Protein S-nitrosylation: A role of nitric oxide signaling in cardiac ischemic preconditioning

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Abstract: Nitric oxide (NO) has been shown as an important signaling messenger involved in cardioprotection of ischemic preconditioning (IPC). To date, most studies suggest that NO might provide its protective effects by regulating the mitochondrial ATP-sensitive potassium (K_{ATP}) channel via the classic NO/cGMP-dependent pathway. However, there is emerging data suggesting that NO might also elicit its physiological role through protein S-nitrosylation. Protein S-nitrosylation, the covalent attachment of an NO moiety to sulfhydryl group(s) of cysteine residue(s) of proteins, is a reversible post-translational protein modification involved in redox-based cellular signaling. IPC has been found to increase S-nitrosothiol content and result in increased S-nitrosylation of proteins, which not only induces the structural and functional changes of modified proteins, but also prevents the target cysteine residue(s) from the further oxidative modification. In addition, S-nitrosothiols could elicit pharmacological preconditioning effect and protect against myocardial ischemia-reperfusion injury. Thus, protein S-nitrosylation is emerging as an important contributor to cardioprotection in IPC, providing protection from cellular oxidative and nitrosative stress.

Key words: S-nitrosylation; ischemic preconditioning; nitric oxide; cardioprotection

1 Introduction

Ischemic preconditioning (IPC) is a cellular adaptive phenomenon whereby brief episodes of ischemia render the heart more resistant to further prolonged ischemic injury[1]. The benefit of IPC composes two windows: acute and delayed IPC, the former occurring within several hours after reperfusion, which is relying on post-translational protein modification. However, the delayed IPC is elicited until at least 12 h after reperfusion and could be sustained...
for several days, which is dependent on gene transcription and new protein synthesis\cite{23}. IPC has been shown to activate a number of signaling pathways, such as G protein-coupled receptors and kinase cascades\cite{2,4}, reactive oxygen species (ROS)\cite{16,17}, and nitric oxide (NO)\cite{7,8}.

NO plays an important role in the regulation of cardiac function\cite{4,12}. In addition to activating the cyclic guanosine monophosphate (cGMP)-dependent signaling pathways, NO transduces the cGMP-independent signaling by redox reversible modification on cysteine residues(s), i.e., via S-nitrosylation, the covalent attachment of an NO moiety to protein sulfhydryl group(s) and resulting in the formation of S-nitrosothiol (SNO). In the past decade, protein S-nitrosylation has emerged as a very important post-translational protein modification based on redox mechanism\cite{13,14}. Recent studies suggest that S-nitrosylation not only directly leads to the structural and functional changes of modified proteins, but also provides protection to cells by S-nitrosylation of some critical protein thiols and prevents them from further oxidative modification by ROS\cite{11,15}.

Most studies suggest that the increase in NO formation in IPC hearts is cardioprotective because the infusion of hearts with a NO synthase (NOS) inhibitor before IPC abolishes or attenuates the cardioprotection\cite{16-18}. Although NO has been found to provide its protective effects by regulating mitochondrial ATP-sensitive potassium (KATP) channel via the classic NO/cGMP-dependent pathway\cite{19,20}, there is emerging evidence that NO might also elicit its cardioprotective role through protein S-nitrosylation in IPC hearts\cite{21-23}. In this review, we will elaborate on the current understanding of how protein S-nitrosylation, this NO-related redox signaling, would mediate the cardioprotection in acute IPC.

2 Protein S-nitrosylation and SNO detection in IPC heart

S-nitrosylation possesses high spatial and temporal specificity on targeted cysteine residues\cite{13,24}. In most cases, the specificity of S-nitrosylation is governed by the juxtaposed consensus acid-base motifs controlling targeted thiol pKa and nucleophilicity\cite{13}. Since the hydrophobic environment facilitates the reaction for S-nitrosylation, the relative hydrophobicity of the region surrounding the target thiol may provide a “hydrophobic motif” for protein S-nitrosylation\cite{13}.

