Circulating Nitrite Contributes to Cardioprotection by Remote Ischemic Preconditioning

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ABSTRACT

Rationale: Remote ischemic preconditioning (rIPC) with short episodes of ischemia and reperfusion (I/R) of an organ remote from the heart is a powerful approach to protect against myocardial I/R injury. The signal transduction pathways for the crosstalk between the remote site and the heart remain unclear in detail.

Objective: To elucidate the role of circulating nitrite in cardioprotection by rIPC.

Methods and Results: Mice were subjected to 4 cycles of no-flow ischemia with subsequent reactive hyperemia within the femoral region and underwent in vivo myocardial I/R (30 min/5 min or 24 h). The mouse experiments were conducted using genetic and pharmacological approaches. Shear stress dependent stimulation of endothelial nitric oxide synthase (eNOS) within the femoral artery during reactive hyperemia yielded substantial release of nitric oxide (NO’), subsequently oxidized to nitrite and transferred humorally to the myocardium. Within the heart, reduction of nitrite to NO’ by cardiac myoglobin (Mb) and subsequent S-nitrosation of mitochondrial membrane proteins reduced mitochondrial respiration, reactive oxygen species (ROS) formation and myocardial infarct size. Pharmacological and genetic inhibition of NO’/nitrite generation by eNOS at the remote site or nitrite bioactivation by Mb within the target organ abrogated the cardioprotection by rIPC. Transfer experiments of plasma from healthy volunteers subjected to rIPC of the arm identified plasma nitrite as a cardioprotective agent in isolated Langendorff mouse heart preparations exposed to I/R.

Conclusions: Circulating nitrite derived from shear stress dependent stimulation of eNOS at the remote site of rIPC contributes to cardioprotection during I/R.

Keywords:
Endothelial nitric oxide synthase, inorganic nitrite, myoglobin, remote ischemic preconditioning, myocardial infarction

Nonstandard Abbreviations and Acronyms:

AAR  area at risk
cGMP  cyclic guanosine monophosphate
eNOS  endothelial nitric oxide synthase
I/R  ischemia and reperfusion
LDPI  Laser Doppler perfusion imaging
Mb  myoglobin
NO’  nitric oxide
rIPC  remote ischemic preconditioning
ROS  reactive oxygen species
INTRODUCTION

Remote ischemic preconditioning (rIPC) has been demonstrated to reduce myocardial ischemia/reperfusion (I/R) injury by short episodes of I/R of an organ distant to the heart, e.g. the arm, prior to a sustained ischemic myocardial ischemia. Previous studies support a translation into clinical practice, and recent studies even demonstrated improved prognosis in patients with acute myocardial infarction or undergoing elective interventional or surgical coronary revascularization. Three components of rIPC can be distinguished: the signal generation, the transfer of the signal to the target organ, and its response to the transferred signal resulting in cardioprotection. The underlying mechanisms of rIPC have not been identified in detail so far. Both, humoral factors and neuronal transmission as well as their interaction have been hypothesized to forward the protective signal to the heart. Given that the cardioprotective effects can be simulated by transfusing blood from preconditioned donor animals to unconditioned recipients in both in vivo and ex vivo preparations, the contribution of a blood-borne signaling factor appears likely. We have previously observed in humans that a short episode of forearm I/R through blood pressure cuff in- and deflation is capable of modulating the composition of nitric oxide (NO•)/nitrite levels in the blood compartment. Nitrite is not only the oxidation product of NO•, but also a key reservoir for NO• in blood and cellular compartments and detectable at stable levels over time. The largest component of the bodily nitrite provision derives from endogenous NO• generation and a smaller part is related to nutritional sources, in particular dietary inorganic nitrate. The exact half-life of nitrite in plasma is not known, but calculated for humans to be approximately 35 min. We and others have recently demonstrated that exogenous supplementation with near physiological nitrite doses has implications for tissue protection under pathological conditions. This pertains to single nitrite injections, chronically administered nitrite, and remarkably also to nitrite administered before the onset of ischemia. Finally, we recently demonstrated that nitrite contributes at physiological levels to the mechanisms causing vessels to dilate when challenged with hypoxia.

To assess whether the rIPC maneuver has a significant impact on the circulating nitrite pool with a potential benefit for tissue protection, we first tested in healthy volunteers whether the ischemic phase or the reactive hyperemia with the resulting shear stress during rIPC are responsible for the modulation of plasma nitrite levels using 4 cycles of arm ischemia/reperfusion. In an experimental approach combining pharmacological and genetic techniques, we determined that endothelial NO synthase (eNOS) in the endothelium is responsible for nitrite generation during reactive hyperemia, which is then readily transported to the myocardium. In a further approach, we assessed the response of the target organ to the transferred signal. Taking advantage of the myoglobin (Mb) knockout mouse (complete genetic Mb knockout, Mb/−) we demonstrated that the nitrite generated during reactive hyperemia is converted to bioactive NO• with subsequent modification of mitochondria by S-nitrosation with then ultimately confer the cardioprotective effects. Transfer experiments with plasma from healthy volunteers subjected to rIPC in the arm identified nitrite as a cardioprotective agent in isolated mouse hearts with I/R.

METHODS

Human subjects.
Only male participants were enrolled in this study. The protocol was approved by the institutional review board as an amendment of the study Int. No. 3276 (Ethikkommission of the Medical Faculty, Heinrich-Heine-University Duesseldorf). This study is listed on Clinicaltrials.gov (NCT01259739). All participants were healthy and without cardiovascular risk factors (smoking, hypertension, hyperlipidemia) and gave written consent to the participation of the study. Subjects were asked to fast for 12 h before the study. All tests were conducted between 7.00 and 11.00 am. Upon arrival at the laboratory, the participants were requested to rest in supine position before a blood sample was drawn from the cubital vein. Fifteen min
later the protocol of rIPC was conducted by placing a standard blood pressure cuff on the right upper arm. Arm occlusion was achieved by cuff inflation to 200 mmHg and confirmed by Laser Doppler perfusion imaging (LDPI) and ultrasound (Arteria brachialis, 12 MHz linear probe, GE vivid I to measure diameter, peak and mean flow velocity). All ultrasound measurements were undertaken on longitudinal sections. Based on these measurements we calculated the wall shear stress. Calculations for blood flow were conducted by multiplication of the mean flow velocity and area. Upon completion of the protocol, a second blood sample was drawn.

