Chapter 5

Real-Time Monitoring of Nitric Oxide Dynamics in the Myocardium: Biomedical Application of Nitric Oxide Sensor

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Additional information is available at the end of the chapter

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Abstract

Nitric oxide (NO) is an important physiological mediator that regulates a wide range of cellular processes in many tissues. Therefore, the accurate and reliable measurement of physiological NO concentration is essential to the understanding of NO signaling and its biological role. Most methods used for NO detection are indirect including spectroscopic approaches such as the Griess assay for nitrite and detection of methemoglobin after NO reaction with oxyhemoglobin. These methods cannot accurately reflect the changes in NO concentration in vivo and in real time. Therefore, direct methods are necessary for investigating biological process and diseases related to NO in biological conditions. There is a growing interest in the development of electrochemically based sensors for direct, in vivo, and real-time monitoring of NO. Electrochemical methods offer simplicity, good sensitivity, high selectivity, fast response times, and long-term calibration stability compared to other techniques including electron paramagnetic resonance, chemiluminescence, and fluorescence. In this article, we present real-time NO dynamics in the myocardium during myocardial ischemia-reperfusion (IR) utilizing electrochemical NO microsensor. And applications of electrochemical NO sensor for the evaluation of cardioprotective effects of therapeutic treatments such as drug administration and ischemic preconditioning are reviewed.

Keywords: nitric oxide, real-time detection, myocardial ischemia-reperfusion, electrochemical sensor, therapeutic treatments

1. Introduction

Nitric oxide (NO) is one of gaseous cellular-signaling molecules, which regulates a wide range of physiological and cellular processes in various tissues. In particular, it plays a vital role in a variety of biological processes including immune defense, neurotransmission, regulation of cell death (apoptosis), and cell motility [1–4]. NO has some key features that make this molecule suited to its cellular-signaling functions. NO is a lipophilic diatomic gas under atmospheric conditions. As it has a relatively small Stokes radius and neutral charge, it can rapidly diffuse the cell membrane. The presence of an unpaired electron in NO supports its high reactivity with oxygen (O_2), superoxide (O_2^-), transition metals, and thiols [5, 6]. The removal of the unpaired electron in NO generates the nitrosonium cation NO^+, while the addition of an electron forms the nitroxyl anion (NO^-). These different forms of NO represent distinct chemical reactivities [6, 7]. And NO reacts with O_2^- to form peroxynitrite ($OONO^-$), a particularly destructive molecule within biological systems [8].

NO is known to play a major role in vascular biology and heart failure. NO is a double-edged sword; NO inhibits ischemia-reperfusion (IR) injury, represses inflammation, and prevents left ventricular remodeling, whereas excess NO and coexistence of reactive oxygen species (ROS) with NO are injurious [6]. In that, low concentration of NO has beneficial effects on heart function, while high concentration of NO has opposite effects. Recently, it was reported that the final action of NO is not only regulated by its concentration and cellular confinement but also strongly depends on the level of oxidative stress in the myocardium [4]. But, the cardioprotective mechanism of NO is not yet clear, and it is not known whether NO effectively acts during ischemia or during reperfusion. Therefore, the accurate and quantitative detection of physiological NO concentration is crucial to the understanding of NO signaling and its biological role. This review focuses on the role of NO in myocardial IR injury. In addition, we will summarize the studies from our laboratory, which evaluates the cardioprotective effects of therapeutic treatments such as drug administration and ischemic preconditioning in the myocardium during myocardial IR utilizing electrochemical NO sensor.

2. Production of nitric oxide

In general, NO is produced from the conversion of L-arginine to L-citrulline, a reaction catalyzed by a family of enzymes called NO synthases (NOSs). Endothelial NOS (eNOS, also known as NOS3) and neuronal NOS (nNOS, also known as NOS1) are constitutive and low-output enzymes, whereas the macrophage-type NOS isoform, known as inducible NOS (iNOS, also known as NOS2), is an inducible and high-output enzyme [9]. NOS is a homodimeric oxidoreductase containing iron protoporphyrin IX (heme), flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin (BH_4), which is a cofactor essential for the catalytic activity of all three NOS isoforms [6, 10, 11]. NO biosynthesis

by the three NOS isoforms can be suppressed by several small-molecule inhibitors: N^G-methyl-L-arginine (L-NMA) inhibits all NOS isoforms, and L-N^G-nitroarginine methyl ester (L-NAME) has some selectivity for the constitutive NOS isoforms (i.e., nNOS and eNOS), whereas other inhibitors such as aminoguanidine, 1400 W, and many others show selectivity for iNOS [9].

Although NOS had been generally considered to be the primary source of NO in biological systems, nonenzymatic NO synthesis also occurs. NO can be produced in tissues by either direct disproportionation or reduction of nitrite to NO under the acidic and highly reduced conditions which occur in disease states, such as ischemia [12]. Tissue acidosis occurring during ischemia increases NO production independent from eNOS [13], and even at normal pH, xanthine oxidase in the presence of low pO_2 and high nicotinamide adenine dinucleotide (NADH) concentration is capable of producing NO from nitrite [14]. Besides, in the isolated rat heart [15] and in rabbit hindlimb muscle [16], the NO concentration is still increased during ischemia after complete NOS inhibition by Nw-nitro-L-arginine (L-NNA).

