

Concepts of hypoxic NO signaling in remote ischemic preconditioning

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Author contributions: Totzeck M, Hendgen-Cotta U and Rassaf T conceived, drafted and approved the manuscript.

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors contributed their efforts in this manuscript.

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Received: May 29, 2015
Peer-review started: June 1, 2015
First decision: August 4, 2015
Revised: August 17, 2015
Accepted: September 7, 2015
Article in press: September 8, 2015
Published online: October 26, 2015

Abstract

Acute coronary syndromes remain a leading single cause of death worldwide. Therapeutic strategies to treat cardiomyocyte threatening ischemia/reperfusion injury are urgently needed. Remote ischemic preconditioning

(rIPC) applied by brief ischemic episodes to heart-distant organs has been tested in several clinical studies, and the major body of evidence points to beneficial effects of rIPC for patients. The underlying signaling, however, remains incompletely understood. This relates particularly to the mechanism by which the protective signal is transferred from the remote site to the target organ. Many pathways have been forwarded but none can explain the protective effects completely. In light of recent experimental studies, we here outline the current knowledge relating to the generation of the protective signal in the remote organ, the signal transfer to the target organ and the transduction of the transferred signal into cardioprotection. The majority of studies favors a humoral factor that activates cardiomyocyte downstream signaling - receptor-dependent and independently. Cellular targets include deleterious calcium (Ca^{2+}) signaling, reactive oxygen species, mitochondrial function and structure, and cellular apoptosis and necrosis. Following an outline of the existing evidence, we will furthermore characterize the existing knowledge and discuss future perspectives with particular emphasis on the interaction between the recently discovered hypoxic nitrite-nitric oxide signaling in rIPC. This refers to the protective role of nitrite, which can be activated endogenously using rIPC and which then contributes to cardioprotection by rIPC.

Key words: Remote ischemic preconditioning; Ischemia/reperfusion injury; Nitrite; S-nitrosation; Mitochondria

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Core tip: Therapeutic strategies to treat ischemia/reperfusion injury are urgently needed. Remote ischemic preconditioning (rIPC) appears to exert beneficial effects for patients. The underlying signaling remains incompletely understood. Following an outline of the existing evidence, we will characterize the existing knowledge and discuss future perspectives with particular emphasis on the interaction between the recently discovered hypoxic nitrite-

nitric oxide signaling in rIPC.

Totzeck M, Hendgen-Cotta U, Rassaf T. Concepts of hypoxic NO signaling in remote ischemic preconditioning. *World J Cardiol* 2015; 7(10): 645-651 Available from: URL: <http://www.wjgnet.com/1949-8462/full/v7/i10/645.htm> DOI: <http://dx.doi.org/10.4330/wjc.v7.i10.645>

INTRODUCTION

Current guidelines of both European and United States cardiac societies emphasize one primary goal for patients with acute myocardial infarctions - the timely and successful reperfusion^[1]. This concept among many additional pharmaceutical treatments has significantly reduced mortality and morbidity in patients suffering from acute coronary syndromes. Experimental evidence, however, has largely implicated that rapid restoration of coronary perfusion, in turn, may paradoxically harm cardiomyocytes^[2,3]. This phenomenon has been named ischemia/reperfusion (I/R) injury. The underlying signaling is complex and yet incompletely understood. Current concepts for the modulation of myocardial I/R injury have particularly emphasized three major contributors to the final I/R injury: reactive oxygen species (ROS), Ca²⁺, and mitochondria. Particularly the latter are now not only regarded as targets during I/R but also as mediators of cellular injury and death. For a detailed description of the signaling in myocardial I/R injury the reader is kindly referred to the recently published excellent review sources^[2-7]. Experimental and initial clinical studies implicate that all three signaling components of I/R injury may serve as potential targets in attempt to reduce I/R injury. Although early reperfusion therapy has led to a marked decrease in mortality following acute myocardial infarction, application of such cardioprotective strategy might further reduce the burden of cardiovascular disease.

