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Roles of the nitric oxide signaling pathway in cardiac ischemic preconditioning against myocardial ischemia-reperfusion injury

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Summary

Nitric oxide (NO), a vasoactive gas that can freely diffuse into the cell, has many physiological effects in various cell types. Since 1986, numerous studies of ischemic preconditioning against ischemia-reperfusion (I/R) injury have been undertaken and the roles of the NO signaling pathway in cardioprotection have been explored. Many studies have confirmed the effect of NO and that its relative signaling pathway is important for preconditioning of the cardioprotective effect. The NO signaling against I/R injury targeted on the mitochondria is believed to be the end-target for cardioprotection. If the NO signaling pathway is disrupted or inhibited, cardioprotection by preconditioning disappears. During preconditioning, signaling is initiated from the sarcolemmal membrane, and then spread into the cytoplasm via many series of enzymes, including nitric oxide synthase (NOS), the NO-producing enzyme, soluble guanylyl cyclase (sGC), and protein kinase G (PKG). Finally, the signal is transmitted into the mitochondria, where the cardioprotective effect occurs. It is now well established that mitochondria act to protect the heart against I/R injury via the opening of the mitochondrial ATP-sensitive K^+ channel and the inhibition of mitochondrial permeability transition (MPT). This knowledge may be useful in developing novel strategies for clinical cardioprotection from I/R injury.

key words: **nitric oxide • ischemic preconditioning • ischemia/reperfusion injury • heart**

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Background

Acute myocardial infarction (MI) is one of the major causes of death worldwide [1]. Acute MI occurs when the large epicardial coronary artery is occluded by a thrombotic blood clot or lipid accumulation. When the occlusion lasts over 20–40 minutes, ischemic tissues become irreversibly damaged, resulting in infarction [2]. The precise time to the development of infarction varies between species. For example, faster development is observed in small rodents with a high heart rate and low collateral blood flow, and slower development is found in larger animals [3]. The progressive and irreversible damage incurred during myocardial ischemia can only be stopped by an immediate reperfusion. Despite this fact, severe and irreversible myocardium damage during the ischemic phase could be caused by reperfusion itself, referred to as reperfusion injury [4]. Irreversible reperfusion injury is defined as an injury caused by reperfusion after the ischemic episode, and results in the death and loss of cells that had only been reversibly injured and primed for death during the preceding ischemic episode [2].

During ischemia, the cardiomyocytes become depleted of oxygen and energy. Higher CO_2 and lactate production in the cell is associated with greater acidosis. The increased acidosis that is produced by anaerobic metabolism increases the influx of Na^+ via the Na^+/H^+ exchanger and the depletion of ATP for Na^+/K^+ ATPase activity, which in turn inhibits the eradication of Na^+ from the cell. This results in the accumulation of Na^+ in cytoplasm [5]. The large amount of Na^+ in cytoplasm drives the reverse mode of the Na^+/Ca^{2+} exchanger, leading to the overload of Ca^{2+}. Finally, the elevated cytosolic Ca^{2+} levels contribute to the opening of a mitochondrial permeability transition pore (mPTP) and result in the death of cells [6]. Large infarct size and failure of infarct tissues to perform a physiological function may lead to heart failure. Protecting the heart from these harmful consequences of coronary occlusion has been the goal of ongoing research by a number of investigators for many decades [7–12]. In recent years, cardiac ischemic preconditioning (i.e., the brief sequences of coronary occlusion and reperfusion before prolonged occlusion) has been extensively studied and found to be cardioprotective against ischemia/reperfusion (I/R) injury. Its various roles in cardioprotection involve many factors, including the nitric oxide signaling pathways [13]. In this review, factors involving the cardioprotective process of ischemic preconditioning, particularly nitric oxide (NO), are presented, the mechanisms of this process are explained, and clinical implications for future therapeutic approaches involving the role of nitric oxide (NO) are discussed.

Ischemic Preconditioning

Ischemic preconditioning (IPC) is the induction of a brief episode of ischemia and reperfusion in myocardium to markedly reduce tissue damage induced by prolonged ischemia [7]. Ischemic preconditioning as a potent cardioprotective method against I/R injury was first reported by Murry et al. in 1986 [8]. In their study using dogs, four sequential episodes of 5-minute occlusion each followed by 5-minute reperfusion were induced in the left circumflex coronary artery, preceding a sustained 40-minute prolonged occlusion period. Another group of dogs were left on a prolonged coronary ligation without IPC. After 4 days of reperfusion, the infarction generated in the former group was reduced by 75% compared to that of the latter group. In their study there was no significant difference in the collateral blood flows of both groups. These results suggested that the tolerance of cardiac tissue to I/R injury is not related to the change of regional blood flow. This remarkable cardioprotection process has also been demonstrated in various species of experimental animals, including rats, mice, rabbits, chicken, and swine [9–13].