3. Nitric oxide measurements

It is difficult to directly measure NO concentration in vivo because NO is present at nanomolar concentrations in the body and highly reactive with numerous endogenous species including free radicals, oxygen, peroxides, transition metals, and metalloproteins. Indeed, the half-life of NO in biological milieu is <10 s [17].

3.1. Indirect method (Griess assay)

Indirect methods measure the stable metabolites of NO such as nitrites (NO_2^-) and nitrates (NO_3^-). The most widely used method for NO detection is based on Griess assay reagents, which can react with nitrite to form a purple azo dye. This method requires that nitrate first be reduced to nitrite and then nitrite determined by the Griess reaction [18]. Briefly, the Griess reaction is a two-step diazotization reaction. First, the NO-derived nitrosating agent, dinitrogen trioxide (N_2O_3), generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO) reacts with sulfanilamide to produce a diazonium ion. And then it is coupled to N-(1-napthyl)ethylenediamine to form a chromophoric azo product that strongly absorbs at 540 nm [19]. This method has some disadvantages including its sensitivity and its ability to detect nitrate. Therefore, nitrate must be converted to nitrite before the total nitrite is detected. The reduction of nitrate to nitrite can be achieved by using bacterial nitrate reductase or reducing metal such as cadmium [20]. However, these methods often fail to accurately reflect the spatial and temporal distributions of NO in biological environments. In addition, the concentration of nitrate and nitrite metabolite may not show the accurate amount of NO in the specific site because other parts of the body can also produce these compounds. Therefore, direct measurement strategies are necessary for investigating the physiological origin and action of endogenously produced NO.

3.2. Direct methods (nitric oxide sensor)

Several methods exist for directly measuring NO including electron paramagnetic resonance spectroscopy [21], chemiluminescence [22], fluorescence [23, 24], and electrochemical sensing [17, 25, 26]. Of these approaches, miniaturized electrochemical (e.g., amperometric and voltammetric) sensors represent the most promising means for determining the spatial and temporal distributions of NO near its physiological source [17]. Electrochemical methods provide simplicity, fast response times, good sensitivity, high selectivity, and long-term calibration stability [27, 28]. The most simple detection scheme to date involves the electrochemical oxidation of NO at a metal (e.g., platinum and gold) or carbon electrode [27, 29]. However, it is necessary to use a relatively high working potential (+0.7 to +0.9 V vs. Ag/AgCl) for the direct electrooxidation of NO. In this condition, several interferences from other readily oxidizable biological species such as nitrite, ascorbic acid, uric acid, and acetaminophen often disturb selective detection of NO [25, 27]. Therefore, further surface modification with permselective membranes is required to achieve the desired selectivity for NO via size exclusion or electrostatic repulsion [4]. Indeed, several polymeric materials have been evaluated as gas-permeable or permselective membranes including nafion, collodion, polycarbazole, o- and m-phenylenediamine, poly(tetrafluoroethylene), cellulose acetate, and multilayer hybrids of these polymers [27, 30–36]. In particular, Shin et al. reported that sol-gel-derived electrochemical sensors showed good sensitivity and selectivity for NO detection [17, 27].

4. Nitric oxide in myocardial ischemia-reperfusion injury

Myocardial infarction (MI) is one of the major causes of morbidity and mortality in industrialized countries, despite the improvement in clinical management of the disease. MI is caused by sudden stoppage of blood supply to the heart that leads to tissue necrosis. The normal myocardium produces more than 90% of its adenosine triphosphate (ATP) by oxidative metabolism and less than 10% by anaerobic glycolysis [4, 37]. After the induction of ischemia, the myocardium can be completely recovered to its normal state if blood supply is adequately restored. But cellular necrosis eventually occurs if ischemia persists [4]. 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 [4, 38].

NO has been extensively studied in the setting of myocardial IR injury. Previous studies demonstrate that the deficiency of eNOS deteriorates myocardial IR injury [39], whereas the overexpression of eNOS [40], the administration of NO donors [41], and inhaled NO gas therapy [42] significantly protect the myocardium. NO possesses several physiological properties that make it a potent cardioprotective-signaling molecule, as follows [43]: First, NO is a potent vasodilator in the ischemic myocardium which enables an essential perfusion of injured tissue. Second, NO reversibly inhibits mitochondrial respiration during early reperfusion. It leads to a decrease in mitochondrial-driven injury by extending the zone of adequate tissue cellular oxygenation away from vessels [43–45]. It is known that restoration of oxygen at reperfusion

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leads to a lethal burst of reactive oxygen species (ROS) generation. An important source of ROS is the mitochondria. In mitochondria, electrons from intermediary metabolism move down the electron transport chain (ETC) and transferred to oxygen at complex IV [46]. When oxygenation is normal, complex I activity is high because a cysteine residue on its ND3 subunit is protected from modification. During ischemia (without oxygen), electrons accumulate along the ETC [46]. Reperfusion leads to a burst of ROS production from multiple sites which can attack proteins, lipids, and DNA, as well as lethal activation of the mitochondrial permeability transition pore [46]. NO inhibits mitochondrial complex I by S-nitrosation (or S-nitrosylation) of cysteines, which subsequently prevents damage during IR injury [47]. Reversible S-nitrosation of complex I slows the reactivation of mitochondria during the crucial first minutes of the reperfusion, thereby decreasing ROS production, oxidative damage, and tissue necrosis [48]. Third, NO is a potent inhibitor of neutrophil adherence to the vascular endothelium which is a significant event initiating further leukocyte activation and superoxide radical production [43, 49, 50]. Fourth, NO prevents platelet aggregation [51], and this effect attenuates capillary plugging together with the anti-neutrophil actions of NO [52]. Finally, NO inhibits apoptosis either directly or indirectly by inhibiting caspase-3-like activation via a cGMP-dependent mechanism [43, 53] and by direct inhibition of caspase-3-like activity through protein S-nitrosylation [43, 54]. In summary, 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 the endothelium, and possibly modulation of myocardial oxygen consumption.