**Statistical analyses.**

The results are presented as mean±SD. Data were analyzed by ANOVA and post-hoc Tukey’s or Bonferroni’s comparison tests with GraphPad Prism 6 software to compare differences between multiple groups and Student’s unpaired *t*-test when analyzing two groups. A value of *P*<0.05 was considered statistically significant.

Details on chemicals, mice, rIPC and I/R protocols, determination of nitrite levels and the circulating and tissue NO• pool, mitochondrial analyses and transfer experiments are presented in the Online Data Supplement.

**RESULTS**

*Nitrite generation by rIPC in healthy volunteers depends on shear stress during reactive hyperemia.*

To investigate the ability of rIPC to generate bioactive amounts of nitrite in humans, male healthy volunteers were subjected to a standard rIPC protocol (Figure 1A-1C). A blood pressure cuff was placed around the upper right arm and inflated to 200 mmHg for 5 min followed by 5 min of cuff deflation for a total of 4 cycles. Before and after the procedure, blood was taken from the contralateral antecubital vein to measure plasma nitrite levels using gas-phase chemiluminescence. The tissue perfusion during the rIPC maneuver was controlled by LDPI on the distal forearm. In addition, a 12MHz ultrasound probe was placed on the brachial artery distal to the cuff. We compared controls to individuals undergoing rIPC with full reactive hyperemia and individuals undergoing rIPC without reactive hyperemia (reactive hyperemia was inhibited by reducing the diameter of the brachial artery by gentle pressure with the ultrasound probe to at least 50% of its diameter with venous flow left unaltered) (scheme in Figure 1B). Prevention of reactive hyperemia was used to inhibit an increase in shear stress and subsequent eNOS-derived nitrite generation. As compared to baseline, rIPC increased blood volume flow and was associated with a calculated wall shear stress of 106.5 ± 7.78 dynes/cm² in the brachial artery, while inhibition of reactive hyperemia was associated with a shear stress of 49.27 ± 5.44 dynes/cm². Compared to controls and those without reactive hyperemia, only individuals undergoing rIPC with full reactive hyperemia had significantly increased nitrite levels (basal vs. rIPC: 18 ± 8 nM vs. 29 ± 5 nM, *P*<0.05, Figure 1D-1F). The data without reactive hyperemia by a mechanical maneuver to reduce reperfusion point to endothelial shear stress as the main generator of nitrite with rIPC. To explore the involvement of the myocardial eNOS in the generation of nitrite we investigated the phosphorylation status of the serine/threonine protein kinases Akt and Erk after the rIPC maneuver which promotes eNOS activation. rIPC did not trigger the phosphorylation of Akt and Erk. Likewise, administration of cPTIO and nitrite did not lead to any detectable changes in the myocardium prior to ischemia (data not shown). If cardiac eNOS was responsible for the observed effects, then we would have expected an increase in the phosphorylation levels of Akt and Erk. However, this does not preclude a role of Akt/Erk signaling in the reperfusion phase.
Nitrite formation from eNOS-derived NO• and accumulation in the myocardium.

For further mechanistic insight upon the contribution of reactive hyperemia induced eNOS activity we performed experiments using the NO• scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or eNOS−/− mice. Intubated and mechanically ventilated mice were studied in supine position, and a small cuff was placed tightly around the right upper hindlimb. A digital manometer permitted control of occlusion pressure. The same rIPC protocol as in humans was applied (Figure 2A and 2B). Figure 2C shows that plasma nitrite was markedly increased in mice undergoing rIPC (rIPC vs. control: 511 ± 132 nM vs. 362 ± 82 nM, P<0.05). Pharmacological NO•-scavenging by prior intravenous (1 mg/kg) cPTIO abolished this effect by prevention of NO• oxidation to nitrite. If nitrite is generated at the remote site and then transferred humorally to the heart, we would expect a subsequent increase of myocardial nitrite. Remarkably, myocardial nitrite levels were elevated after the stimulus (rIPC vs. control: 884 ± 168 nM vs. 532 ± 146 nM, P<0.05). In eNOS−/− mice this increase in plasma and myocardial nitrite levels was completely abolished (Figure 2D). These studies point to eNOS as an indispensable mechanism for nitrite generation and accumulation of nitrite in the myocardium.

Requirement for nitrite in rIPC-induced infarct size reduction.

To address the relevance of the rIPC-induced nitrite formation with subsequently increasing cardiac nitrite levels, we next determined infarct size in vivo.29 Experiments were performed in mice undergoing 30 min regional myocardial ischemia and 24 h reperfusion with or without rIPC and using the NO•-scavenger cPTIO and mice without eNOS. Infarct size was determined morphologically (representative micrographs in Online Figure I). An overview of the experimental approaches is given in Figure 3A. We first tested the effects of rIPC on infarct size reduction in both wild-types. rIPC reduced infarct size in NMRI wild-type mice (Figure 3B, control vs. rIPC: 37 ± 4% vs. 17 ± 3% of area at risk (AAR), P<0.001). In mice were NO• was scavenged, no effect on infarct size was determined. Accordingly, rIPC reduced infarction in C57BL/6 wild-types, while removal of the femoral nerve had no detectable effect on infarct size and rIPC-mediated cardioprotection was preserved (Online Figure II, control vs. rIPC vs. rIPC after femoral nerve dissection: 41 ± 5% vs. 24 ± 3% vs. 23 ± 3% of AAR, P<0.001 comparing the two rIPC groups with controls). Finally, genetic ablation of eNOS completely abolished cardioprotection by rIPC (Figure 3C). These results also show that cPTIO alone does not affect infarct size. We next determined whether equivalent amounts of exogenous nitrite matching the increase of endogenous nitrite levels with rIPC would mimic cytoprotection. We administered 4.8 nmoles of nitrite intravenously (tail vein) as a 100 µl bolus 5 min before ischemia. This demonstrates that supplementation with rIPC-matched nitrite levels simulates cardioprotection seen with rIPC (Online Figure III) eNOS−/− mice treated with exogenous nitrite also had decreased infarct size and thus remained responsive to nitrite (Online Figure IV).