Although many investigations have documented preconditioning as an intervention capable of protecting the heart against I/R injury, the cardioprotective effect of IPC is relatively short-lived and the underlying mechanism is not completely understood. More recently it was found that the fundamental processes and the end-target of cardioprotection of IPC mechanisms are related to the prevention of mitochondrial permeability transition (MPT) [1]. Any drug that blocks MPT, such as cyclosporine A, is able to mimic the IPC effect [6]. The MPT and intramitochondrial signaling against I/R injury will be described later in this review.

Even after IPC was first described by Murry et al. in 1986, its mechanisms remained largely unknown for several years. The only clue at the time from studies in various species was that IPC was not involved in the coronary blood flow through the damaged area [14]. However, in 1991 Liu et al. discovered that stimulation of cardiac G_i-coupled adenosine receptor type 1 (A_1) was necessary for the preconditioning effect [14]. The study also showed that IPC's protection could be attenuated by an adenosine receptor antagonist, whereas the infusion of adenosine, or the A_1-specific agonist N6-1-(phenyl-2R-isopropyl) adenosine (R-PIA), could reduce the infarct size. Therefore, cardioprotection of IPC was achieved as ischemic myocardium rapidly degraded ATP to adenosine, which then accumulated in this area [7].

Bradykinin and opioid receptors are also involved in the IPC process [15–17]. During the preconditioning ischemia, bradykinin and endogenous opioid are released from the heart, together with the production of adenosine as a result of metabolic breakdown of ATP [7]. These 3 ligands activate their respective G-protein coupled receptors (GPCRs), which work on the other pathways to protect the heart from ischemic conditions [7]. Nevertheless, the protection of IPC could not be completely blocked if 1 of these 3 receptors is inhibited. On the other hand, it was suggested that combining 2 or more of these receptors produces an incremental cardioprotective effect against I/R injury of IPC [18]. Further research has shown that increasing the number of preconditioning cycles leads to greater resistance of the heart to I/R injury [19]. It was proposed that the additional brief I/R cycles produced more of the trigger substances, and more activities of these receptors could be observed [19]. In addition to these substances and receptors, nitric oxide (NO) has been shown to play an important role in the cardioprotective process of IPC during myocardial ischemia, which will be described later in this review.

Nitric Oxide in the Heart

NO is a signaling molecule that affects the cardiovascular system as a vasodilatator [20]. In hypertensive patients the level of NO metabolites (nitrite and nitrate) are found to be attenuated from their normal levels and inversely correlated

with blood pressure [21]. In the heart, NO is known to be an important regulator of cardiac contractility in physiological condition [22]. In cardiomyocytes all 3 isozymes of NO synthase (NOS) are expressed – neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3) [22]. Despite the fact that NO is a highly diffusible signaling molecule that can diffuse freely across the membranes, many studies have shown that the regulation of nNOS and eNOS are localized due to their compartmentalization. nNOS is located in the sarcoplasmic reticulum (SR), whereas eNOS is located in the caveolae of the sarcolemma [22]. Each type of NOS performs a different cardiac contraction modulation. While nNOS signaling potentiates the response of β-adrenergic stimulation [23], eNOS signaling depresses the functional regulation of β-adrenergic stimulation [24]. Although nNOS and eNOS are membrane-bound enzymes and regulate cardiomyocytes under physiological condition, iNOS is a soluble enzyme and produces less expression in physiological condition [24]. Many factors appear to be involved in the regulation of iNOS induction in response to cytokines or cardiac stress [25].

NOS produces NO from the conversion of L-arginine into citruline, using a large number of cofactors, including nicotinamide adenine dinucleotide phosphate (NADPH) and flavin mononucleotide (FMN) [25]. Then, the activation of soluble guanylyl cyclase (sGC) by NO leads to the formation of cGMP from the nucleotide GTP [25]. cGMP-dependent protein kinase (PKG) is further activated, initiating numerous physiological regulations in cardiomyocytes. As a result, L-type Ca^{2+} channel (LTCC) activity is depressed by NO via cGMP-dependent pathways [26–28]. Moreover, a phosphorylation of α_{1C} subunit at position Ser^{533} of LTCC can be developed by the activated PKG, causing an inhibition of L-type Ca^{2+} current ($I_{Ca,L}$) [26]. The rate of Ca^{2+} reuptake into SR, by the increased phosphorylation of phospholamban, can be increased by NO signaling [29]. NO that is released from nNOS when it binds with the superoxide anion, can form peroxynitrite, which exerts this specific effect on phospholamban [29]. Apart from the studies of the effects of NO and cGMP on the Ca^{2+} channel, other cardiac ion channels have also been shown to be regulated by this pathway. The ATP-sensitive K^+ channel is activated by NO donors, and this involves both activation of PKG and the cGMP-independent effect [30,31]. The hyperpolarization-activated pacemaker current (I_f) is also activated by NO and the cGMP-dependent pathway in isolated guinea pig sinoatrial node cells and in human right atrial appendage cells [32,33].