In addition, the protein S-nitrosylation also depends on the colocalization of NO sources such as NOS with the targeted proteins and other nitrosylating equivalents\cite{13,24-26}. Once formed, the SNO moiety of an S-nitrosylated protein can be transferred to other thiols through a process termed transnitrosylation. Reducing glutathione (GSH) is the principal low molecular weight thiol that can be transnitrosylated, leading to the denitrosylation of those formed SNO proteins. IPC has been reported to increase the formation of NO\cite{18,27}, which provides the source of NO moiety for S-nitrosylation. The low oxygen condition during IPC might also facilitate the reaction of S-nitrosylation\cite{29}. On the other hand, IPC could change the redox equilibrium of subcellular organelle\cite{5,6} and ROS formation, leading to a decreased intracellular GSH pool, which might stabilize the SNO formation by attenuating GSH-mediated trans-/de-nitrosylating equivalents. Thus, IPC provides an environment that favors S-nitrosylation, including NO and SNO formation, ion content, pH, and redox equilibrium.

Compared to other post-translational protein modification, the liability of the SNO bond makes S-nitrosylated proteins cumbersome to measure and identify accurately\cite{29,30}. Using NO-based (following breakdown of SNO by mercury) chemical reaction such as 2,3-diaminonaphthylene fluorescence assay, Ghelardoni et al. have found that SNO production was increased to ~20 pmol/mg protein in rat heart membranes after exposure to NO donors, although SNO content in perfusion control could not be detected because of the sensitivity limit in their study\cite{31}. Using the same method with careful preparation of samples to prevent SNO decomposition, such as protecting from light and including EDTA and neocuproine (copper chelator), we detected about ~3-4 pmol SNO/mg protein in membrane fractions isolated from perfused mouse hearts\cite{32}, while SNO content was increased nearly three folds in IPC hearts\cite{33}. By using the photolytic/ozone chemiluminescence technique, Burwell et al. found that IPC significantly increased SNO content in mitochondrial proteins isolated from IPC rat hearts, ~15 pmol SNO/mg protein in mitochondrial proteins isolated from IPC hearts versus less than 1 pmol SNO/mg protein in perfusion control\cite{32}. Recently, the biotin switch method developed by Jaffrey and Snyder\cite{34}, has become a widespread technique in combination with proteomic approaches\cite{35}. By using these new techniques, we have identified several candidate S-nitrosylated proteins in IPC hearts, involving the regulation of intracellular Ca\textsuperscript{2+} handling, myocardial energetics, and contractile ultrastructure\cite{33}.

3 S-nitrosylated proteins and their cardioprotective relevance to IPC

Accumulating evidence suggests that protein S-nitrosylation
could mediate important redox signaling in the cardiovascular system, and products of S-nitrosylation, SNO, might play key roles in human health and disease\cite{11,36,37}.

3.1 Intracellular Ca\(^{2+}\) handling

Although NO-mediated regulation is dependent at least in part on cGMP and the subsequent modification of the phosphorylation state of channels, NO could also participate in the regulation of intracellular Ca\(^{2+}\) handling through cGMP-independent redox mechanisms\cite{10,38}. All of the major cardiac Ca\(^{2+}\) handling proteins, such as the L-type Ca\(^{2+}\) channel, the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA2a) and Ca\(^{2+}\) release channel/ryanodine receptor (RyR2), possess multiple free cysteine residues and are subjected to redox regulation\cite{39,40}.

Recent studies suggest that NO regulates cardiac Ca\(^{2+}\) signaling by spatial confinement of NOS isoforms to subcellular organelles where those Ca\(^{2+}\) handling proteins are localized\cite{9,41-45}. The Ca\(^{2+}\)-dependent activation of constitutive isoforms of NOS and colocalization with those redox-sensitive Ca\(^{2+}\) handling proteins provide a spatialized physiological environment for protein S-nitrosylation. Although it has been shown that exogenous SNO compounds could poly-S-nitrosylate RyR2 in isolated canine cardiac SR vesicles and activate the channels \textit{in vitro}\cite{46}, we never could detect the occurrence of S-nitrosylation of RyR2 in IPC mouse hearts by the biotin switch method. Instead, we have found that the L-type Ca\(^{2+}\) channel \(\alpha 1\) subunit is one of the predominant S-nitrosylated proteins in cardiomyocytes. S-nitrosylation of the L-type Ca\(^{2+}\) channel inhibits the Ca\(^{2+}\) current in cardiomyocytes under adrenergic stimulation and such a “\(\beta\)-blocker” effect would lead to an attenuation of Ca\(^{2+}\) overload in ischemic-reperfused hypercontractile hearts\cite{32}. In a more recent study, we have found that IPC increases S-nitrosylation of the L-type Ca\(^{2+}\) channel\cite{31}. Since the L-type Ca\(^{2+}\) channel is a well-known therapeutic target for cardiovascular disease\cite{47}, our finding of S-nitrosylation and inhibition of this channel might bring insight and invention for new drug development.