Intracellular activation of the transfer signal nitrite by Mb.

We next analyzed the response caused by the transferred nitrite signal within the target organ and the subsequent cardiomyocyte signaling. We have previously shown that Mb is indispensable for the reduction of nitrite in the reperfused myocardium.29,34 We therefore subjected Mb−/− mice to rIPC followed by in vivo I/R (experimental scheme in Figure 4A). Transgenic Mb knockout mice were reported to be viable, fertile, without any obvious signs of functional limitation and behave normally. As compensatory mechanisms Mb−/− mice exhibit elevated hemoglobin concentrations, increased coronary flow as well as higher density of capillaries compared to their wild-type littermates.35 Figure 4B and 4C shows an increase of nitrite in plasma and myocardium of Mb−/− mice. However, despite such increase of nitrite, rIPC had no infarct-sparing effect in Mb−/− mice (Figure 4D), demonstrating that Mb is indispensable to activate the transfer signal nitrite to protective NO•. To demonstrate that Mb−/− mice are responsive to well-established cardioprotective stimuli, mice were treated with cyclosporine A (CSA at 10 mg/kg prior to reperfusion). This caused a significant reduction in infarct size (Online Figure V).
Nitrite is reduced to bioactive NO’ by Mb to regulate mitochondrial respiration in the reperfused myocardium.

While these previous results show that Mb significantly contributes to nitrite bioactivation, we next investigate the downstream effects of the resulting NO’. The formation of NO’ and the subsequent activation of the soluble guanylate cyclase was confirmed by an increase of the resulting product cGMP. This was increased from 51 ± 20 fmol/mg to 144 ± 26 fmol/mg myocardial tissue of rIPC treated mice (Figure 5A, n=4/5, P=0.02). It was recently demonstrated that NO’-dependent modulation of mitochondrial respiration is indispensible to the cardioprotective NO’-signaling in the reperfused myocardium. Remarkably, rIPC increased S-nitrosation of mitochondrial complex I in the myocardium of wild-type mice from 0.18 ± 0.12 pmol/mg protein to 1.96 ± 0.42 pmol/mg protein (Figure 5B, n=4/5, P=0.008). In parallel to this modification, we observed a decrease in the activity of complex I from 305 ± 14 nmol/min/mg protein to 240 ± 8 nmol/min/mg protein (Figure 5C, n=4/5, P=0.004). This resulted in a significantly reduced formation of potentially harmful ROS as measured by the myocardial content of H2O2, which decreased from 1.58 ± 0.04 nmol/mg wet tissue to 1.36 ± 0.06 nmol/mg wet tissue (Figure 5D n=5/4, P=0.02). No such effects were detectable in Mb-/- mice (Figure 5E-5H), consistent with our previous results for exogenous nitrite.29

Human plasma nitrite from remote conditioning protects from experimental I/R injury, To address the relevance for a transferable blood-borne factor by rIPC, ex vivo experiments using mouse hearts (Langendorff preparations) subjected to conditioned human blood were conducted. As demonstrated by us and others, circulating nitrite levels in the investigated species range at around 20-50 nM in humans and around 300-500 nM in mice.36,37 Considering that after 40 min (i.e. 4 cycles of ischemia and 4 cycles of reperfusion of each 5 min duration) most nitrite will escape the circulation and accumulate in tissue and organs, as seen in the mouse myocardium in vivo, a modified protocol had to be applied. We therefore applied a single cycle of rIPC (5 min ischemia) in humans and took blood from the ipsilateral antecubital vein after 1 min reperfusion (Figure 6A). Arguably, this approach allowed us to collect all generated nitrite from the draining vein before it is readily distributed throughout the circulation and every organ. However, this would also apply to all other potential rIPC mediators which have previously been proposed. On the ipsilateral arm, nitrite in plasma increased to a concentration of 162 ± 58 nM, which has been demonstrated to be protective in mice. Plasma, processed by filtration, was then perfused via a perfusion line in admixture with Krebs-Henseleit buffer into the isolated mouse hearts. To determine whether rIPC-generated nitrite in human plasma accumulates into the isolated hearts, we measured cardiac nitrite levels and detected an increase of nitrite after rIPC plasma perfusion as compared to controls; an effect which was inhibited by pretreatment of conditioned plasma with the nitrite-scavenger sulfanilamide (Figure 6B) again pointing to an increased uptake of nitrite into the heart. Perfusion of the isolated hearts with the conditioned human plasma reduced infarct size (47 ± 10% to 31 ± 4%, P<0.05, Figure 6C). The cardioprotective effect of the plasma was abolished after pretreatment with sulfanilamide (Figure 6C). Using plasma from subjects without reactive hyperemia and less shear stress did not lead to any detectable changes in infarct size when transferred to the isolated mouse heart (54 ± 13%) while plasma with exogenous nitrite matched to the concentration achieved during full reactive hyperemia reduced infarct size (25 ± 3%, Figure 6C).

To further confirm the relevance of generated nitrite for cardioprotection, we increased the endogenous nitrite levels by dietary nitrate supplementation27 and performed the rIPC procedure. Administration of the conditioned plasma to mouse hearts reduced infarct sizes to 25 ± 3% and nitrite scavenging with sulfanilamide again abolished these protective effects (data not shown). To prove the relevance of Mb-mediated nitrite reduction, conditioned human plasma was subjected to hearts of Mb-/- mice, which cannot reduce nitrite to bioactive NO’. This did not result in a detectable cardioprotection (46 ± 8% to 42 ± 4%, P=n.s.) (data not shown).
DISCUSSION

The rIPC maneuver by brief I/R episodes in a remote organ provides significant myocardial protection from potentially lethal myocardial I/R injury. Several clinical studies have demonstrated that upon application of rIPC, periprocedural myocardial damage was significantly reduced as evidenced by a marked decrease in the release of myocardial biomarkers during vascular, valve and bypass surgery and during percutaneous coronary interventions. We and others were furthermore able to show that in conjunction with a decrease in myocardial injury biomarkers, rIPC may provide a significant prognostic effect for these patients. Although the cardioprotective properties of rIPC were first reported two decades ago, the underlying mechanisms remain incompletely understood with respect to the potential source, the transfer, and to the activated intracellular target-signaling in the myocardium.