Role of Nitric Oxide Signaling Pathway in the Cytosolic Signaling of IPC

NO and its signaling pathway have been shown to be important in cardioprotection against I/R injury [1]. Numerous studies have shown the cardioprotective effect of both endogenous and exogenous NO on IPC (Table 1). In 1995 it was shown for the first time that NO has a cardioprotective effect against I/R injury, but another report in the same year showed the opposite result [34,35]. Williams et al. reported that endogenous NO plays a critical role in the reduction of infarct size after a 30-minute period of ischemia and a 120-minute period of reperfusion in rabbit hearts in an *in vivo* model [34]. Using L-nitro-arginine (L-NA), a non-specific NOS inhibitor, to treat one group before coronary occlusion and another group during reperfusion (with the same dose administered in each group), increased infarct areas in both groups were observed, compared to a vehicle-treated group. In addition, there was no significant difference in infarct sizes between the L-NA-treated groups before and after coronary occlusion [34]. Despite this result, Woolfson et al. reported that when the isolated rabbit heart was perfused with N (G)-nitro-L-arginine methyl ester (L-NAME), the non-specific NOS inhibitor, at 10 min before the coronary occlusion and continued for 15 min of reperfusion period in 45 min of ischemia and 180 min of reperfusion episode, the infarct size was decreased compared to the untreated group [35]. These inconsistent findings could be due to use of different study models as well as differences in the duration of the treatment with NOS inhibitors and/or the duration of I/R episodes. Moreover, NO is claimed to be a pro-apoptotic factor when it reacts with superoxide anion to form peroxynitrite [36]. This result may be the cause of the myocardial injury in I/R condition found by Woolfson et al. [35], who also found that the NOS inhibitor could reduce infarct size in non-preconditioned hearts after I/R injury [35]. Nevertheless, the mechanisms of NO in the heart are still unclear and need to be investigated further.

The cardioprotective effect of NO on I/R injury was also supported by Zhao et al. in 1997 [37], who suggested that monophosphoryl lipid A (MLA) has an IPC-mimetic effect due to an increase in iNOS activity after coronary occlusion and reperfusion in rabbit hearts [37], as well as discovering the important role of iNOS in the cardioprotective effect against I/R injury when aminoguanidine (AMG), the specific-iNOS inhibitor, was used. In groups treated with AMG alone or with MLA, the infarct size was significantly increased compared to the MLA-only treated group and was not different from the vehicle-treated group [37]. These results suggested that when iNOS is inhibited, the cardioprotective effect of MLA disappears. In a pacing-induced preconditioning model of the rat heart, N^G-nitro-L-arginine (L-NNA), a non-specific NOS inhibitor, increased the release of lactate dehydrogenase, a marker of necrotic cell death, from the coronary-occluded ischemic area [38]. Yang et al. found that L-NAME could abolish the cardioprotective effect of adenosine receptor agonist 5'-(N-ethylcarboxamido) adenosine (NECA) or bradykinin in the I/R model in rabbit hearts [39]. Prendes et al. demonstrated in 2007 that IPC increased cardiac contractility after global ischemia and reperfusion in isolated rat hearts [40]; however, the effect of IPC disappeared when treated with L-NAME [40]. All of these studies demonstrated the cardioprotective effect of NO against I/R injury.

The role of exogenous NO in cardioprotection during ischemia was demonstrated by Nakano et al. [41], who found that S-nitroso-N-acetylpenicillamine (SNAP), an NO donor that serves as the resource of exogenous NO, was able to mimic preconditioning by decreasing infarct size in rabbit hearts after a long period of I/R without IPC, compared to those without SNAP infusion. The study also reported that protein kinase C (PKC) and free radicals or reactive oxygen species (ROS) were important for cardioprotection of the preconditioning-mimetic effect of exogenous NO. When chelerythrine, an inhibitor of PKC, or N-(2-mercaptopropionyl)-glycine (MPG), a ROS scavenger, were combined with SNAP,

Table 1. Effects of sources of NO in cardioprotection against ischemia-reperfusion injury.