But, excessive generation of NO is detrimental to cardiovascular function as exemplified in septic shock where burst generation of iNOS-derived NO causes hypotension, cardiodepression, and vascular hyporeactivity [55]. The detrimental effect of excess NO is attributed to the action on mitochondria. NO inhibits the mitochondrial respiratory chain, resulting in inhibition of ATP production, increased oxidant production, and increased susceptibility to cell death [56]. Inhibition of mitochondrial respiration by NO and its derivatives stimulates production of reactive oxygen and nitrogen species by mitochondria [56], which contribute to cell death in excess.

In conclusion, NO can preserve blood flow in the ischemic tissues and reduce platelet aggregation and neutrophil-endothelium interaction following IR. Besides, low concentrations of NO improve cardiomyocyte function. On the contrary, higher NO concentrations diminish cardiomyocyte function, mediate inflammatory processes following IR, worsen mitochondrial respiration, and even induce cardiomyocyte death. Therefore, it seems that NO can mediate both protective and detrimental myocardial effects which are crucially dependent upon the experimental conditions. Consensus is being reached in the debate regarding a NO protective effect, with most studies reporting its protective effects. However, the role of their product, NO, in the process of IR is still not well defined mainly because of the difficulty in measuring NO concentration in the body tissue.

In the next section, we summarize real-time NO dynamics in the myocardium during myocardial IR. And applications of electrochemical NO sensor for the evaluation of cardioprotective effects of therapeutic treatments such as hypothermia, drug administration, and ischemic preconditioning are summarized.

5. Real-time monitoring of nitric oxide dynamics in the myocardium during myocardial ischemia-reperfusion

5.1. Hypothermia

In general, hypothermia is thought to reduce the metabolic needs of cells, specifically perhaps by reducing the oxygen demand in the hypothermic tissues [57]. Besides, in isolated heart perfusion system, hearts were placed in ice-cold buffer as quickly as possible to avoid any detrimental effects of hypoxia. Therefore, myocardial hypothermia might be a useful technique to limit ischemic damage during infarction or as adjunctive therapy during minimally invasive cardiac surgery [58]. Lee et al. reported changes in NO dynamics during myocardial IR utilizing a sol-gel-modified electrochemical NO sensor and isolated heart perfusion system [4]. They attempted to clarify the role of endogenous NO release by comparing intact and cardioprotected hearts, in which cardioprotection was conferred by hypothermic treatment of the hearts. For the hypothermic group, hearts were immediately immersed in ice-cold perfusion buffer for 3 min after harvest. In the ischemic myocardium, NO showed a time-dependent change during the 40 min ischemic episode. After myocardial ischemia and early reperfusion, the restoration level of NO was decreased below the pre-ischemic level (**Figure 1**). However, the myocardium with hypothermic treatment (151 ± 37 nM) generated more NO during the ischemic period than that without any treatment (59 ± 15 nM). Besides, the restoration level of NO in the hypothermic group (−57 ± 26 nM) was significantly higher than that of the intact group (−170 ± 50 nM, $p < 0.05$) [4]. As a result, they inferred that hypothermic treatment of the heart would promote endogenous NO production in the ischemic myocardium. It might be a helpful therapeutic strategy for protecting the myocardium from IR injury [4].

5.2. Myocardial oxygen dynamics

Because oxygen plays a critical role in the pathophysiology of myocardial injury during subsequent reperfusion, as well as ischemia, the accurate measurement of myocardial oxygen tension is crucial for the assessment of myocardial viability by IR injury. Lee et al. reported a sol-gel-derived electrochemical oxygen microsensor to monitor changes in oxygen tension (pO_2) during myocardial IR [59]. And they analyzed differences in oxygen tension recovery in the post-ischemic myocardium depending on ischemic time to investigate the correlation between recovery parameters for oxygen tension and the severity of IR injury. **Figure 2** shows the nine parameters for pO_2 dynamics during myocardial IR and the maximum and restoration values of pO_2 at different ischemic times. As a result, they observed that if ischemia was stopped within 20 min, the pO_2 in the myocardium after the onset of reperfusion restored to pre-ischemic levels. However, the pO_2 in the myocardium did not recover to its pre-ischemic state, if the ischemic time was >30 min [59]. These results show that the maximum and restoration values of pO_2 in the post-ischemic myocardium were closely related to the infarct size [59]. In summary, they demonstrated that the degree of reoxygenation in the post-ischemic myocardium was an important index of IR injury and myocardial viability, utilizing a sol-gel-derived electrochemical oxygen microsensor and recovery parameters.