We here describe a role for endothelium-derived nitrite to contribute to the transfer of the protective signal from the remote site to the myocardium at risk, where nitrite is reduced by Mb to cardioprotective NO•. Indeed, many different pathways have been proposed to be activated by rIPC, including neuronal and humoral signal transduction mechanisms. Our present observations specifically assess a role for nitrite in rIPC, but it is likely that multiple overlapping enzymatic and non-enzymatic pathways for signal transduction are present in the circulation and the different blood compartments to allow for the graded protection response of the heart. These data therefore also highlight the potential for blood cells and potentially undiscovered mediators to be involved in rIPC signaling. Further investigations are needed to compare each of their relative contributions. The key evidence that supports a major contribution of nitrite signaling in rIPC is: (i) Nitrite is the stable product of NO• and transported as such in the blood in much higher concentrations as compared to nitroso species. Genetic ablation of eNOS abolished the cardioprotective nature of rIPC. (ii) While this may also be related to eNOS in cardiomyocyte and eNOS-dependent nitrite production in the myocardium itself, our results relating to the activation of the Akt pathway demonstrate that rIPC has no detectable impact on the levels of phospho-Akt. This would be expected if eNOS in the cardiomyocyte itself was activated by rIPC and caused the observed increase in cardiac nitrite. However, this does not rule out a role for Akt/Erk signaling in the reperfused myocardium. (iii) In our experiments, plasma from conditioned participants pretreated with sulfanilamide which scavenges all nitrite abolished cardioprotection while replenishment with exogenous nitrite simulated the rIPC effects. (iv) In Mb−/− mice, which cannot activate nitrite to NO•, no effect on infarct size was detectable.

rIPC-dependent nitrite generation and signal transfer to the target organ.

The first studies to test how the protective signal is transferred from the remote tissue to the target organ involved pharmacological ganglion blockade and defined a role for a nerval signal transmission. However, porcine studies argued against a mere transmission via the nervous system by demonstrating that the cardioprotective effects of rIPC are active in animals with transplanted and thus denervated hearts. This was further evidenced by studies that transfused conditioned plasma to naive recipient hearts with subsequent reduction of myocardial injury, thus arguing in favor of a humoral factor. Arguably, the rIPC maneuver exerts direct and indirect effects on the vasculature by repetitive sets of short ischemia and reactive hyperemia in the reperfusion phases. Increased blood flow to the arm or hindlimb, respectively, in conjunction with increased shear stress is the major stimulus for eNOS to generate NO• and nitrite, the more stable oxidation form. In a first strategy, we aimed to investigate whether the rIPC maneuver producing high levels of shear stress can modulate nitrite in the blood of healthy volunteers and whether this is related to the reactive hyperemia phase. Inhibition of reactive hyperemia markedly reduced the level of shear stress and nullified the generation of nitrite in the course of rIPC, thus precluding a direct contribution of the ischemic phase to the increase of plasma nitrite, e.g.
through ischemia-induced tissue damage. Taking advantage of the eNOS−/− mouse as well as through pharmacological scavenging, we provided evidence that abrogation of nitrite generation through NO• production abolished the cardioprotective effects from rIPC. Furthermore, a specific role for cardiac eNOS in producing tissue nitrite can be excluded by lack of change in phosphorylation of Akt in the myocardium after the rIPC maneuver. These results argue against an eNOS-derived NO• production within the cardiomyocytes. Nitrite, in turn, has been demonstrated to modulate vascular functions. However, this relates particularly to higher pharmacological doses, while the effects of the nitrite levels at baseline and as modulated by rIPC do not change vascular tone and the corresponding blood pressures. The effects of these endogenous moderately increased levels are limited to events of ischemia and hypoxia where nitrite serves as a selective hypoxic ‘NO• donor’. This argues in favor of a direct effect of nitrite on the myocardium during I/R rather than one secondary to more favorable hemodynamics. One limitation of our current study is the lack of nitrite measurements in the myocardium of humans. Arguably, the absolute circulating values in humans and mice differ in our study. However, after the rIPC protocol consisting of 4 phases of limb ischemia (5 min) followed by 5 min of reperfusion, we determined a significant increase in circulating nitrite levels in both our experimental approaches and the intervention trial, which in relative terms was comparable. The generated nitrite is readily distributed throughout all organs and thus escapes the circulation. In our experimental studies in mice, we showed that nitrite accumulates in the heart. We furthermore showed, in a proof-of-concept approach, that nitrite generated by rIPC in humans is cardioprotective. After 4 cycles of rIPC, most nitrite will have escaped the circulation. We therefore decided to obtain the plasma after one cycle from the ipsilateral arm. However, this does not preclude any other potential mediators generated in the course of rIPC. Although the protective effects of rIPC can be transferred with plasma between men and mice - with abrogation of cardioprotection due to prior nitrite scavenging – differences in nitrite levels between human participants and mice must be acknowledged. Future studies on the cardioprotective effects of nitrite are currently undertaken and dose responses of nitrite on cardioprotection in humans remain to be defined. As we show here relatively high concentrations of nitrite are necessary in order to be cardioprotective. This may be one reason why in an early report from the Nitrites In Acute Myocardial Infarction (NIAMI) trial no evidence for a benefit of a five-minute intravenous infusion of 70 µmol sodium nitrite before reperfusion in the general population has been seen.

Signal transfer and transduction into cardioprotection.

We next aimed to assess whether the nitrite signal is effectively transported to the heart leading to a nitrite-dependent cardioprotection signaling during myocardial I/R. Nitrite is regarded as a ‘reservoir’ for NO• which is selectively activated under physiological and pathophysiological conditions. We here demonstrate that the rIPC stimulus leads to a transfer of nitrite to the myocardium where it is then reduced by Mb to bioactive NO• confirming our previously published results for exogenously supplemented nitrite. Subsequent S-nitrosation of mitochondrial complex I finally led to a reduction of ROS in the reperfused myocardium at risk as downstream correlate. This is therefore also the first demonstration that rIPC activates a signal transduction machinery to down-regulate mitochondrial function by S-nitrosation, recently emerging as a key event for cardioprotection. A potential impact on I/R-induced mitochondrial damage or turnover would also be an important aspect and future studies are needed.

