Sympathetic nervous response to ischemia-reperfusion injury in humans is altered with remote ischemic preconditioning

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Sympathetic nervous response to ischemia-reperfusion injury in humans is altered with remote ischemic preconditioning. Am J Physiol Heart Circ Physiol 311: H364–H370, 2016. First published June 10, 2016; doi:10.1152/ajpheart.00369.2016.—Sympathetic neural activation may be detrimentally involved in tissue injury caused by ischemia-reperfusion (IR). We examined the effects of experimental IR in the forearm on sympathetic nerve response, finger reactive hyperemia, and oxidative stress, and the protection afforded by applying remote ischemic preconditioning (RIPC). Ischemia was induced in the forearm for 20 min in healthy volunteers. RIPC was induced by applying two cycles, 5 min each, of ischemia and reperfusion to the upper leg immediately before IR. We examined muscle sympathetic nerve activity (MSNA) in the contralateral leg using microneurography, finger reactive hyperemia [ischemic reactive hyperemia (RHI)], erythrocyte production of reduced glutathione (GSH), and plasma nitric oxide (NO) concentration. In controls (no RIPC; n = 15), IR increased MSNA in the early and late phase of ischemia (70% at 5 min; 101% at 15 min). In subjects who underwent RIPC (n = 15), the increase in MSNA was delayed to the late phase of ischemia and increased only by 40%. GSH increased during ischemia in the control group (P = 0.05), but not in those who underwent RIPC. Nitrate and nitrite concentration, taken as an index of NO availability, decreased during the reperfusion period in control individuals (P < 0.05), while no change was observed in those who underwent RIPC. Experimental IR did not affect RHI in the control condition, but a significant vasodilatory response occurred in the RIPC group (P < 0.05). RIPC attenuated ischemia-induced sympathetic activation, prevented the production of an erythrocyte marker of oxidative stress and the reduction of NO availability, and ameliorated RHI.

LOCAL ISCHEMIC PRECONDITIONING is an established method to protect the heart against tissue damage induced by prolonged ischemia and subsequent reperfusion (9). Although recently disputed in large-scale studies (5, 24), data from smaller size clinical trials indicate that brief periods of ischemia applied at an easily accessible remote site, usually a limb, before an injurious ischemic event [remote ischemic preconditioning (RIPC)] ameliorates myocardial injury following coronary artery bypass graft surgery (6) and lowers all-cause mortality (30). Experimental studies indicate that the protection afforded by RIPC can also impact other organs, such as the kidneys, brain, and liver (10). The pathway generating distant organ protection remains unclear, but is believed to involve both humoral and neural mechanisms. Humoral mediators, such as adenosine, bradykinin, and opioids, have been proposed as necessary triggers for preconditioning the heart. RIPC may also act by improving endothelial dysfunction and reducing inflammation and reactive oxygen species (ROS) formation (20). Indeed, studies in humans have shown that RIPC can either improve endothelial function (22), or prevent ischemia-reperfusion (IR)-induced endothelial dysfunction as well as prevent systemic neutrophil activation (15). Sympathetic nervous activation and noradrenaline overflow is a key component of the IR-induced organ injury (11), and pharmacological sympathoinhibition was found to prevent the ischemic kidney injury in rats (32), thereby indicating the potential deleterious effect of ischemia-induced sympathetic activation. In addition, it was found that local ischemic preconditioning suppressed the enhanced renal sympathetic nerve activity during ischemia, probably through activation of nitric oxide (NO) production (33). However, the effects of IR and preconditioning on sympathetic nervous system activation have not been fully explored in humans. We evaluated sympathetic nerve response by direct microneurographic recordings, finger reactive hyperemia, noradrenaline, NO, and ROS release to forearm IR injury in healthy humans and determined whether RIPC performed in the leg altered these responses.

METHODS

Thirty healthy volunteers (13 men, 17 women; mean age, 21 yr; range, 18–39 yr) were recruited. The study protocol conformed to relevant guidelines of the National Health and Medical Research Council of Australia and was approved by The Alfred Hospital Human Research Ethics Committee. All participants gave written, informed consent before their participation.
IR and RIPC. Participants were studied in the supine position. The IR injury was performed in the left upper arm (occluding the brachial artery). Ischemia was induced by inflating a 9-cm-wide blood pressure (BP) cuff to a pressure of 200 mmHg for 20 min. Reperfusion was induced by deflating the cuff. The RIPC was performed in the upper part of the left leg using a 9-cm-wide BP cuff. The cuff was inflated to 200 mmHg for 5 min (ischemia), followed by a 5-min deflation (reperfusion). The inflation/deflation cycle was performed two times. RIPC was initiated immediately before the IR injury induction. Participants were randomly assigned to be in a control group (no RIPC, n = 15) or RIPC (n = 15).