Source of NO	Model of I/R	Animal Models	Effect of NO	References
Endogenous	Isolated heart (coronary ligation) Isolated heart (global ischemia)	Rabbit	Reduce infarct size (in both models of I/R injury)	[34]
Endogenous	Cultured embryonic ventricular myocyte	Chick	Decrease cell death	[58]
Endogenous	Isolated heart (coronary ligation)	Rat	Decrease cell death by decreasing of lactate dehydrogenase released	[38]
Endogenous	Isolated heart (global ischemia)	Rat	Improve cardiac function but do not change in cell viability	[40]
Endogenous	*In vivo* (coronary ligation)	Rabbit	Reduce infarct size	[62]
iNOS	*In vivo* (coronary ligation)	Rabbit	Reduce infarct size	[37]
iNOS	Isolated heart (global ischemia)	Mouse	Reduce infarct size	[44]
iNOS	*In vivo* (coronary ligation)	Mouse	Reduce infarct size	[82]
iNOS, eNOS	Isolated heart (global ischemia) Isolated cardiomyocyte	Rat	Reduce infarct size Decrease cell death	[61]
eNOS, iNOS and exogenous	Isolated heart (global ischemia)	Mouse	Reduce infarct size	[43]
eNOS	Isolated heart (global ischemia)	Mouse	No response	[48]
eNOS	Isolated heart (global ischemia)	Mouse	Reduce infarct size	[50]
Exogenous	Isolated heart (coronary ligation)	Rat	Reduce infarct size	[59]
Exogenous	Heart slice	Rat	Decrease necrosis and apoptosis	[60]
Exogenous	Cultured neonatal cardiomyocyte	Rat	Decrease cell death	[85]
Exogenous	Isolated heart (coronary ligation)	Rabbit	Reduce infarct size	[41]
Exogenous	Isolated heart (coronary ligation)	Rat	Reduce infarct size	[42]

the cardioprotective effect of SNAP was eliminated, as shown by the enlargement of infarct size as compared to the SNAP-only treated group [41].

The cardioprotective effects of NO have also been supported by other studies [42,43]. Kanno et al. in 2000 showed that iNOS was overexpressed in eNOS knockout mice after 30 minutes of ischemia followed by 60 minutes of reperfusion; however, nitrite formation and infract area were not different from that of wild type mice [43]. The role of iNOS was also reported by Zhao et al. in 2000 [44], who found that an increase in iNOS expression could be induced by treating with 2-chloro-N^6-cyclopentyladenosine (CCPA), an adenosine A_1 receptor agonist, while the effect disappeared when treated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an adenosine A_1 receptor antagonist [44]. CCPA was also found to reduce infarct size after I/R, while DPCPX or S-methylisothiourea (SMT), a specific iNOS inhibitor, cancelled this effect [44]. Results of a study by Wang et al. in 2002 supported the dominant effect of iNOS in preconditioning [45]. It was discovered that IPC, with a sequence of 6 cycles of 4-minute coronary occlusion and 4-minute reperfusion, increased the expressions of iNOS mRNA and protein at 3 hours after the last cycle of IPC [45]; however, the expression of eNOS remained unchanged [45]. Although iNOS has been shown to protect the heart against myocardial injury after I/R, in 2008 Heinzel et al. found that the treatment of iNOS inhibitor aminoguanidine (AG) in the sustained moderate regional ischemia by a reduction in coronary artery pressure to ~45 mmHg for 6 h in miniswine, AG caused an increase in the cell shortening compared to a non-treated group combination with L-arginine in an isolated cardiomyocyte model [46]. This result suggests the role of iNOS in the attenuation of cardiac function after hypoperfused ischemia [46]. The role of iNOS in the late phase of IPC was also observed. Guo et al. found that iNOS knockout mice had larger infarct size than wild-type mice after being subjected to 30-min coronary occlusion and 24-h reperfusion in the presence of IPC (6 episodes of 4-min occlusion and 4-min reperfusion cycles) [47]. Moreover, at 24 h after I/R, wild-type mice with IPC had smaller infarct size than both non-IPC wild-type and iNOS knockout mice at 24 h after IPC [47]. This cardioprotective effect of iNOS was supported by the increased level of iNOS expression in the IPC group compared to the non-IPC group at 24 h after IPC [47].

The significance of eNOS on cardioprotection against I/R injury has also been investigated. Bell et al. reported that the infarct size in eNOS knockout mice was not different from that of wild-type mice after IPC [48]. This finding is consistent with a 2008 study by Guo et al., which reported that both eNOS-knockout mice and wild-type mice have a