Taken together, the present findings imply a significant contribution for eNOS-derived nitrite to transfer the protective signal from the remote region to the heart, where it is reduced by Mb to bioactive NO• ultimately contributing to infarct size reduction by rIPC. While many mechanisms that have previously been forwarded may work along-side the proposed one, our present studies show a marked effect on infarct size reduction and may also explain why the effect of rIPC in humans is not as pronounced, given that endothelial function is partly impaired in patients with atherosclerosis and subsequent coronary artery disease.
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DISCLOSURES
None.

REFERENCES


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Figure 1. Remote ischemic preconditioning (rIPC) generates nitrite. Nitrite generation by rIPC was assessed in healthy human volunteers. (A) Scheme illustrating the experimental setting. A blood pressure cuff was placed on the upper right arm and perfusion was monitored distally by Laser Doppler perfusion imaging (LDPI). The cuff was inflated to 200 mmHg for 5 min (Ischemia, I), followed by 5 min of deflation (Reperfusion, R). An ultrasound probe was placed on the brachial artery. Using this approach, three groups were studied (protocol in B). Group 1 served as control (n=6), while groups 2 (n=6) and 3 (n=6) received the rIPC stimulus. In contrast to group 3 with full R, in group 2 R and thus reactive hyperemia was limited by manual compression with the ultrasound probe. (C) Exemplary registration showing brachial artery diameter and relative volume flow increase over the first 120 s of reperfusion. (D) In control subjects (CTRL), no differences in LDPI signal (at reperfusion), flow velocity (basal vs. levels after the rIPC protocol. However, regular reperfusion with reactive hyperemia significantly increased plasma nitrite levels after rIPC. However, regular reperfusion with reactive hyperemia significantly increased plasma nitrite levels after rIPC (n=6, *P<0.05, unpaired Student’s t-test).

Figure 2. Remote ischemic preconditioning (rIPC) generates nitrite in mice in an eNOS dependent manner. (A) Experimental set-up for in vivo rIPC experiments. Mice were anesthetized, mechanically ventilated, a pressure cuff was placed around the right upper hindlimb, and perfusion distal to the cuff was assessed by Laser-Doppler perfusion imaging (LDPI). We assessed the generation of nitrite after the complete rIPC protocol. This consisted of 4 cycles of 5 min I/5 min R (B, protocol and exemplary LDPI registration showing complete arrest of perfusion during hindlimb ischemia to establish a valid rIPC protocol). (C) rIPC increased nitrite levels in plasma and heart of wild-type mice. Pre-treatment with the NO• scavenger cPTIO abolished the effects of rIPC (n=5 - 10, *P<0.05, one-way ANOVA). (D) In eNOS−/− mice, the increase of nitrite was not observed in either plasma or heart.

Figure 3. Remote ischemic preconditioning (rIPC) and reduction of infarct size – role of nitrite. Anaesthetized mice were subjected to the rIPC stimulus by 4 cycles of hindlimb I/R or served as controls and were then challenged with 30 min coronary occlusion followed by 24 h of reperfusion. Infarct sizes were determined histologically. cPTIO was used to scavenge NO during rIPC. In an additional group, the femoral nerve was dissected prior to the rIPC stimulus. Scheme in A. Results in Online Figure II. (B) In wild-type mice (WT), rIPC reduced infarct size, and the NO• scavenger cPTIO abolished this effect (n=5, ***P<0.001, one-way ANOVA compared to CTRL). (C) Genetic ablation of endothelial NO• synthase in eNOS−/− mice abrogated the infarct size reduction by rIPC (n=4 - 5)

Figure 4. Nitrite reduction via myoglobin (Mb) is required for cardioprotection by rIPC. Mice without Mb (Mb−−) were subjected to rIPC (A). rIPC increased plasma (B) and myocardial nitrite (C) (n=5, *P<0.05, t-test). (D) A) In Mb−− mice, rIPC even worsened I/R injury (n=6 CTRL vs. rIPC: 32 ± 3 vs. 38 ± 5 %, *P=0.02, Student’s two-tailed unpaired t-test).

Figure 5. Regulation of mitochondrial respiration by nitrite from rIPC. In wild-type mice, rIPC increased cGMP levels (A), S-nitrosation of mitochondrial complex I (B), decreased complex I activity (C) and finally reduced H2O2 levels (D). These effects were not observed in Mb−− mice (E-H) (n=4 - 5, *P<0.05, **P<0.01, Student’s t-test).

Figure 6. Transfer of cardioprotection. (A) Blood was obtained from human volunteers after one cycle of the rIPC stimulus on the rIPC arm (ipsilateral). The resulting plasma was transferred to isolated mouse hearts. (B) Infusion of ipsilateral human rIPC plasma to isolated hearts increased cardiac nitrite levels compared to controls, while this increase was abolished after pre-incubation of plasma with sulfanilamide to scavenge nitrite (n=3 - 4, *P<0.05, one-way ANOVA). (C) Infarct studies using conditioned human...
plasma. Conditioned human plasma reduced infarction compared to control. This reduction in infarct size was abolished by nitrite-scavenging with sulfanilamide. Plasma from subjects following rIPC without reactive hyperemia did not lead to a detectable protection, while this was the case after nitrite had been added to plasma ($n=3 - 5$, *$P<0.05$ compared to non-nitrite-supplemented plasma, one-way ANOVA).
Novelty and Significance

What Is Known?

- Remote ischemic preconditioning (rIPC) with brief cycles of blood pressure cuff in- and deflations at the upper or lower limb protects against myocardial ischemia and reperfusion (I/R) injury.

- This maneuver increases circulating nitric oxide (NO) species (nitrite, nitroso species) via an unknown mechanism.

- Application of exogenous nitrite during myocardial I/R protects the heart from I/R injury.

What New Information Does This Article Contribute?

- The rIPC maneuver activates endothelial nitric oxide synthase (eNOS) through enhanced shear stress during reactive hyperemia, resulting in increased formation of NO, which subsequently is oxidized to nitrite and transferred humorally to the myocardium.