Experimental protocol. The experimental protocol is illustrated in Fig. 1. First, a venous catheter was placed in an antecubital vein of the right arm. Microneurographic recording of muscle sympathetic nerve activity (MSNA) was then commenced. After a robust MSNA site was found, participants were allowed to rest for 20 min to obtain baseline measurements. Endothelial function measurements commenced after the baseline period. RIPC or no intervention (control group) was conducted for a total period of 20 min and was followed by a 20-min period of ischemia and another 20-min period of reperfusion. Endothelial function assessment was repeated immediately after the IR injury. Blood samples were collected from participants within the last 2 min of the following periods: 1) baseline, 2) forearm ischemia; and 3) forearm reperfusion.

MSNA and hemodynamic recordings. Recording of multiunit postganglionic MSNA in humans was performed as previously described (17). Briefly, recordings were made from a tungsten microelectrode (FHC, Bowdoinham, ME) inserted directly into the right peroneal nerve just below the fibular head. During MSNA recording, beat-to-beat BP was measured continuously using the Finometer system (Finapress Medical System BV, Amsterdam, The Netherlands). Brachial BP was assessed every 15 min (Dinamap model 1846SX, Critikon, Tampa, FL) to ensure correct readings. Heart rate (HR) was extracted from lead III ECG. All of these parameters were digitized with a sampling frequency of 1,000 Hz (PowerLab recording system, model ML 785/8SP, ADI Instruments). Sympathetic bursts were counted manually and expressed as burst incidence (bursts/100 heartbeats) and burst frequency (bursts/min). Average MSNA, BP, and HR were performed over a period of ~5 min at the end of baseline, just before IR (end of RIPC period), in the early (4–8 min) and late (16–20 min) phase of ischemia, and in the early and late phase of reperfusion.

Finger reactive hyperemia. Finger pulse-volume amplitude was measured to calculate reactive hyperemia index (RHI), assessed as an indicator of total NO production (31). This method is based on the principle that GSH concentration was achieved in a two-step process. The first step involved the conversion of NO3 to NO2 using nitrite reductase. In the second step, addition of the Griess reagent resulted in the conversion of NO2 into a deep purple compound, which was photometrically measured using a Benchmark Plus Microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The Griess reaction is the most frequently used analytic method for quantification of the major metabolites of NO (NO3 and NO2) in blood (31).

Assessment of NO release. From plasma, we measured NO3 and NO2 as an indicator of total NO production (31). We used the commercial colorimetric kit from Cayman Chemical (Ann Arbor, MI) and followed the manufacturer’s instructions. The final products of NO in vivo are NO3 and NO2, with the best index of NO production being the sum of both NO3 and NO2. Measurement of total NO3/NO2 concentration was achieved in a two-step process. The first step involved the conversion of NO3 to NO2 using nitrite reductase. In the second step, addition of the Griess reagent resulted in the conversion of NO2 into a deep purple compound, which was photometrically measured using a Benchmark Plus Microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The Griess reaction is the most frequently used analytic method for quantification of the major metabolites of NO (NO3 and NO2) in blood (31).

Assessment of erythrocyte markers of oxidant stress. Erythrocyte production of reduced glutathione (GSH) was determined by the method of Beutler et al. (1). This method is based on the principle that GSH reduction of 5,5-dithiobis-(2-nitrobenzoic acid) forms a yellow colored anion, which can be measured spectrophotometrically at 412 nm. A GSH standard was prepared by adding 50 mg GSH and deionized water to a final volume of 100 ml. Two hundred microliters of the washed cell suspension or standard, 1.8 ml deionized water, and 3 ml of precipitating solution (8.35 g glacial metaphosphoric acid, 1 g di-potassium EDTA, 150 g sodium chloride in 500 ml deionized water) were added to a test tube. The mixture was then left to stand for 5 min to allow proteins to precipitate. Proteins were then filtered using a glass funnel, and 1 ml of the sample filtrate or GSH standard was added to 4 ml phosphate solution containing 0.3 M di-sodium hydrogen phosphate. Five hundred microliters of 5,5-dithiobis-2-nitrobenzoic acid solution (0.5 mM in 1% tri-sodium citrate) was then added, and the absorbance was measured at 412 nm. The GSH concentration of the samples (mg GSH/100 ml erythrocytes) was determined using the absorbance value of the GSH standard, and the hematocrit was determined on the washed cell suspension.

Plasma noradrenaline concentration. Noradrenaline was extracted from plasma using alumina absorption, and the amount was quantified using high-performance liquid chromatography with colorimetric detection, as previously described by our laboratory (18). Intra-assay coefficient of variation in our laboratory is 1.3%, and interassay coefficient of variation is 3.8%.