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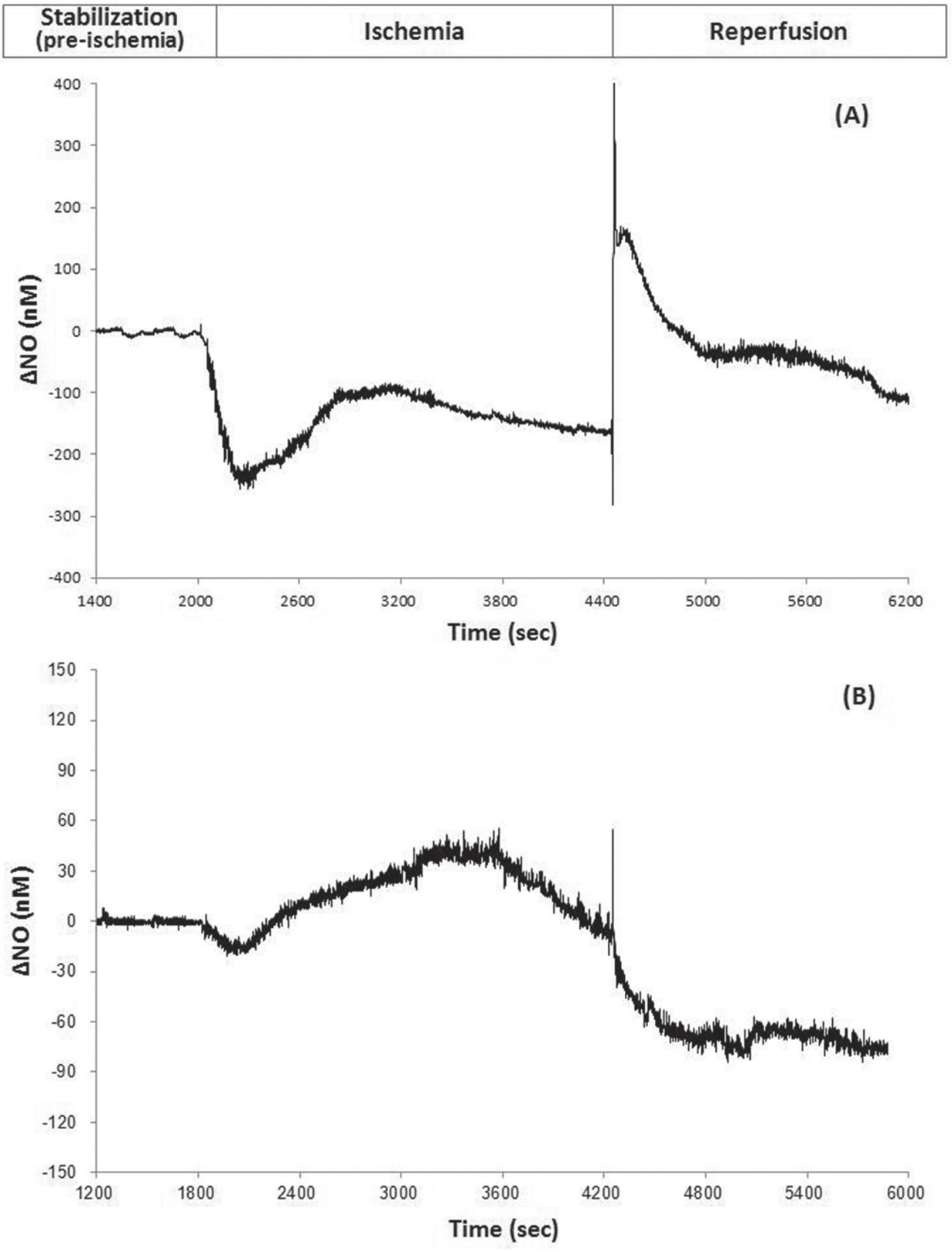

Figure 1. Representative real-time measurement of NO in intact (A) and hypothermic (B) groups during myocardial ischemia-reperfusion of Langendorff-perfused rat hearts. Reproduced with permission from Lee et al. [4]. © 2011 Elsevier B.V.