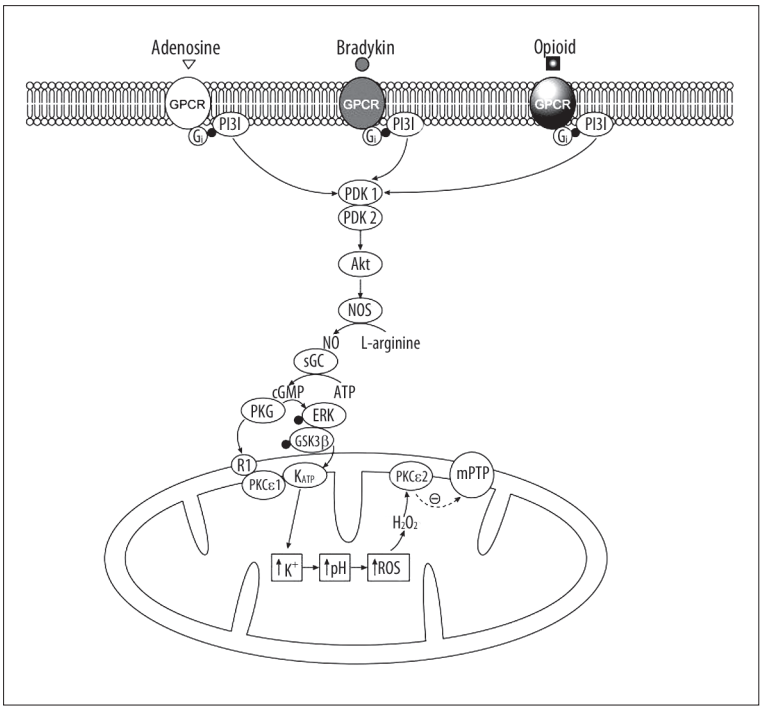

Figure 1. The schematic diagram represents the ischemic preconditioning pathway in cardiomyocytes. IPC induces cardiomyocytes to release adenosine, bradykinin and endogenous opioid which occupy their specific G-protein coupled receptors. After that, the signal will pass into the cytosol via the activation of the enzyme series including PI3K, PDKs, Akt and NOS. The latter enzyme produces NO which acts as signaling molecule to activate sGC and resulted in the cGMP formation. Then, cGMP activates PKG which has the separated mechanism on the mitochondria, the direct and indirect mechanism. The direct mechanism of PKG is on the R1 protein on outer mitochondrial membrane which then activates the opening of mitoK$_{ATP}$ channel on the inner mitochondrial membrane via the phosphorylation of PKCε1. The indirect mechanism of PKG is the phosphorylation of ERK and GSK3β which then act on mitoK$_{ATP}$ channel. However, the mechanism of how GSK3β activates the opening of mitoK$_{ATP}$ channel is still unclear. After the mitoK$_{ATP}$ channel opening, K^+ then enters the mitochondrial matrix and H^+ is ejected out of the matrix to balance the positive charge. When H^+ level decreases, the electron transport chain is interrupted and leads to the formation of superoxide anion and then H_2O_2. Finally, H_2O_2 will act as signaling molecule to activate PKCε2 which then inhibits the opening of mitochondrial permeable transition pore (mPTP). This inhibition of mPTP opening helps to protect mitochondrial damage during I/R injury. (Modified with permission from [63]).

similar infarct size after I/R injury in the non-preconditioned state [49]. In eNOS knockout mice, these findings suggested that the role of eNOS in preconditioning is less prominent than that of iNOS. Although the infarct size was reduced in the overexpressed eNOS compared to wild type mice, du Toit et al. found that there was no difference in the infarct size between IPC and non-IPC in this overexpressed eNOS mouse model [50]. It has been proposed that the heart with eNOS overexpression may be already maximally protected against I/R injury by elevated endogenous NO levels produced by eNOS in this model [50]. Nevertheless, it is not known whether exogenous NO would be beneficial in overexpressed eNOS mice, and further studies are needed to investigate this possibility.

In biological systems, NO has also been found to be produced from enzyme-independent generation [51]. Under acidic conditions, such as intracellular acidosis from ischemia, NO can be generated in the tissues from the reduction of nitrite, NO_2^- [52]. Zweier et al. found that although all NOS isoforms in the heart were totally blocked by L-NAME, the NO formation was partially inhibited in the hearts after 30 min of ischemia [53]. They also reported that in the I/R condition of 30-min ischemia followed by 45-min reperfusion, the time course of the recovery of rate pressure product in L-NAME-treated hearts were higher than in the untreated group; however, when L-NAME was combined with nitrite, the time course of the recovery of rate pressure product decreased [53]. This finding suggested that nitrite can reverse the inhibitory effect of NOS inhibitor, indicating the enzyme-independent generation of NO as a possible mechanism protecting against myocardial I/R injury.