Statistics. All data are expressed as means ± SE. MSNA, BP, HR, RHI, plasma noradrenaline concentrations, and GSH were evaluated using two-way repeated-measures analysis of variance, testing for protocol effects (control vs. RIPC), RIPC and IR effects, and interactions. As NO data were not normally distributed, a Friedman repeated-measures ANOVA on ranks was used. GSH changes be-
between the two groups observed during ischemia were assessed as percent change and analyzed using an independent Student’s t-test. Differences were considered significant at \( P < 0.05 \). Statistical analysis was performed using SigmaStat for Windows version 3.5.

**RESULTS**

All participants tolerated the procedure without complication. BP did not change during the procedure, as shown in Fig. 2. Mean resting systolic and diastolic BP was 111 ± 3 and 67 ± 1 mmHg and 112 ± 4 and 65 ± 3 mmHg for the control and RIPC group, respectively. Average baseline HR was 64 ± 3 and 61 ± 3 beats/min, for the control and RIPC group, respectively. HR remained unchanged during the entire protocol in the two groups, with the HR being 66 ± 4 and 62 ± 3 beats/min at the end of the study in the control and RIPC group, respectively.

*Sympathetic nerve response.* Microneurographic recordings were maintained for the duration of the study in 18 subjects (8 controls and 10 RIPC); hence MSNA data are presented in this cohort only. An example of MSNA response in one control subject and one RIPC subject is presented in Fig. 3. In the control group, MSNA was 20 ± 2 and 22 ± 2 bursts/min at baseline and just before IR injury, respectively. Ischemia was associated with an increase in MSNA to 31 ± 2 bursts/min in the early phase (\( P = 0.033 \) vs. baseline) and to 35 ± 2 bursts/min in the late phase (\( P < 0.001 \) vs. baseline). During reperfusion, MSNA was no longer different from the baseline values (28 ± 2 and 21 ± 2 bursts/min in the early and late phase of reperfusion, respectively). In the group that underwent RIPC, MSNA was 21 ± 2 bursts/min at baseline. The RIPC did not significantly affect MSNA (16 ± 2 bursts/min). In the early phase of ischemia, MSNA remained unchanged (22 ± 2 bursts/min), but significantly increased during the late phase (29 ± 2 bursts/min, \( P < 0.001 \) compared with RIPC values). The increase in MSNA during the early phase of ischemia was significantly less in the RIPC group compared with the control one [change (\( \Delta \)) = 8.3 bursts/min, \( P = 0.028 \), RIPC vs. control group].

When expressed as percent change from baseline, the increase in MSNA (bursts/min) was 70 and 101% during the early and late phase of the ischemia, respectively, and 55% during the early phase of reperfusion in the control group. These changes were significantly different from the changes observed before IR (\( P = 0.018 \), \( P < 0.01 \), and \( P = 0.15 \), respectively). In the RIPC group, MSNA changes during RIPC
were not significantly altered compared with baseline (−24%), but the increase in MSNA during the late phase of ischemia was significantly different compared with the changes observed during RIPC (40%, \( P = 0.011 \)). Nevertheless, the changes in MSNA during both the early and late phases of ischemia were less than those seen in the control group (\( P < 0.05 \) compared with controls at both early- and late-phase time points) (Fig. 4). MSNA expressed as burst incidence (bursts/100 heartbeats) displayed similar changes as that expressed as burst frequency (data not shown).

**Finger reactive hyperemia.** Finger reactive hyperemia measurements were successfully obtained both before and after the procedure in 11 subjects in each group. In the control group, the IR injury did not significantly affect finger reactive hyperemia (RHI; 1.68 ± 0.15 vs. 1.86 ± 0.12 at baseline), whereas, in subjects who underwent RIPC, finger reactive hyperemia was significantly improved compared with baseline (2.28 ± 0.20 vs. 1.81 ± 0.08, \( P = 0.002 \)) (Fig. 5). After IR injury, RHI was also significantly higher in the RIPC group compared with the control group (\( P = 0.028 \), Fig. 5).

**Erythrocyte marker of oxidant stress.** In the control group, erythrocyte production of GSH increased during ischemia (77.6 ± 1.1 vs. 73.4 ± 1 mg/100 ml red blood cells, \( P = 0.05 \)), and this increase was absent in those subjected to RIPC (75.1 ± 1.2 vs. 77.8 ± 1.2 mg/100 ml red blood cells; Fig. 6A). Increases in GSH during ischemia reached 6% in the control group. This was significantly different from the change observed in the RIPC group (−4%, \( P = 0.006 \), Fig. 6B).