Ischemic conditioning regimens are among the most effective cardioprotective approaches. These techniques are capable of reducing the final infarct size by up to approximately 30%-60%^[8-10]. Brief episodes of sublethal I/R applied to the index organ [ischemic preconditioning (IPC)] - or a remote organ (rIPC) - before, during or after a main ischemic event initiate a powerful cellular signaling cascade rendering the cardiomyocyte capable of protecting itself. The majority of relevant clinical phase II trials have produced favorable results for ischemic conditioning techniques. However, a definitive implementation into the clinical routine is still warranted. The underlying signaling in the scope of rIPC remains incompletely understood. Here we first outline the events leading to myocardial I/R injury, followed by an introduction to ischemic conditioning. Finally, we will put a major focus on the recently discovered nitrite-nitrogen oxides (NO)

signaling, which targets mitochondria during I/R and protects the heart from lethal injury.

MYOCARDIAL I/R INJURY

Reperfusion therapy either by invasive coronary intervention, bypass surgery or pharmacological lysis causes a rapid increase in cellular oxygen levels^[2]. One key pathological consequence of this is an insufficient mitochondrial respiration in consequence of an incomplete electron transport over mitochondrial membranes. This can cause an excess formation of ROS^[11]. While ROS at physiological concentrations contribute to the general cellular homeostasis, higher levels may initiate a deleterious signaling. This contributes to an increase in cell death by necrosis or apoptosis^[12].

The reperfusion phase is not only characterized by increased ROS levels, but also by a reduction in the bioavailability of NO. NO is a gaseous signaling molecule that regulates a wide variety of cardiomyocyte functions including scavenging of radicals, cardiac immune response, improvement of blood flow and left ventricular function^[13-17]. By consequence, these functions are impaired during reperfusion. In addition, interactions between the ROS system and NO signaling may further contribute to the final myocardial I/R injury. This relates to the increase formation of peroxynitrite (an oxidant) in the reperfusion period^[18], which in turn may contribute to nitrosative stress-related cell injury to membranes^[19,20]. The exact role of peroxynitrite and whether peroxynitrite signaling can be effectively modulated in patients remains to be determined.

The second major contributor to cardiomyocyte dysfunction in I/R is a deteriorated Ca²⁺ signaling^[21-24]. Elevated intracellular Ca²⁺ levels can not only trigger arrhythmias, and cardiomyocyte hypercontracture^[25], they may also activate Ca²⁺-dependent signaling pathways. This pertains especially to Ca²⁺-dependent proteases named calpains, which then cleave cellular elements involved, *e.g.*, in apoptosis, mitochondrial respiration or mitochondrial turnover^[26-28]. Hypercontracture and necrosis are not limited to one cell but can also be distributed from cell to cell either by direct contracture or by cell-to-cell progression through intercellular gap junctions^[29]. Off note, high calcium levels may also directly target and disrupt cell membranes^[20]. While both ROS and intracellular Ca²⁺ elevation mark the initial events of myocardial I/R injury, the central target of reperfusion-associated impaired signaling is the mitochondrion.

MITOCHONDRIA IN I/R INJURY

Mitochondrial injury in I/R comprises at least three relevant entities: (1) Destruction of mitochondrial membrane integrity by permeabilization; (2) deficits in mitochondrial structural dynamism, the recently termed mitochondrial fusion and fission and, finally; and (3) the deterioration of mitochondrial respiration^[30].

The exact events leading to mitochondrion-driven cell death by mechanisms of necrosis, apoptosis, or autophagy are incompletely understood. Dysregulated Ca^{2+} signals and ROS concentrations, as found in early reperfusion, provide excellent circumstances to deteriorate mitochondrial integrity^[31].

Apoptosis causes cell shrinkage, cellular fragmentation, and finally phagocytosis. The general characteristics of necrosis include cellular swelling and rupture, and a marked depletion of energy resources. Newer studies argue that apoptosis as well as necrosis are regulated by a complex signaling machinery with overlapping processes^[32]. The critical step for apoptosis is the permeabilization of the mitochondrial outer membrane pore (MOMP). This occurs by activation of pro-apoptotic BH3 proteins, *e.g.*, Bax^[31]. Subsequently, a release of apoptogenic factors is initiated, *e.g.*, cytochrome c or apoptosis-inducing factor (AIF)^[33,34]. By contrast, the key characteristic of necrotic cell death is the permeabilization of the inner mitochondrial membrane causing the formation of a yet incompletely identified mitochondrial permeability transition pore (mPTP).