The cardioprotective mechanisms of NO beyond the cGMP/PKG-dependent pathway have also been reported. Since NO can modify the structure of protein through the mechanism called S-nitrosylation, some recent studies have also demonstrated the cardioprotective effect of NO via this mechanism [54,55]. S-nitrosylation occurs when NO, from NO sources such as NOS, acts through the post-translational modification of cysteine thiol on the targeted proteins that colocalized with it to form S-nitrosothiol (SNO), resulting in a change in their protein functions [56,57]. A study by Sun et al. in 2007 reported that when treated with S-nitrosoglutathione (GSNO) (i.e., the exogenous source of NO for S-nitrosylation), the infarct area was smaller than in the untreated group after 20 min of no-flow ischemia and 20 min of reperfusion in isolated mouse hearts [54]. Lin et al. in 2009, using female ovariectomized mice, demonstrated that 17β-estradiol (E2) and the estrogen receptor-β-selective agonist 2,2-bis(4-hydroxyphenyl)-proprionitrite (DPN) decreased the infarct size in the heart after being subjected to 20 min of ischemia followed by 30 min of reperfusion [55]. Proteomic analysis also demonstrated that an increase in S-nitrosylation of a number of proteins could be found in the DPN- and E2-treated hearts, as well as in the preconditioning group [55]. This finding suggests that the increased SNO protein from DPN and E2 treatment after

I/R could lead to cardioprotection against myocardial injury [55]. Therefore, the S-nitrosylation by NO could be one of the major targets for cardioprotection against I/R injury.

Nitric Oxide and Intramitochondrial Signaling of IPC

A growing body of evidence demonstrates the important relationship among NO, ROS, and ischemic preconditioning[41,58–62]. In 2003 Lebuffe et al. found that H_2O_2 and NO were important for preconditioning-like cardioprotection [58]. Furthermore, when the mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel was inhibited by 5-hydroxydecanoate (5-HD) in the IPC model, the cardioprotective effect of IPC disappeared [58], suggesting the importance of the mitoK_{ATP} channel in cardioprotection against I/R injury.

Recent research on the mechanisms of IPC suggests that during a brief episode of ischemia, 3 ligands are released from the cardiomyocytes, and long sequences of activities are found here [7]. These ligands (bradykinin, endogenous opioid, and adenosine), occupying their respective G-protein coupled receptors (GPCRs), result in activations of phosphatidylinositol 3-kinase (PI_3K) and series of phospholipid-dependent kinase (PDK) (Figure 1). PDK causes phosphorylation and activation of Akt, where the latter induces further phosphorylation onto NOS, causing NO to be generated. After that, sGC, activated by NO, transforms GTP into cGMP, in which PKG is finally activated. In the last step of cytosolic signaling, PKG then reacts on mitochondria, resulting in the opening of the mitoK_{ATP} channel (Figure 1) [63]. The opening of the mitoK_{ATP} channel leads to the inhibition of the mitochondrial permeability transition pore (mPTP), resulting in the protection of mitochondria from damage during ischemia. Thus, a cGMP-dependent mechanism has been proposed as the main pathway to activate the mitoK_{ATP} channel via phosphorylation by PKG [60,62].

In mitochondria, the mPTP has also been shown to play a critical role in I/R injury. The mPTP is the megachannel that is the formation of 3 specific components, including the voltage-dependent anion channel (VDAC), the adenosine nucleotide transporter (ANT), and cyclophilin D [64]. Currently, the molecular structure of this pore is still under debate [64]. A high concentration of calcium, including calcium overload due to myocardial ischemia, has been shown to trigger the opening of mPTP [65]. Previous studies demonstrated that cyclosporine A, the mPTP inhibitor, could protect the heart from myocardial I/R injury [66,67]. In 2002, Hausenloy et al. proposed that the inhibition of mPTP opening could be the end-target of IPC [68]. Therefore, if mPTP opening is inhibited or interrupted by the process of mitoK_{ATP} channel opening, mitochondrial damage from I/R injury could be prevented, resulting in cardioprotection against I/R injury.

Exogenous NO by SNAP can increase ROS generation in isolated rat cardiomyocytes [59]. However, when SNAP was co-incubated with 5-HD, the ROS production decreased. On the other hand, when treated with diazoxide, the mitoK_{ATP} channel opener, without the treatment of SNAP, the ROS production was instead increased [59]. These findings suggested a relationship between NO, mitoK_{ATP} channel, and ROS production, in which ROS production via the opening of mitoK_{ATP} channel is activated by NO.

Growing evidence supports the proposed hypothesis that the NO-cGMP-PKG pathway causes the mitoK_{ATP} channel opening [58,60,62,69,70]. In this pathway, PKG was proposed as the last step in the signaling process before the involvement of mitochondria (Figure 1). This hypothesis was established when it was shown that exogenous PKG plus cGMP, when added to the isolated mitochondria, causes the increase of mitochondrial matrices due to the opening of the mitoK_{ATP} channel [71]. Furthermore, this effect could be reversed by several substances such as KT5823 (the specific PKG inhibitor), 5-HD or glibenclamide (the mitoK_{ATP} channel inhibitors), and εV_{1-2} (the protein kinase C ε isoform [PKCε]-specific inhibitor) [71].