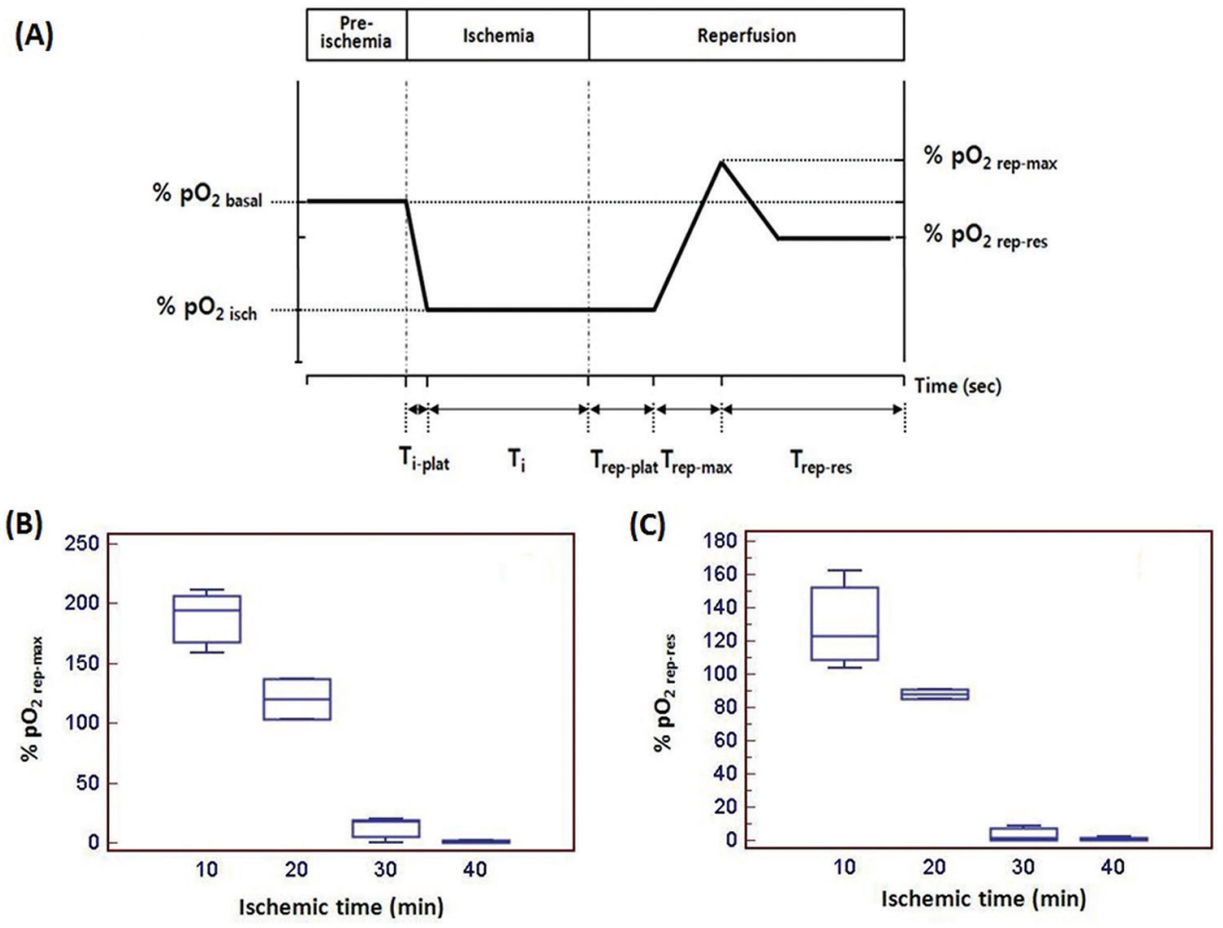

Figure 2. (A) Definition of analysis parameters proposed for changes in oxygen tension throughout the experimental protocol and box and whisker graph depicting maximum (B) and restoration levels (C) of oxygen tension during the reperfusion period as a percentage of pre-ischemic levels at different ischemic times (n = 3 per group). Reproduced from Lee et al. [59]. © The Royal Society of Chemistry 2012.

5.3. Remote ischemic preconditioning

Ischemic preconditioning is an adaptive response of briefly ischemic tissues that serves to protect against subsequent prolonged ischemic insults and reperfusion injury [60]. In particular, remote ischemic preconditioning (RIPC) is a novel method where ischemia followed by reperfusion of one organ is believed to protect remote organs either by the release of biochemical messengers into circulation or by the activation of nerve pathways, resulting in the release of messengers that have a protective effect [60–62]. This preserves the target tissue without trauma to major vessels or direct stress to the target organ [63]. Although some studies have demonstrated that endothelial NO is one of the major contributors to the candidate mechanism of RIPC [60, 64], the mechanism of RIPC-induced cardioprotection has not yet been fully elucidated. Lee et al. simultaneously measured NO and O_2 dynamics in the myocardium during myocardial IR utilizing sol-gel-modified electrochemical NO and O_2 microsensors [65]. By comparing control and RIPC-treated hearts, we attempted to clarify the correlation between NO release in the ischemic period and O_2 restoration in the myocardium after reperfusion. **Figure 3** represents the schematic diagrams of experimental design and experimental setup of an isolated heart perfusion system and a real-time monitoring system for NO and oxygen tension dynamics during myocardial IR of the rat.