- Cardioprotection from rIPC is effective only when cardiac myoglobin (Mb) activates nitrite to bioactive NO•, which, in turn then modulates mitochondrial function during myocardial I/R.

- The rIPC-mediated cardioprotection can be transferred with human plasma to mouse hearts and, in turn, is abrogated when plasma nitrite is scavenged.

Remote ischemic preconditioning (rIPC) activates the cardioprotective nitrite signaling pathway. Shear stress during reperfusion of the previously ischemic limb activates eNOS, which produces NO• and subsequently nitrite. This nitrite is humorally transported to the myocardium, where it is activated by Mb to NO• and initiates cardioprotective signaling in the mitochondria. Genetic ablation of eNOS or Mb abolishes cardioprotection by rIPC. Transfer of plasma from humans with rIPC to mouse hearts further support the notion of blood-borne cardioprotection. Nitrite contributes significantly to the signaling which underlies rIPC. These findings help to better understand the signal transduction mechanisms of rIPC and to resolve why rIPC is effective in some patients while others may be resistant.
**Figure 1**

A. Schematic diagram showing the location of blood pressure cuff, LDPI, blood sampling, and ultrasound imaging.

B. Graph showing time of reperfusion (s) vs. volume flow with hyperemia and diameter increase with reactive hyperemia.

C. Graph showing volume flow increase with reactive hyperemia and diameter with reactive hyperemia over time with different markers for volume flow and diameter.

D. Graphs showing LDPI and flow velocity with Stimulus (CTRL) and Basal conditions.

E. Graphs showing LDPI and flow velocity with Stimulus (rIPC - reactive hyperemia) and Basal conditions.

F. Graphs showing LDPI and flow velocity with Stimulus (rIPC + reactive hyperemia) and Basal conditions.

**Legend:**
- **CTRL:** Without reactive hyperemia
- **rIPC:** With reactive hyperemia
- Blue line: Volume flow + hyperemia
- Dotted line: Diameter + hyperemia
- Red line: Volume flow + hyperemia
- Red dashed line: Diameter + hyperemia
Figure 2

A. Schematic diagram of a mouse with a hindlimb cuff and LDPI.

B. Time course of CTRL and rIPC treatments.

C. Nitrite levels in WT Plasma for CTRL, rIPC, and rIPC + cPTIO.

D. Nitrite levels in eNOS−/− Plasma for CTRL and rIPC.

E. Nitrite levels in WT Heart for CTRL, rIPC, and rIPC + cPTIO.

F. Nitrite levels in eNOS−/− Heart for CTRL and rIPC.
Figure 3

A

CTRL

rIPC

CTRL NO• scavenging

cPTIO

NO• scavenging

rIPC nerve dissection

Infarct per AAR (%)

B

WT

WT

rIPC

NO• scavenging (cPTIO)

Infarct per AAR (%)

C

eNOS−/−

rIPC

NO• scavenging (cPTIO)

Infarct per AAR (%)

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Figure 4

A

<table>
<thead>
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<th>min.</th>
<th>0</th>
<th>40</th>
<th>70</th>
<th>75</th>
</tr>
</thead>
</table>

Ischemia 30' Reperfusion 24h

CTRL:

rIPC:

IR IR IR IR IR

NO Pool cGMP mitochondria Infarct size

B

Nitrite [nM] (Mb\(^{-/-}\) Plasma)

CTRL rIPC

C

Nitrite [nM] (Mb\(^{-/-}\) Heart)

CTRL rIPC

D

Infarct per AAR (%)

CTRL rIPC
Figure 5

**WT**

A. cGMP (fmol/mg tissue) in CTRL and rIPC

B. SNO Complex I (pmol per mg protein) in CTRL and rIPC

C. Complex I activity (nmol/min/mg protein) in CTRL and rIPC

D. H$_2$O$_2$ (nmol/mg wet tissue) in CTRL and rIPC

**Mb^{-/-}**

E. cGMP (fmol/mg tissue) in CTRL and rIPC

F. SNO Complex I (pmol per mg protein) in CTRL and rIPC

G. Complex I activity (nmol/min/mg protein) in CTRL and rIPC

H. H$_2$O$_2$ (nmol/mg wet tissue) in CTRL and rIPC

* and ** denote statistical significance compared to CTRL.
Figure 6

A

Signal generation

Blood sampling

Blood pressure cuff

0' 5'

Blood sampling

B

Heart nitrite

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nitrite [nM]</th>
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</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>400 ± 10</td>
</tr>
<tr>
<td>Plasma cycle rIPC</td>
<td>600 ± 10</td>
</tr>
<tr>
<td>Plasma cycle rIPC + nitrite scavenging</td>
<td>500 ± 10</td>
</tr>
</tbody>
</table>

C

Infarct per AAR (%)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Infarct (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rIPC</td>
<td>-</td>
</tr>
<tr>
<td>rIPC w/o reactive hyperemia</td>
<td>-</td>
</tr>
<tr>
<td>nitrite scavenging</td>
<td>-</td>
</tr>
<tr>
<td>exogenous nitrite</td>
<td>-</td>
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</tbody>
</table>

* Indicates statistical significance.
Circulating Nitrite Contributes to Cardioprotection by Remote Ischemic Preconditioning
Tienush Rassaf, Matthias Totzeck, Ulrike B Hendgen-Cotta, Sruti Shiva, Gerd Heusch and Malte Kelm

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SUPPLEMENTAL MATERIAL

Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning

Tienush Rassaf*, Matthias Totzeck*, Ulrike B. Hendgen-Cotta, Sruti Shiva, Gerd Heusch,
Malte Kelm

* contributed equally to this work
Detailed Methods

Chemicals
All chemicals were purchased from Sigma (Seelze, Germany), except for phosphate-buffered saline (PBS, Serag-Wiessner, Naila, Germany), heparin (ratiopharm, Ulm, Germany), ketamine (Pfizer, Berlin, Germany), xylazine (aniMedica, Senden-Bö sensell, Germany), and isoflurane (DeltaSelect, Pfullingen, Germany).