The mitoK_{ATP} channel is located in the mitochondrial inner membrane (MIM); however, PKG is a cytosolic enzyme that is unable to cross the mitochondrial outer membrane (MOM). Therefore, how PKG interacts with the mitoK_{ATP} channel remains unknown. It has been proposed that phosphorylation of serine or threonine by an unknown protein called R1, located on MOM, is a necessary step taken by PKG (Figure 1) [63]. The PKG-dependent mitoK_{ATP} channel opening seems to require the intact MOM, and is reversed by Ser/Thr phosphatase PP2A [63]. The phosphorylation of this unknown protein R1 causes the signal to be transmitted to PKCε on the MIM, leading to phosphorylation and opening of the mitoK_{ATP} channel (Figure 1).

The confirmation of the presence of R1 was demonstrated by Costa and Garlid in 2008 [72], who used mitochondria and mitoplast (mitochondria without MOM) to test the effects of many specific activators and inhibitors on intramitochondrial signaling [72]. By using phorbol 12-myristate-13-acetate (PMA), a PKCε activator that can cross the MOM, they found that it caused mitochondria matrix swelling due to the opening of the mitoK_{ATP} channel in both mitochondria and mitoplast. However, when treated with isolated PKG, the swelling was observed in mitochondria but not in mitoplast [72]. Both the effects of PMA and PKG, nonetheless, could be removed when treated with the PKCε phosphatase PP2A [72]. This clearly shows that the specific protein R1 located in MOM has the ability to interact with PKG, as well as the ability to open the mitoK_{ATP} channel. PKG itself does not directly open the mitoK_{ATP} channel, and does not cause the mitochondrial matrix swelling in the mitoplast [72].

Another mechanism by which PKG mediates the opening of the mitoK_{ATP} channel was demonstrated by Das et al. in 2008 and 2009 [73,74]. The 2008 study demonstrated that the expression of ERK and GSK3β protein in the ischemia-reoxygenation condition was increased in mouse cardiomyocytes treated with sildenafil citrate, the phosphodiesterase type 5 (PDE-5) inhibitor [73]. They also demonstrated that when treated with the combination of PD98059, an ERK inhibitor, and sildenafil citrate, the level of necrosis and apoptosis increased compared to sildenafil citrate treated alone in ischemia-reoxygenation cardiomyocytes [73]. In 2009, their subsequent study demonstrated that after treatment with sildenafil citrate for 24 h, the phosphorylation of ERK and GSK3β increased in the intact mouse heart [74]; however, when treated with PD98059, the phosphorylation of ERK and GSK3β decreased and were not different from the control. These findings suggested that GSK3β is downstream

of ERK [74], and led the researchers to propose a mechanism downstream from PKG, in which ERK and GSK3β protect the heart against I/R injury via the opening of the mitoK$_{ATP}$ channel (Figure 1).

GSK3β, a multifunctional Ser/Thr kinase, is one of the main target signaling cascades in cardioprotection against I/R injury [75]. This protein was originally found to be involved in the disruption of glycogen synthesis via the inhibition of glycogen synthase, and was named glycogen synthase kinase (GSK) [75,76]. The role of GSK3β phosphorylation in the inhibition of mPTP opening in the heart was first reported by Juhaszova et al. in 2004 [77], showing that the threshold for mPTP opening was increased when GSK3β was knockdown via RNAi in isolated neonatal rat cardiomyocytes [77]. The GSK3β inhibitors were also reported to have a cardioprotective effect against I/R injury [78]. A 2002 study by Tong et al. demonstrated that pretreatment with lithium as well as SB216763, the GSK3β inhibitors, could decrease the infarct size after 20-min global ischemia and 30-min reperfusion in isolated rat hearts [78]. A 2006 *in vivo* study in rats by Nishihara et al. also supports the hypothesis that the GSK3β inhibitor SB216763 can limit infarct size after 20-min LAD occlusion and 40-min reperfusion in a dose-dependent manner [79]. Moreover, a study by Gross et al. in 2004 found that SB216763 and another GSK3β inhibitor, SB415286, when administered for 10 min before ischemia or 5 min before reperfusion, could decrease infarct size, and that infarct size was not different between pre- and post-treatment in 30-min ischemia and 2-hr reperfusion in rats [80]. In 2008, Obame et al. demonstrated that the cardioprotective effect of the GSK3β inhibitor against I/R injury was related to the inhibition of mPTP opening [81].