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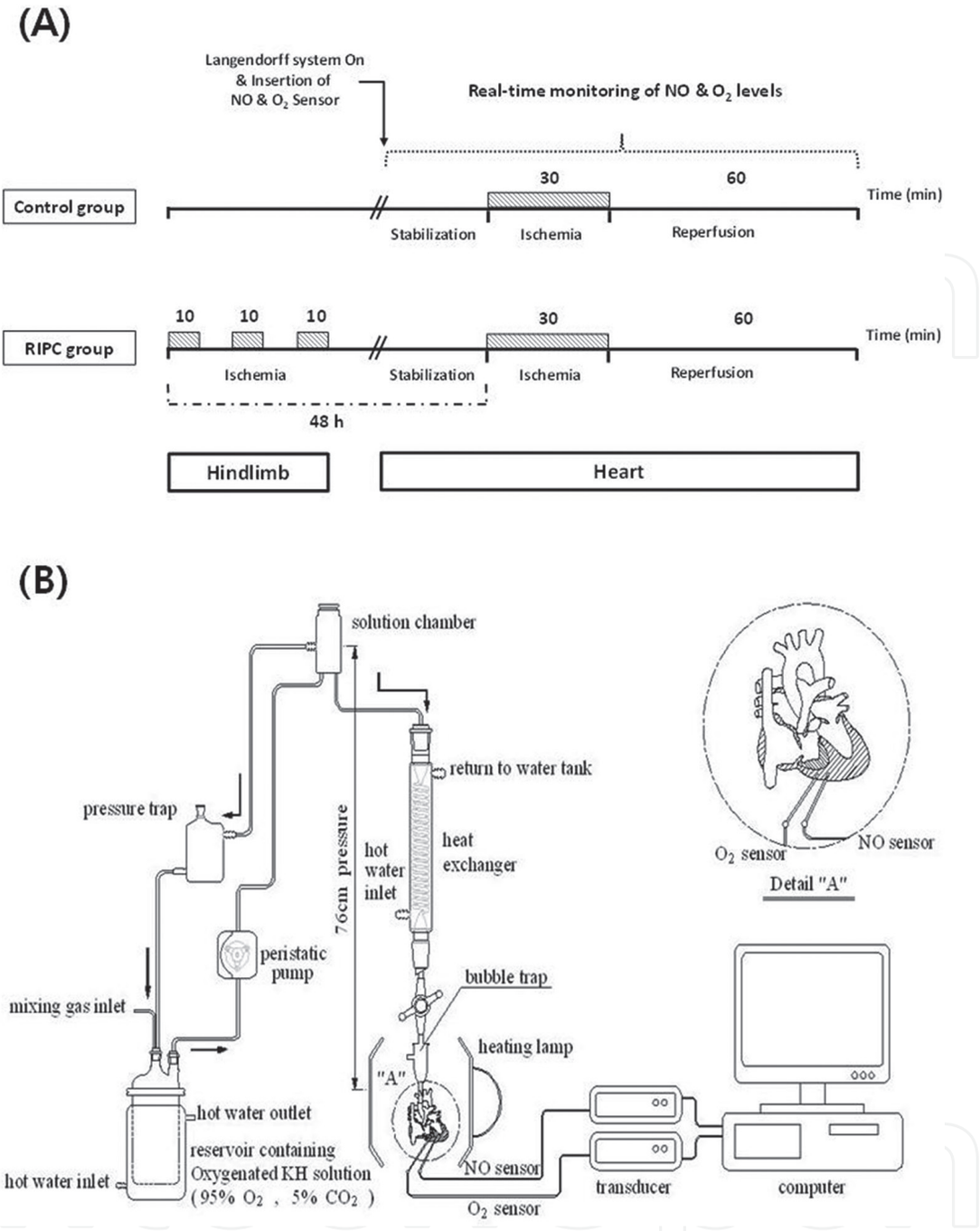

Figure 3. Schematic diagrams of (A) experimental design and (B) experimental setup of an isolated heart perfusion system and a real-time monitoring system for nitric oxide and oxygen tension dynamics during myocardial ischemia-reperfusion of the rat. Reproduced from Kang et al. [65]. © 2013 Elsevier B.V.

As a result, the concentration change of NO in the RIPC group was different from those in the control group during the ischemic period. In the control group, the NO level initially declined but then gradually inclined during the ischemic episode. In contrast, the NO level in the RIPC group rapidly increased after the onset of ischemia and continued to rise throughout the entire ischemic period [65]. When reperfusion was initiated, the pattern of both NO level and pO_2 in the RIPC group was different from that of the control group. As a result, the NO level and the pO_2 of the myocardium in the RIPC group were

restored to pre-ischemic levels, unlike those in the control group that did not recover to their pre-ischemic state (**Figures 4** and **5**). In summary, the endogenous production of NO during the ischemic period appears to be correlated with the restoration of NO and pO_2 in the post-ischemic myocardium after early reperfusion. Additionally, RIPC would promote endogenous NO release against ischemic stimuli and subsequently facilitate reoxygenation in post-ischemic myocardia after reperfusion [65].

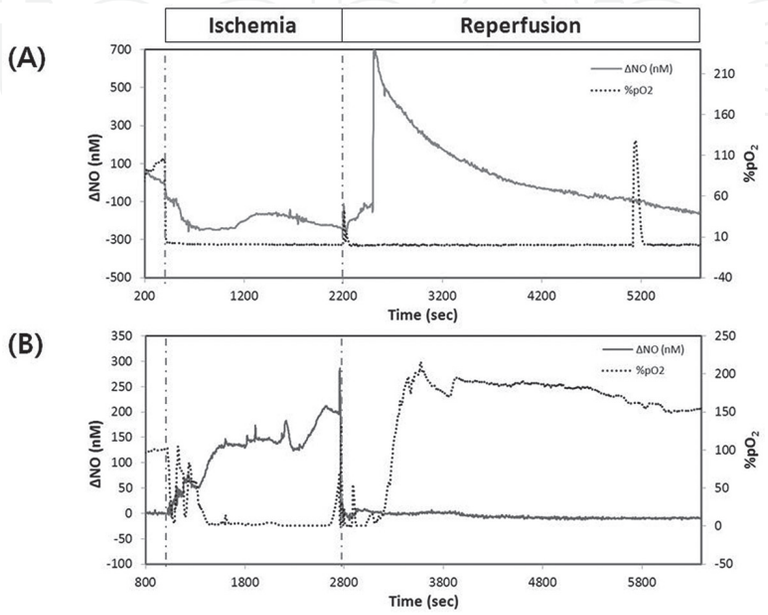

Figure 4. Representative real-time and simultaneous measurement of nitric oxide and oxygen tension in (A) control and (B) remote ischemic preconditioning (RIPC) groups during myocardial ischemia-reperfusion in Langendorff-perfused rat hearts. Reproduced from Kang et al. [65]. © 2013 Elsevier B.V.