Mice
All experiments were approved by the responsible committee according to the ‘European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes’ (Council of Europe Treaty Series No. 123) and animal care was in accordance with institutional guidelines. All mice were male with an average age of 12 ± 3 weeks and a weight of 32 ± 6 g. NMRI wild-type (Naval Medical Research Institute), Myoglobin deficient (Mb/- on a NMRI background), C57BL/6 wild-type and endothelial nitric oxide synthase deficient (eNOS-/- on a C57BL/6 background) mice were obtained from the Duesseldorf animal facility, fed on standard rodent chow, tap water ad libitum and held on a 12/12 h light/dark cycle until the experiments.

In vivo remote ischemic preconditioning protocol
Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (45 mg kg-1) and xylazine (Rompun, 10 mg kg-1). A tracheal tube was inserted and mechanical ventilation initiated according to the individual body weight (Inspira, Harvard Apparatus, Hugo-Sachs, March-Hugstetten, Germany). Isoflurane (1.2 vol%) was supplemented to the respiratory gas to maintain anesthesia. A small vascular occluder (6 mm, Kent Scientific, Torrington, United States) was placed around the right upper hindlimb. Inflation of this occluder to an internal pressure of 200 mmHg, which was measured digitally (Halstrup Walcher, Kirchzarten, Germany), caused a complete arrest of hindlimb perfusion. In order to establish a valid rIPC mouse model, arrest and recovery of perfusion were monitored continuously by LDPI (Perimed, Stockholm, Sweden). The rIPC protocol consisted of 4 cycles of 5 min hindlimb ischemia followed by 5 min of reperfusion. Wild-types of both genetic backgrounds (NMRI and C57BL/6) as well as eNOS-/- and Mb/- mice were subjected to the rIPC protocol. A sub-group of wild-type and eNOS-/- mice received an intravenous (i.v.) bolus injection of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 1 mg kg-1) without or immediately before the rIPC stimulus to scavenge NO• generated in the course of the rIPC maneuver. Pharmacological preconditioning to simulate rIPC was achieved by i.v. injection of 0.166 nmol/g body weight nitrite. Additionally, in a group of C57BL/6 mice, the femoral nerve was removed by surgical excision of a small approximately 3-4 mm long piece at its most proximal part. After wound closure, these mice also underwent rIPC.4 All mice were ultimately sacrificed to analyze NO• species or to measure infarct size after coronary occlusion and reperfusion.

Analysis of NO• species in plasma and myocardium
Plasma was obtained from human subjects and mice before and after completion of the rIPC protocol and collected in heparinized tubes. The resulting plasma as well as myocardial tissue samples were analyzed by HPLC and chemiluminescence with high sensitivity and detection of very small differences over a wide range of matrices as described in detail previously.5-9 For the analysis of myocardial NO• levels,
samples were weighed and homogenized immediately in ice-cold sodium chloride (0.9% supplemented with NEM/EDTA) with a semi-automatic glass-on-glass-homogenizer (Schuett, Göttingen, Germany).

**Determination of Akt and Erk phosphorylation**
The phosphorylation of Akt and Erk\textsuperscript{10} were determined in heart homogenates from control mice and mice exposed to the rIPC maneuver, rIPC maneuver and cPTIO treatment and intravenously applied nitrite (4.8 nmoles). The absolute phosphorylation of Akt was measured using a commercially available immunoassay according to the manufacturer’s instructions (R&D Systems Minneapolis, United States, SUV 887B for Akt and SUV1018B for Erk). In brief, snap-frozen tissues were homogenized in phosphate-buffered saline, supplemented with proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and then lyzed using the buffer included in the kit. After a brief centrifugation, supernatents were diluted in the shipped dilution buffer and processed using the supplied pre-coated 96 well plates. After administration of substrate and stop solution, development of color changes was measured according to the manufacturer’s instructions.

**In vivo myocardial I/R injury**
Following either the rIPC protocol or a 40 min control period, mice underwent an open-chest \textit{in vivo} myocardial I/R protocol as previously described.\textsuperscript{11} Briefly, after skin incision, median sternotomy and pericardiomy, the left main coronary artery was ligated half-way from base to apex with a 7-0 silk suture. After 30 min, the ligation was removed and the myocardium reperfused for either 24 h or 5 min. For analysis of infarct size, hearts were excised and perfused free of blood. The left coronary artery was re-ligated and Evans blue dye was perfused into the heart through the aorta in order to distinguish between the myocardium at risk (unstained area at risk, AAR) and the non-ischemic zones. After storage of the tissues at -20 °C for 60 min, the hearts was serially sectioned and each slice was weighed. The sections were incubated in 2,3,5-triphenyltetrazolium chloride for 15 min at 37 °C to quantify the amount of necrotic and viable tissue by computer-assisted technique. Final infarct sizes are expressed as percentage per AAR. For all biochemical analysis, hearts were perfused free of blood \textit{in situ}, excised, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. To assess the responsiveness of \textit{Mb}\textsuperscript{-/-} mice to cardioprotection, \textit{Mb}\textsuperscript{+/-} mice were treated with 10 mg/kg cyclosporine A (CSA) 5 min prior to reperfusion.\textsuperscript{10}

**Mitochondrial function analyses**
Heart mitochondria were isolated by differential centrifugation in a buffer (250 mM sucrose, 10 mM Tris, 1 mM EGTA, pH 7.4) at 4 °C, as previously described.\textsuperscript{12} To measure S-nitrosation, 10 mg of isolated mitochondria were lysed with a solution of 1% NP-40 and 100 μM diethylenetriamine pentaacetate. Half of the sample was immediately injected into a copper/cysteine-based reductive chemiluminescence apparatus, which is specific for S-nitrosated protein adducts. The other half of the protein was divided into three parts: one left untreated, one treated with 10% acidified sulfanilamide to eliminate nitrite, and one treated with 5 mM mercuric chloride and 10% acidified sulfanilamide (v/v) to eliminate nitrite and RSNOs. The three fractions were injected into a vessel containing triiodine and connected inline to an NO\textsuperscript{•} chemiluminescence detector (CLD 88, Ecophysics, Munich, Germany), as previously described.\textsuperscript{12, 13} Complex I activity was determined in isolated mitochondria.
by spectrophotometry (340 nm), monitoring the oxidation of 100 µM NADH in the presence of 10 µM coenzyme Q1, and in the presence and absence of 25 µM rotenone, respectively.