NO has been reported to modulate the activity of SERCA2a\cite{48}, but the molecular mechanism is not clear. Our recent study has shown that both IPC and pharmacological preconditioning with S-nitrosoglutathione (GSNO) result in S-nitrosylation of SERCA2a and we further show that GSNO treatment increases SERCA2a activity\cite{33}. An increase in SERCA2a activity during ischemia and early reperfusion would provide improved Ca\(^{2+}\) uptake into the SR, which would reduce diastolic Ca\(^{2+}\) levels, making SERCA2a a plausible target for cardioprotection\cite{49}. Indeed the SR has been suggested to be a primary target of reperfusion protection\cite{50}. Furthermore, recent studies also suggest that protein modification mediated by NO carriers could result from S-nitrosylation or from further other secondary oxidative modifications such as S-glutathiolation\cite{51,52}. An allosteric role of NO and superoxide anion in regulating SERCA2a has been recently elucidated, in which SERCA2a is activated by reversible S-glutathiolation at Cys674 via the formation of peroxynitrite in the presence of GSH\cite{51,52}.

Ca\(^{2+}\) overload and oxidative stress are two major factors that might lead to ischemia-reperfusion (I/R) injury\cite{53}. Moreover, oxidative stress could further impair intracellular Ca\(^{2+}\) regulation, and the dysregulation of intracellular Ca\(^{2+}\) homeostasis is thought to be one of the most important reasons inducing many acute and chronic cardiovascular diseases. Our findings of the overall effects of S-nitrosylation of the L-type Ca\(^{2+}\) channel (inhibition and decreased Ca\(^{2+}\) entry) and SERCA2a (activation and increased SR Ca\(^{2+}\) uptake) in IPC will lead to the attenuation of the cytosolic free Ca\(^{2+}\) and reduce Ca\(^{2+}\) overload after I/R, which could be cardioprotective. Indeed, IPC has been shown able to reduce cytosolic Ca\(^{2+}\) levels during ischemia\cite{54}.

3.2 Myocardial energetics

The heart is capable of altering its metabolic rate during exercise or ischemia. It has been reported that ~50% of the ATP generated during ischemia by glycolysis is consumed by reverse mode of the mitochondrial F1-ATPase\cite{55}. Therefore, inhibition of the mitochondrial F1-ATPase during ischemia would conserve ATP. Indeed, inhibition of the mitochondrial F1-ATPase was an early hypothesis to explain the reduced rate of decline in ATP observed during ischemia in IPC hearts\cite{56}. Furthermore, the ATP consumed by the reverse mode of the mitochondrial F1-ATPase is used to maintain the mitochondrial \(\Delta \psi \) and transport Ca\(^{2+}\) into the mitochondria. Thus, inhibition of the mitochondrial F1-ATPase could be beneficial by conserving cytosolic ATP and by reducing Ca\(^{2+}\) uptake into the mitochondria. Of particular interest we have recently observed that pharmacological preconditioning with GSNO results in increased S-nitrosylation of the mitochondrial F1-ATPase \(\alpha 1\) subunit, and GSNO dose-dependently inhibits the mitochondrial F1-ATPase in freshly isolated submitochondrial particles\cite{31}.

It has been reported that IPC prevents the loss of activity of \(\alpha\)-ketoglutarate dehydrogenase (\(\alpha\)-KGDH) induced by I/R\cite{37}. Interestingly we have found that IPC results in an increased S-nitrosylation of \(\alpha\)-KGDH. We further find...
that GSNO treatment increases the enzymatic activity of α-KGDH\[^{33}\]. Therefore, it is tempting to speculate that the S-nitrosylation of α-KGDH could not only increase its activity, but also protect it from oxidative damage during I/R. The original paper describing IPC\[^{1}\] reported that IPC reduced the accumulation of lactate during sustained ischemia. IPC has also been reported to reduce ischemic acidosis\[^{54,58}\]. Weiss et al. have demonstrated that IPC results in the attenuation of glycogenolysis\[^{58}\]. It is therefore of interest that we have recently found that IPC increases S-nitrosylation of glycogen phosphorylase, the enzyme responsible for catabolism of glycogen\[^{33}\].