5.4. Effect of prostaglandin E1

Prostaglandin E1 (Alprostadil®, PGE1), which is an important member of the prostaglandin (PG) family, is a product of arachidonic acid metabolism by cyclooxygenase [66, 67]. Similar to NO, PGE1 has cardioprotective effects during IR [67, 68], as well as vasodilator effects on the systemic and pulmonary circulation [69]. Fang et al. reported that pretreatment of human umbilical vein endothelial cells with PGE1 significantly protected those cells from H_2O_2-induced cell death [66]. And this effect might depend, at least in part, on the upregulation of NO expression [66]. On the other hand, Huk et al. reported that PGE1 prevents the excessive generation of NO, superoxide, and $ONOO^-$ which trigger a cascade of events leading to IR injury [70]. Though it is known that PGE1 has cardioprotective effects against IR injury, its mechanism and the correlation between NO and PGE1 are not yet clear. Lee et al. monitored the changes in NO and O_2 levels in the myocardium during myocardial IR that were induced by PGE1 pretreatment, utilizing sol-gel-modified amperometric NO and O_2 microsensors [67]. They investigated the correlation between endogenous NO and PGE1 in the ischemic episode, as well as oxygen recovery in the post-ischemic myocardium [67]. For statistical and quantitative analysis, they utilized analytical parameters such as %NO and $\%pO_2$, which are defined as the percentage of normalized NO (Eq. (1)) and pO_2 (Eq. (2)), respectively:

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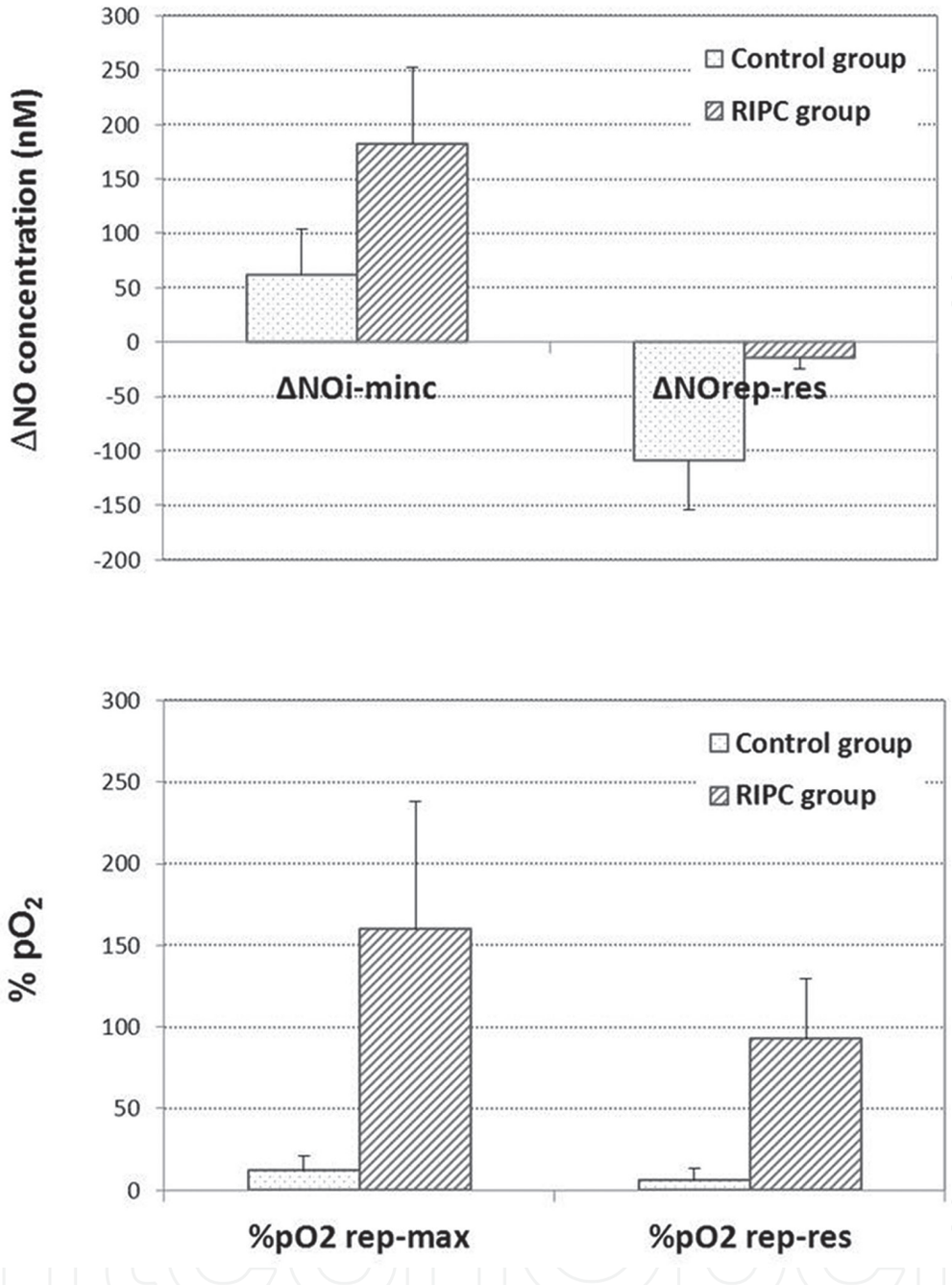

Figure 5. The correlation between ischemia-evoked nitric oxide concentration and reoxygenation parameters of the post-ischemic myocardium in two groups. Error bars represent standard deviation of the mean (n = 5 per group). Reproduced from Ref. [65], Kang SW et al., Anal. Chim. Acta 802, 74 (2013). © 2013 Elsevier B.V.