Mitochondrial morphology and structural dynamism is regulated by fission and fusion^[35]. Interestingly, Bax - previously introduced as major contributor to cell death - is also involved in mitochondrial fusion and fission. As a consequence, Bax supplementation deteriorates mitochondrial structural dynamism in conjunction with a much-increased I/R injury. Taken together both regulation of mitochondria-driven cell death as well as mitochondrial structure is complex but many overlapping yet incompletely defined processes exist that contribute to cardiomyocyte damage in I/R injury.

In case the initial phase of reperfusion is survived the preservation and restoration of mitochondrial function becomes a major goal. Mitochondria comprise one third of the cell mass of cardiomyocytes. Recent evidence suggests that at least three subpopulations exist: Subsarcolemmal (SSM), interfibrillary (IFM) and perinuclear (PNM) mitochondria. Their relative contribution to the function of cardiac cells remains to be completely elucidated^[30,32]. Mitochondria generate a vast amount of adenosine triphosphate per day^[36] and the electron transfer through the complexes of the mitochondrial respiratory chain is under control of a delicate regulatory machinery. I/R may cause a major functional disturbance with a subsequent incomplete respiration and a burst in the generation of ROS^[37]. Many cardioprotective strategies have attempted to reduce these excessive ROS levels. Newer approaches, *e.g.*, rIPC, have focused on regulatory posttranslational modifications of respiratory chain elements and particularly complex I, which we will outline in the following.

THE CONCEPT OF RIPC

rIPC is among the most effective techniques in rendering the myocardium capable of protecting itself against I/R injury^[38]. rIPC-associated protection is

initiated *via* short non-deleterious phases of I/R prior to an index ischemia. The maneuver is applied to an organ or tissue at distance to the one undergoing the main ischemic event^[38,39]. Clinical evidence from recent phase II trials favors a potential translation into clinical routine^[40-42]. The underlying rIPC-related signal mechanism remains under intense debate. Generally, the signal transduction machinery initiated by the rIPC stimulus involves a trigger, the transfer of the trigger to the target organ and a distinct cardiomyocyte signaling leaving the cardiomyocyte protected from I/R^[13]. As triggering pathways both humoral/blood borne factors^[43-45] and neuronal transmission^[46] have been proposed. However, the cardioprotection can be transferred when transfusing blood from rIPC animals to unconditioned littermates^[43,45,47]. This argues in favor of important contribution of a humoral factor. This does not preclude a role for the nervous system to be involved in the modulation of the rIPC response^[48]. However, an intact nervous system is particularly important for the remote and not the target organ^[48]. Off note, the signaling is at least to certain degrees species-specific with some of the pathways that are active in murine studies being irrelevant in large animal models and humans^[48,49].

TRANSFER OF PROTECTION *VIA* HUMORAL FACTORS

It is now generally accepted that rIPC initiates a complex interplay between several mediators not only at the remote site, but also in the circulation as well as in the target cell. Over the past two decades many components have been forwarded, and it is presently unlikely that one single mediator is responsible for the complete protection associated with rIPC. Among the factors which have been evaluated in experimental studies are adenosine, bradykinin, opioids, interleukins, stromal cell-derived factor, hypoxia-inducible factor 1 α and members of the so-called RISK pathway. These studies have in part revealed conflicting results especially when comparing experimental results with those from trials in patients^[6].

We and other groups have recently investigated a potential involvement of hypoxic NO signaling in the course of rIPC. Using a mouse model of warm liver I/R, it was demonstrated that ablation of endothelial NO synthase (eNOS) abrogates the protective effects seen with rIPC on microscopic liver damage^[50]. eNOS generates NO which can then modulate cardiovascular functions either at the place of synthesis or at a distance when transported as nitrite or nitroso species (nitrosated proteins)^[51]. Changes in shear stress, *e.g.*, due to an increase in blood flow as seen in hyperemia after short phases of ischemia, are the strongest physiological stimulus of eNOS activity, which is mirrored in higher circulating NO metabolites^[13]. This led to the hypothesis that nitrite-NO signaling is involved in the pathways

