbidity in industrialized nations [9]. Myocardial infarction is caused by sudden stoppage of blood supply to the heart that leads to tissue necrosis. Normal myocardium produces more than 90% of its ATP by oxidative metabolism and less than 10% by anaerobic glycolysis [10]. After the induction of ischemia, the myocardium enters a phase of reversible tissue injury, from which complete recovery is possible if blood supply is adequately restored. Cellular necrosis eventually occurs if ischemia persists. During severe cardiac ischemia, cardiac myocytes must drastically reduce ATP demand or utilization to meet the needs for survival, and thus balance the reduced ATP supply with reduced demand during severe ischemia [11].

NO is an important physiological mediator produced from larginine by nitric oxide synthase (NOS). It was known that the release of low concentrations of NO by constitutive NOS played a role in the regulation of coronary blood flow, inhibition of platelet aggregation, adherence to endothelium, and possibly modulation of myocardial oxygen consumption [12]. Endogenous NOS-derived NO could play an important role for initiating and mediating the delayed phase of ischemic preconditioning protection [13]. Recent reports suggested that endogenous NO synthesis during ischemia and reperfusion protected against apoptotic cell death [36]. NO produced during ischemia was involved in the preservation of cellular ATP during ischemia [14]. The maintenance of the energy balance and of stable steady-state ATP concentrations in hypoxiatolerant cells is a sign of an effective defense against anoxia or ischemia [15]. Therefore, it is very important to evaluate endogenous NO for vascular and myocardial function in myocardial ischemia/reperfusion.

Oxygen plays a critical role in the pathophysiology of myocardial injury during subsequent reperfusion, as well as

ischemia. Additionally, the concentration of oxygen in the myocardium is an important parameter for the assessment of myocardial viability. Because cardiac function is critically dependent on the ability of the myocardium to consume oxygen, the accurate measurement of myocardial oxygen concentration is crucial to the assessment of myocardial viability by IR injury.

In this study, we successfully measured the endogenous NO and oxygen dynamics in myocardial tissue during ischemia-reperfusion utilizing a sol–gel derived microsensor. Our results may be explained by changes in NOS expression observed during myocardial ischemia reperfusion [13]. Under normal perfusion, the cardiac interstitial NO concentration is constant within the nanomolar range and maintains the homeostatic state. The activity of NOS in the heart was stimulated by ischemia. During ischemia, NOS3 activity increases within a few minutes, and subsequently the NO concentration increases during early ischemia. However, with prolonged myocardial ischemia, NOS3 protein expression decreases and the increased tissue acidosis attenuates NOS3 activity. NO concentrations sharply increase within the early seconds of reperfusion and subsequently decrease below baseline values. In particular, it seemed that hypothermic treatment might lead the ischemic myocardium to make more endogenous NO against ischemia for cardioprotection. Therefore quantitative measurements of endogenous NO dynamics would be helpful for evaluation of the cardioprotective effects of therapeutic treatments such as drug administration. And an ischemic time lasting > 30 min, causes a decrease in nutritive organ perfusion and the impairment of microcirculation during the reperfusion period.

5 CONCLUSIONS

In summary, our NO-permselective microsensor has good sensitivity and specificity for detecting biologically released NO in vivo and can be applied for real time monitoring of NO dynamics in various organs. Our results suggest that the promotion of endogenous formation and inhibition of the time-course alteration of NO during an ischemic episode might be helpful as a therapeutic strategy for protecting the myocardium from ischemic injury. Quantitative measurements of oxygen tension dynamics in the myocardium would be helpful for evaluation of the cardioprotective effects of therapeutic treatments such as drug administration.

ACKNOWLEDGEMENTS

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