NO has been found to inhibit mitochondrial ATP generation via inhibition of the mitochondrial respiratory chain, mainly at complex I and IV, leading to a switch from apoptosis to necrosis\[^{59}\]. A recent study using isolated rat heart mitochondria has shown that the 75 kDa subunit of complex I is S-nitrosylated by exogenously added GSNO and results in a significant inhibition of the complex\[^{22}\]. Another study using endothelial cells has reported that mitochondrial complex IV/cytochrome c oxidase could also be persistently inhibited by S-nitrosylation at two active cysteine (Cys196 and Cys200) residues\[^{60}\].

There are other metabolic enzymes found to be subjected to NO-mediated S-nitrosylation modification, including aconitase\[^{63}\], glyceraldehyde-3-phosphate dehydrogenase\[^{61}\], mitochondrial aldehyde dehydrogenase\[^{62}\], and creatine kinase\[^{63}\]. Whether IPC could induce S-nitrosylation of these proteins and the function of modification is worth further characterization.

### 3.3 Myocardial remodeling

Myocardial infarction induces contractile dysfunction and remodeling that can lead to heart failure. NO has been proposed as one of the major players of this pathophysiologic process\[^{64-66}\]. On a cellular level, cardiac myofilament dysregulation, myocyte apoptosis, fibroblast proliferation and changes in the extracellular matrix are some of the principal alterations underlying the remodeling process.

In IPC hearts, we have found that cardiac myosin heavy chain, myosin light chain 1, and myomesin undergo increased S-nitrosylation\[^{33}\]. The myosin-actin crossbridge kinetics is an important determinant for cardiac function. And myomesin, an ultrastructural protein localized in the M line of the sarcomere, has been suggested to function as an integral structural linker of the thick filaments into the sarcomere\[^{67}\]. So far, the most important mechanism of myofilament regulation is through altered post-translational modifications, in particular, phosphorylation. Our study suggests that other modifications, including oxidation and S-nitrosylation, may also play a role\[^{33,68}\]. However, the physiological relevance of S-nitrosylation of these myofilament proteins to IPC is still not clear and needs further investigation.

Apoptosis contributes to ventricular remodeling after myocardial infarction. NO/S-nitrosylation has been implicated in both apoptotic and necrotic cell death\[^{69,70}\]. S-nitrosylation of the redox-sensitive thiol in the catalytic site of caspase plays an essential role in the apoptotic signal cascade by inhibiting apoptotic cell death\[^{70,71}\]. A recent study using neonatal rat cardiomyocytes has demonstrated that the inhibition of apoptosis by S-nitrosylation of caspase-3 plays an important role in cardiomyocyte apoptosis\[^{72}\]. It has been suggested that the prevention of caspases activation by IPC may be important steps in protecting the heart against I/R injury in vivo\[^{73}\]. Thus, it will be interesting to study whether IPC results in S-nitrosylation of caspase, thereby protecting cardiomyocyte apoptosis and myocardial infarction.

Cardiac fibroblasts appear to be important in producing and maintaining the extracellular matrix of the heart. Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme in prostaglandin synthesis, which is induced in animal models of ischemic injury and involved in inflammatory cell infiltration and fibroblast proliferation in the heart\[^{74,75}\]. It has been demonstrated that IPC upregulates the expression and activity of COX-2 in the heart, which mediates the protective effects of the delayed IPC against both myocardial stunning and myocardial infarction\[^{75}\]. Recently, Atar et al. have found in rat heart that atorvastatin-induced cardioprotection is mediated by increasing inducible NOS, which activates COX-2 in the heart by S-nitrosylation\[^{23}\]. Tissue transglutaminase exhibits a Ca\(^{2+}\)-dependent transglutaminase activity that cross-links proteins involved in wound healing, tissue remodeling, and extracellular matrix stabilization. Poly-S-nitrosylation of tissue transglutaminase results in the inhibition of transglutaminase activity and platelet aggregation\[^{76}\].