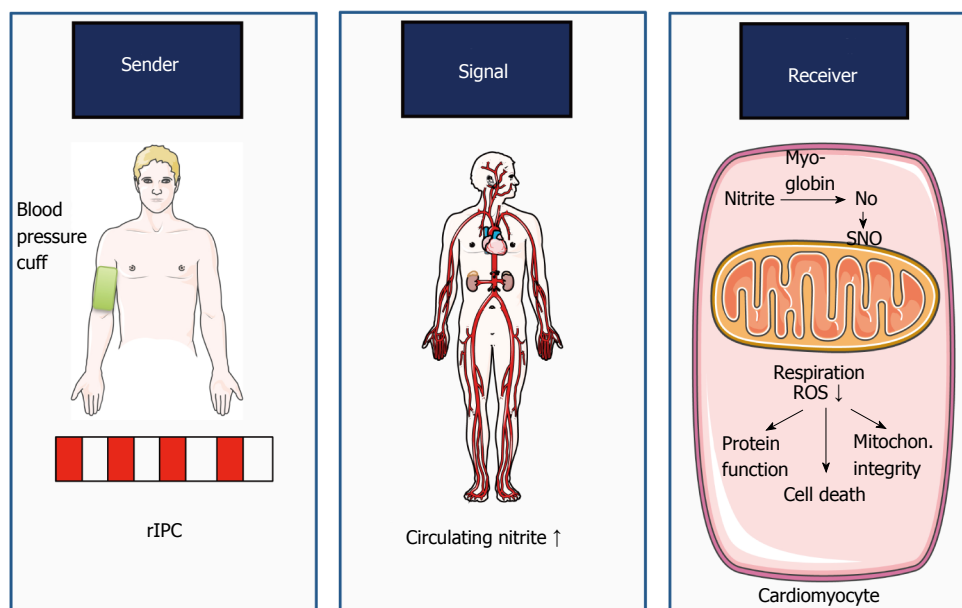


Figure 1 Nitrite/nitrogen oxides related signaling in remote ischemic preconditioning - proposed mechanism. The nitrite/NO signaling involves three components. The sender of the signal is shown in the left panel. In this case the upper extremity receiving rIPC via repetitive in-/deflations of a blood pressure cuff. During this maneuver, the classical L-arginine-NOS-nitrogen oxides (NO) pathway is activated. This includes the activation of eNOS, which generates NO from L-arginine while using several cofactors. Off note, the eNOS system is under tight control of several systems, e.g., the calcium-calmodulin pathway. The middle panel represents the signal transmitted from the sender (remote organ) to the receiver (cardiomyocytes), as nitrite is transmitted from the site of generation via the circulation to the heart. In the receiving target organ, this signal is translated into cardioprotection via myoglobin-dependent nitrite reduction and S-nitrosation of mitochondrial complex I with a protective regulation of mitochondrial functions and reduced reactive oxygen species (ROS). The generation of NO in the last panel activates the alternative pathway for NO generation, which reduces nitrite to NO via hemoglobins, e.g., myoglobin in the heart or hemoglobin in the circulation. The reduction of ROS in turn may help reduce cellular stress as indicated. This relates to an improvement of protein function, cell death and mitochondrial integrity^[8]. rIPC: Remote ischemic preconditioning; NOS: NO synthase.

leading to cardioprotection from the rIPC stimulus.

THE ROLE OF NITRITE/NO SIGNALING IN REMOTE ISCHEMIC PRECONDITIONING

NO is a signaling molecule with a wide variety of functions in the cardiovascular system. Generally, NO protects the heart from I/R injury except for excessive levels during inflammation. However, in the course of I/R the canonical NO generation pathway via the classical L-arginine-NOS-NO signaling pathway becomes impaired due to a lack of molecular oxygen^[52]. Nitrite, formerly regarded a mere NO oxidation product, may serve as an alternative source under these conditions^[14,53]. The majority of the bodily nitrite provision derives from the oxidation of NO, which is enzymatically formed by one of at least three NO synthase (NOS) isoforms^[54]. The remainder of nitrite in the circulation and tissues has been related to nutritional sources^[55]. The half-life of nitrite is quite long in contrast to NO - approximately 35-60 min depending on the models and compartments used to evaluate nitrite distribution^[56]. We have previously shown that the endogenous levels of nitrite can be modified by intravenous infusion of nitrite and certain diets^[14,57,58]. An adequate nitrite production along with sufficient endogenous levels has implications for numerous cardiovascular functions. We have shown that trained athletes with higher nitrite levels performed

superior in exercise tests as compared to those with lower concentrations^[59].