$$\%NO = \frac{\text{NO level on ischemic or reperfusion period (nM)}}{\text{NO level on pre-ischemic period (mM)}} \times 100 \quad (1)$$

$$\%pO_2 = \frac{pO_2 \text{ level on ischemic or reperfusion period (mmHg)}}{pO_2 \text{ level on pre-ischemic period (mmHg)}} \times 100 \quad (2)$$

As a result, there were significant differences in the NO dynamics during ischemia and reperfusion between the control and PGE1-treated rat hearts (**Table 1** and **Figure 6**) as follows

[67]: In the control group, the initial decrease in %NO was 56.0 ± 12.9, and the maximum NO level was 86.7 ± 18.5 during the ischemic period. However, in the PGE1 group, %NO rapidly declined to 19.9 ± 5.8% of the pre-ischemic levels, and this was maintained throughout the 30 min ischemic episode. In addition, after the onset of reperfusion, NO level inclined to a maximum of 82.0 ± 6.4 but did not exceed the pre-ischemic basal NO level. In the control group, the maximum %NO response (164.9 ± 41.0) to reperfusion was approximately double than that of the PGE1 group ($p < 0.01$, $n = 5$). They suggest that the cardioprotective effect of PGE1 might be attributed to a reduction in excessive NO production during early reperfusion.

Parameters	Control group (n = 5)	PGE1 group (n = 5)	*p* value
Level of baseline during pre-ischemic period	101.4 ± 0.9	101.0 ± 2.9	0.767
Level of initial decrement after the onset of ischemia	56.0 ± 12.9	19.9 ± 5.8	<0.001
Maximum level during ischemia	86.7 ± 18.5	26.0 ± 13.1	<0.001
Maximum level during the reperfusion period	164.9 ± 41.0	82.0 ± 6.4	0.01
Restoration level after 60 min of reperfusion	98.7 ± 42.1	45.6 ± 10.1	0.046

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Table 1. Changes in %NO level during myocardial ischemia-reperfusion of the control and PGE1-treated groups.

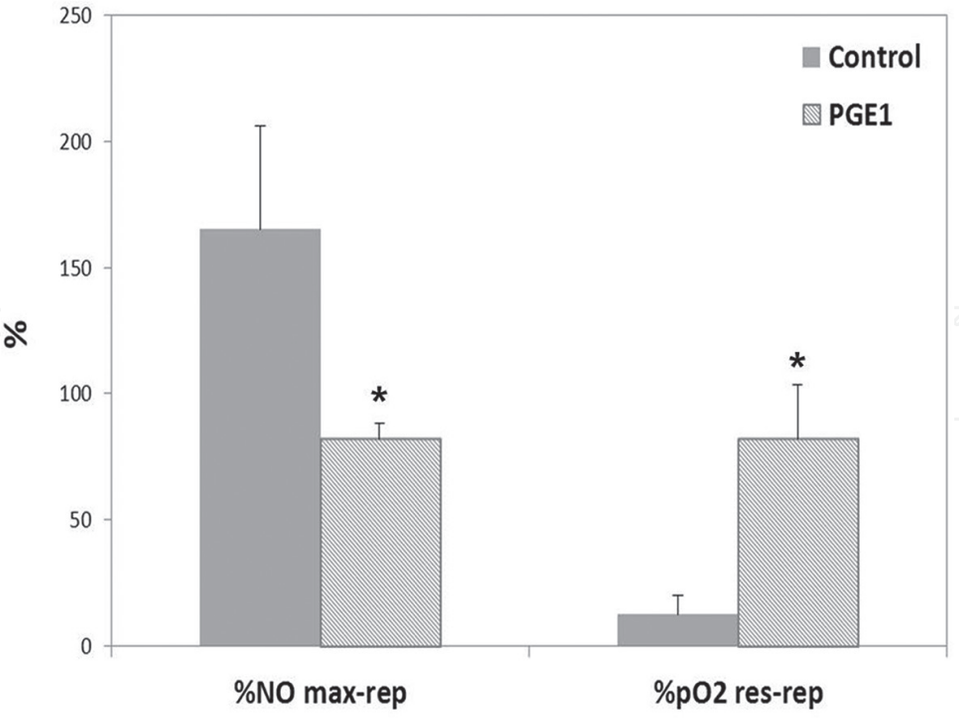

Figure 6. The correlation between the maximum %NO and restoration $\%pO_2$ during 60 min of reperfusion in the two groups. Error bars represent the standard deviation from the mean (n = 5 per group). Reproduced from Kang et al. [67]. © 2014 Elsevier B.V.

6. Conclusions

NO plays important roles in the cardiovascular system by mediating various physiological and pathophysiological processes. From the real-time measurement of endogenous NO dynamics in the myocardium, we summarize as follows: (1) NO concentration was definitely decreased after myocardial ischemia; (2) there was endogenous NO formation as a protective response against ischemia during the ischemic episode, but it was not enough to restore pre-ischemic NO level; (3) 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; and (4) the reduction of excessive NO production in early reperfusion period might also be helpful as a therapeutic strategy to protect the myocardium from IR injury. And NO permselective microsensors have good sensitivity and specificity for detecting biologically released NO dynamics in vivo and can be applied in real-time monitoring of NO dynamics in various organs.

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