### 3.4 Other candidate signaling

Thioredoxin is a class of small redox-regulating protein that appears to play a crucial cardioprotective role in I/R\[^{77}\]. Tao et al. have found that exogenously applied human thioredoxin reduces I/R injury in mouse hearts and this cardioprotective effect could be further enhanced by S-nitrosylated thioredoxin\[^{38}\]. It has been shown that shear stress increases the S-nitrosylation and the reductase activity of thioredoxin\[^{79}\]. In addition, S-nitrosylation of
thioredoxin also contributes to its anti-apoptotic function, possibly by transnitrosylation of proteins such as caspases, thereby inhibiting their activity\[80\]. On the other hand, it has been found that $S$-nitrosylation of thioredoxin at active-site Cys32/Cys35 leads to the dissociation and activation of apoptosis signal-regulating kinase 1, suggesting that $S$-nitrosylation of thioredoxin may also play a role in pro-apoptotic signaling under certain oxidative stresses\[81,82\].

Endothelial NADPH oxidase is a major source of superoxide in blood vessels and has been implicated in the oxidative stress accompanying cardiovascular disease. NADPH oxidase is an enzyme composed of up to 5 subunits. Among them, the organizer subunit p47phox has been recently found to be $S$-nitrosylated by NO, leading to sustained suppression of the activity of NADPH oxidase and superoxide production\[83\]. Although ROS produced under oxidative stress is detrimental to postischemic hearts, it could conversely be cardioprotective in IPC\[5,6\]. For example, superoxide anion produced during IPC could activate survival kinase cascade phosphoinositide 3-kinase (PI3K)/Akt\[4,84,85\]. A recent study has shown that the activation of PI3K/Akt by superoxide is dependent on $S$-nitrosylation of PTEN (phosphatase and tensin homologue deleted on chromosome 10)\[86,87\]. By dephosphorylating the 3-position of the inositol ring of phosphoinositides such as PtdIns(3,4,5)P(3), PTEN’s lipid phosphatase activity is an important counter-acting mechanism in the PI3K/Akt signaling. Moreover, GSNO has been found to activate Akt in vascular endothelial cells via $S$-nitrosylation of PTEN, which stabilizes hypoxia-inducible factor-1 (HIF-1) in normoxic cells\[87\].

HIF-1 is a transcription regulator that responds to oxygen. HIF-1 is a heterodimer composed of subunits HIF-1$\beta$ and

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HIF-1α. Under normoxic condition, HIF-1 activity is usually suppressed due to the rapid degradation of HIF-1α. A recent study has shown that normoxic HIF-1 activity can be upregulated through NO-mediated S-nitrosylation and stabilization of HIF-1α [89]. Another transcription factor, nuclear factor κB (NF-κB), playing a pivotal role in inflammation and cell survival, could be inhibited by NO via S-nitrosylation [90]. In addition, Reynaert et al. have shown that NO represses inhibitory NF-κB kinase by S-nitrosylation, providing a mechanism for S-nitrosylation to be involved in the upstream regulation of NF-κB-mediated inflammatory responses [90].

NO regulates cardiovascular physiology in part by controlling protein trafficking. It has been previously shown by our group that G protein-coupled receptor internalization signaling is required for cardioprotection in IPC [91]. These findings raised an interesting question: could G protein-coupled receptor internalization be regulated by S-nitrosylation? Recent studies suggest that NOS generates NO locally and regulates compartmentalized S-nitrosylation and protein trafficking in the cardiovascular system [26,36]. Some important regulating proteins for protein trafficking, such as glypican-1 [92], Ras [93], N-ethylmaleimide-sensitive factor [94], protein-disulphide isomerase [95], dynamin [96,97] and G protein-coupled receptor kinase 2 [37], have been identified to be S-nitrosylated and S-nitrosylation of these proteins play important role in protein trafficking.

4 Summary

In summary, IPC results in increased S-nitrosylation of a number of proteins, involving multiple cellular signaling transduction including intracellular Ca²⁺ handling, myocar-dial energetics, myocardial I/R remodeling, antioxidant defense, transcriptional regulation and protein trafficking (Table 1). The feasibility of pharmacological preconditioning with SNO has provided an intriguing therapeutic strategy for protecting against myocardial I/R injury [23,98]. Taken together, protein S-nitrosylation is emerging as an important contributor to cardioprotection in IPC.

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