In hypoxia or ischemia, nitrite may be reduced to bioactive NO, thus providing an alternative pathway to the enzymatic formation of NO, which is inactive under these circumstances. Nitrite activation may occur through reaction with hemoglobins such as myoglobin^[60]. With decreasing oxygen gradients, cardiac myoglobin changes its function from an oxygen storage and NO scavenger to an NO producer by reducing nitrite to bioactive NO^[13,15,37,60,61]. This can significantly reduce myocardial I/R injury^[37].

The downstream mechanism following a formation of NO from nitrite largely involves mitochondria. In an experimental approach using exogenous nitrite during myocardial I/R injury we identified an S-nitrosation modification of mitochondrial complex I. This, in turn, regulates myocardial energetics via the modulation of mitochondrial respiration and formation of mitochondria derived ROS. S-nitrosation of complex I is furthermore associated with an adaption of myocardial functions to a reduced O₂ supply. This mechanism has been recognized as hibernation. Finally, nitrite reduction to NO and the downstream signaling cascade contributes to a decrease in myocardial necrosis and apoptosis^[16,37,60,62].

In our recent study using rIPC as protective regimen we revealed that this maneuver also involves a protective nitrite/NO signaling with similarities to the

one described above for exogenous nitrite^[13]. Figure 1 summarizes the general concept. This involves a sender, the transfer of the signal to the target organ, and a receiver - the cardiomyocyte. We propose the sender to be the endothelium of the extremity treated with rIPC. Repetitive phases of in- and deflations of the blood-pressure cuff cause high blood flow and a subsequently increased shear stress. This results in shear-stress dependent eNOS activation and NO formation, which converts to nitrite - the stable oxidation product.

This circulating nitrite is transmitted as signal of cardioprotection to the heart. The stability of nitrite and its half time argues in favor of nitrite^[56]. In transfer experiments, the effects were preserved when infusing conditioned human plasma to isolated mouse hearts. Nitrite scavenging in conditioned human plasma abolished the transmission of cardioprotection to the conditioning-naïve Langendorff hearts.

The receiver of protection is the cardiomyocyte. In the myocardium, cardiomyocyte myoglobin reduces nitrite to NO during I/R with subsequent S-nitrosation modifications of mitochondrial complex I finally leading to cardioprotection. Presently, it is not known whether other signaling molecules regulating for instance apoptosis, necrosis and mitochondrial structure, *e.g.*, the BH3 proteins, are also affected by S-nitrosation modifications by rIPC.

Our recent findings were further evidenced by studies by a group who demonstrated that ischemic conditioning applied locally leads to a wide variety of proteins being S-nitrosated^[63,64]. However, this appears to be a selective process in which SSM mitochondria are favored. This furthermore required connexin-43^[64] to be active. It is tempting to speculate that particularly the subgroup of SSM are largely responsible for cardioprotection during I/R injury. Taken together, current evidence from experimental studies currently implicates a substantial contribution of nitrite/NO to cardioprotection by ischemic conditioning. This involves a specific modulation of mitochondria a targeted S-nitrosation and a reduction in the formation of ROS as well a distinct signaling *via* connexins.

CONCLUSION

In summary, rIPC has been demonstrated to protect the myocardium from I/R injury. Several clinical trials have demonstrated beneficial results for patients. Arguably, the underlying signaling is delicate involving many signaling processes. This pertains to humoral factors, blood cells, neurohumoral mediators and presumably a complex interplay. The data from ours and several other groups implicate a role for hypoxic NO signaling in the course of rIPC^[13,50,64,65]. This requires particularly an S-nitrosation modification of mitochondrial elements. Naturally, many previously forwarded mechanisms will work along-side the proposed one based on NO^[6]. Further studies will be required to elucidate the complex interplay involved in signal generation, signal transfer

and final cardioprotection in the target organ. It will be of particular interest to evaluate the decisive signal transduction pathway in patients treated with rIPC. This is naturally a limitation of the current studies in the field of nitrite-NO signaling in rIPC^[13,50,64,66-68], which involve healthy mice without significant co-morbidities and relevant medications.

ACKNOWLEDGMENTS

The figure was produced using Servier Medical Art.

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