In mitochondria, the opening of the mitoK$_{ATP}$ channel leads to an increase in K^+ influx, causing swelling and alkalinization of the mitochondrial matrix. The increased K^+ influx then replaces H^+ in the electron transport chain, and as a result, superoxide anion, the first ROS that appears in the mitochondria, is produced [63]. The superoxide anion then reacts with H_2O to form H_2O_2, which directly activates PKCε on the MIM. Two isoforms of PKCε are found in MIM – PKCε1 and PKCε2 [63]. PKCε1, located close to the mitoK$_{ATP}$ channel, causes the phosphorylation and opening of the mitoK$_{ATP}$ channel. The other isoform, PKCε2, is located close to mPTP, and its activation causes the inhibition of mPTP opening [63]. A growing body of evidence currently supports this cardioprotective effect against I/R injury by the ROS production in mitochondria from the opening of the mitoK$_{ATP}$ channel [72,82,83]. The end-target effect of mPTP inhibition allows cells to survive due to an inhibition of the released apoptotic signal from mitochondria by the MPT process (the summarized diagram of the pathway is shown in Figure 1) [1,84]. Moreover, the irreversible mPTP opening can lead to the reduction of ATP production by abolishing mitochondrial membrane potential, which finally results in necrotic cell death from energy depletion [69]. Despite the inhibition of MPT by the opening of mitoK$_{ATP}$ channels, a 2008 study by Rickover et al. demonstrated that exogenous NO from SNP decreased intracellular Ca^{2+} overload in cardiomyocytes after ischemia-reoxygenation [85]. Since the intracellular Ca^{2+} overload can directly activate mPTP opening, the reduction of intracellular Ca^{2+} could attenuate the activation of mPTP in I/R condition [86].

Research Translation of Nitric Oxide against I/R Injury

NO-mediated signaling is intimately involved in the pathway that triggers protection against I/R injury. The preconditioning pathway initiates on the sarcolemmal membrane of cardiomyocytes, and carries messages to intracellular enzyme cascading, and, finally, to mitochondria. During the IPC process, myocardial resistance to hypoxic conditions can occur as a result of a brief ischemia before the reperfusion episode. Unfortunately, IPC is not feasible in patients presenting at the hospital with an acute MI. The goal of effective treatment of acute MI in humans, therefore, is to induce an IPC-like effect that can help myocardium to survive the prolonged ischemic process, similar to that in preconditioning.

Enhancing the NO signaling pathway could become a widely accepted method for mimicking the preconditioning effect, as this signaling plays an important role in both physiological and pathological conditions. Drugs such as NO donor or PDE-5 inhibitor are widely used to interrupt the NO signaling pathway. Many studies have found that sildenafil citrate, the PDE-5 inhibitor used for treatment of erectile dysfunction, has abilities to attenuate ischemic cardiomyopathy and reduce cell death from hypoxic conditions [87–90]. Ockaili et al. demonstrated that sildenafil citrate was able to decrease infarct size after coronary artery occlusion in rabbits, and that the effect was blocked by inhibiting mitoK$_{ATP}$ channel (5-HD) [87]. Another study by this team showed that the expression of iNOS and eNOS mRNA and protein in isolated mouse hearts could be increased by sildenafil citrate [88]. The level of eNOS mRNA increased transiently and peaked at 45 minutes after the sample was treated with the drug [88]. The rate of iNOS mRNA expression was slower, but the peak was higher than that of eNOS. Moreover, a significant increase in iNOS and eNOS proteins was detected 24 hours after treatment with sildenafil citrate. The cardioprotective effect against I/R injury induced by sildenafil citrate was eliminated by 1400W, the specific iNOS inhibitor [88]. Das et al. demonstrated that, in an isolated ventricular myocyte, necrosis and apoptosis were reduced, whereas the expression of iNOS and eNOS was elevated, when sildenafil citrate was applied after an ischemia-reoxygenation [89].

Conclusions

Since sildenafil citrate and other PDE-5 inhibitors have been approved by the U.S. Food and Drug Administration (FDA) as a vasoactive drug for the treatment of erectile dysfunction, the drugs may be useful for cardioprotection against I/R injury. Moreover, other pharmacological agents that are related to the NO signaling pathway, including NO donors such as nitroglycerin or drugs that have been shown to have positive effects on NO signaling in β-blockers such as nebivolol, could also impart the NO-related cardioprotection against ischemic cell death [91,92]. According to our understanding of the IPC mechanism, the drugs that regulate some parts of its cascade, including adenosine, bradykinin, opioid agonists, or PI3K-Akt activators, could be applied in clinical practice as possible cardioprotective agents against I/R injury [93]. However, clinical trials of these agents against I/R injury have not yet been conducted. Future demonstration

of the cardioprotective effect of pharmacological IPC in patients should be explored, and this could have an enormous impact on development of drugs for clinical application of pharmacological preconditioning.

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