**Analysis of H₂O₂ and cGMP in reperfused myocardium**
Cardiac samples were processed according to the respective manufacturer’s guidelines as previously described. Whole heart homogenates were used for each assay. cGMP was measured using an enzyme immuno-linked assay (GE Healthcare, Buckinghamshire, U.K.). ROS formation was detected spectrophotometrically by measuring the oxidation of amplex red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to its product resofurin at 560 nm using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (manufacturer).

**Ex vivo experiments with transfer of human plasma to isolated mouse hearts**
Plasma from healthy volunteers was obtained before and after one ischemic cycle with and without reactive hyperemia (60 s after the beginning of arm reperfusion on the ipsilateral/rIPC arm). We used plasma from the vein that drains the rIPC arm to obtain the highest available concentration of nitrite to match the levels of nitrite in the mouse circulation and to limit the time that isolated hearts must be perfused to 20 min. Arguably, the use of ipsilateral plasma provides not only higher concentrations of nitrite but also higher levels of any other potential blood borne trigger of rIPC. Plasma was prepared as previously described and then centrifuged using 20 kDa filters (Centrisart, Sartorius, Göttingen, Germany). As compared to many other filters and dialysis membranes, these specific filters have been tested to exhibit no detectable nitrite contamination. We furthermore used dietary nitrate (0.15 mmol/kg bodyweight, single dose) to increase the levels of nitrite in plasma as previously described. For control experiments, sulfanilamide was added to scavenge all plasmatic nitrite, while exogenous nitrite was supplemented to samples to simulate rIPC induced nitrite increases. Isolated wild-type mouse hearts were prepared as previously described. After an equilibration period with perfusion of Krebs-Henseleit buffer via the main infusion line, plasma was co-infused for 20 min through a side arm in conjunction with Krebs-Henseleit buffer. The following I/R protocol consisted of 30 min of global ischemia and 60 min of reperfusion with subsequent assessment of infarct sizes. In control experiments, nitrite was further increased in human plasma by dietary nitrate ingestion and subsequent transfusion after the rIPC protocol. For every participant, a separate isolated mouse heart was taken as bioassay.
Online Figure IA

CTRL

rIPC

CTRL + cPTIO

rIPC + cPTIO

+ nitrite
Online Figure IB

CTRL

rlPC

CTRL
+cPTIO

rlPC
+cPTIO

+ nitrite
Online Figure II

Infarct per AAR (%)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infarct per AAR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rIPC -</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>+</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>+</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>femoral nerve dissection -</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Online Figure III

![Bar graph showing the effect of exogenous nitrite on infarct per AAR (%). The graph compares the percentage of infarct per AAR with and without exogenous nitrite. The graph indicates a significant difference (***) between the two conditions.]
Online Figure IV

![Bar chart showing infarct per AAR (%) for different levels of exogenous nitrite.](chart.png)

- exogenous nitrite: -  and +  

Infarct per AAR (%)
Online Figure V

![Bar graph showing Infarct per AAR (%) for CTRL and CSA groups. The graph indicates a statistically significant difference between the two groups, marked with **.](image-url)
Figure Legends

Online Figure I. Representative micrographs of mouse hearts after in vivo ischemia/reperfusion injury (30 min ischemia and 24 h reperfusion). The figure shows exemplary hearts of rIPC conditioned animals. (A) NMRI wild-type mice with and without rIPC with and without prior administration of carboxy PTIO (cPTIO) after exogenous nitrite administration. (B) eNOS knockout mice with and without rIPC with and without prior administration of carboxy PTIO (cPTIO), and after exogenous nitrite administration. (C) C57BL/6 wild-type mice with and without rIPC and with rIPC after previous dissection of the femoral nerve. (D) Myoglobin knockout mice with and without rIPC and with administration cyclosporine A (CSA, 10 mg/kg) 5 min prior to reperfusion.

Online Figure II. rIPC-induced cardioprotection is not inhibited by previous femoral nerve dissection. In wildtype mice, resection of the femoral nerve does not abolish cardioprotection by rIPC (n=4 - 6, ***P<0.001, one-way ANOVA compared to CTRL).

Online Figure III. Exogenous nitrite matched to the endogenous levels observed with rIPC reduced infarct size wild type mice 4.8 nmoles of nitrite were administered 5 min before the 30 min ischemia period followed by 24 h of reperfusion. Infarct sizes were decreased from 37 ± 4% to 35 ± 3% of area at risk (AAR) (means±S.D., n=5, ***P=0.0004, unpaired Student’s t-test).

Online Figure IV. Exogenous nitrite matched to the endogenous levels observed with rIPC reduced infarct size in endothelial NO-synthase deficient mice (eNOS−/−). 4.8 nmoles of nitrite were administered 5 min before the 30 min ischemia period followed by 24 h of reperfusion. Infarct sizes were decreased from 44 ± 7% to 34 ± 1% of area at risk (AAR) (means±S.D., n=5, *P=0.0118, unpaired Student’s t-test).

Online Figure V. Cyclosporine A (CSA) reduces myocardial I/R injury in myoglobin knockout (Mb−/−) mice. Mice were treated with 10 mg/kg CSA 5 min prior to reperfusion following 30 min
ischemia. Infarct sizes after 24 h of reperfusion showed a significant reduction from 31 ± 2% to 26 ± 2% (mean±S.D., n=6 and 4, **P=0.0014, unpaired Student’s t-test).

Online Table I – Hemodynamics in mice investigated during in vivo myocardial I/R

<table>
<thead>
<tr>
<th>Strain</th>
<th>Systolic pressure (mmHg)</th>
<th>Diastolic pressure (mmHg)</th>
<th>Mean arterial pressure (mmHg)</th>
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</thead>
<tbody>
<tr>
<td>NMRI Wild-type</td>
<td>105 ± 6</td>
<td>82 ±7</td>
<td>89±6</td>
</tr>
<tr>
<td>C57BL/6 Wild-type</td>
<td>102 ± 10</td>
<td>84 ±10</td>
<td>90 ±10</td>
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<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>134 ± 6</td>
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Supplemental References