**NO production.** In the control group, NO was not affected during the end of the ischemic period, but significantly decreased during reperfusion (79 ± 10 vs. 71 ± 8 \( \mu \)M, \( P = 0.018 \)). In subjects submitted to RIPC, no change in NO was noted at either ischemic or reperfusion periods (62 ± 3 vs. 62 ± 2 \( \mu \)M) (Fig. 7).

**Plasma noradrenaline concentration.** In the control group, plasma noradrenaline concentrations did not change during the protocol (253 ± 17 pg/ml at baseline, 215 ± 17 pg/ml during ischemia, and 216 ± 17 pg/ml during reperfusion). In the RIPC group, there was a small decrease in plasma noradrenaline concentration during the reperfusion period (279 ± 16 pg/ml at baseline, 223 ± 16 pg/ml during ischemia, and 209 ± 16 pg/ml during reperfusion; \( P = 0.016 \) compared with baseline). Overall, the change in noradrenaline seen during the reperfusion period in RIPC subjects (−21%) was not significantly different from that of the controls (−2%, \( P = 0.241 \)).

**DISCUSSION**

To our knowledge, this is the first study to demonstrate that forearm IR in humans is associated with sympathetic nervous activation, and that RIPC attenuates and delays sympathetic activation seen during the ischemic period. RIPC-induced sympathetic attenuation during ischemia was associated with improved RHI, a surrogate marker of endothelial function, and prevention of an indirect marker of oxidant stress formation and prevention of decreased of \( \text{NO}_3^- \) and \( \text{NO}_2^- \), an index of NO availability. Our study highlights that the protection offered by RIPC in healthy humans may involve the attenuation of the sympathetic nervous system activity and associated endothelial mechanisms.

During myocardial (11, 28) or renal (29) ischemia, a significant amount of norepinephrine is released from sympathetic nerve terminals, causing myocardial or renal hemodynamic and histological damage. In a rat model, Tsutsui et al. (33) documented that renal sympathetic nerve activity increased by 109% immediately after the start of renal ischemia, and that this was accompanied by increased renal venous plasma norepinephrine after reperfusion and severe lesions in the medulla, medullary congestion, hemorrhage, and tubular necrosis. In humans, brief (elective coronary angioplasty) or severe prolonged ischemia (acute myocardial infarction) are accompanied by elevated plasma norepinephrine levels (27). Indeed,
norepinephrine release is thought to be critically involved in the progression of ischemic cell damage and the occurrence of ventricular fibrillation in early ischemia (28). In the present study, direct sympathetic nerve recording was performed at a site remote from the ischemic organ. Ischemia rapidly induced sustained sympathetic activation, in the order of 101% after 15 min. This sympathoexcitation subsided during the reperfusion phase. This profile of response was not associated with hemodynamic change, in line with that previously described (15). Moreover, plasma noradrenaline concentrations were unchanged. This suggests that the sympathoexcitation we observed may not be generalized, and/or other vascular beds may not be affected similarly. We must, however, point out that plasma noradrenaline has limited ability to reflect sympathetic drive compared with muscle sympathetic nerve traffic (4). Our model elicits a moderate ischemic injury as sympathetic responses were similar to that expected during a very short challenge combining muscle contraction and ischemia (23).

The RIPC performed in the upper part of the leg did not change MSNA significantly. Nevertheless, the sympathetic activation induced by IR was still present in the late phase of the ischemia, albeit to a lower level, indicating that RIPC was able to reduce the immediate muscle sympathetic activation induced by ischemia, but did not abolish it completely, particularly in the latter phase. Separately, we observed that, during the reperfusion period, plasma noradrenaline concentration slightly decreased in those who underwent RIPC, but the change was not statistically different from that of the control group. Interestingly, in a rat model of renal ischemia (33), it was found that ischemic preconditioning at the site of ischemia almost completely reduced renal sympathetic nerve activation during renal IR injury and attenuated the IR-induced renal dysfunction and histological damage. Taken together, this suggests that sympathoinhibitory effects may partly be involved in the protective mechanisms of ischemic preconditioning.

Changes in endothelial function have been reported in diverse tissues after IR (8). Reestablishment of blood flow induces blood to rapidly diffuse the vasculature, allowing the rapid reintroduction of molecular oxygen triggering the generation of oxygen-derived free radicals and decreased NO bioavailability (19). Given that endothelial cells are susceptible to IR injury, enhanced or preserved endothelial function may play a role in tissue protection during IR. We noted that RHI, an index of endothelial function, was significantly higher following IR in the subjects submitted to RIPC, suggesting improved local endothelial function. This finding is in line with a recent report showing improvement in the endothelial function of patients undergoing ischemic preconditioning before primary percutaneous coronary intervention (22). In the present study, the possibilities to explain the enhanced RHI induced by RIPC may include the protection of the availability of NO (26), the reduced formation of ROS, such as was recently demonstrated in a rat model of mesenteric ischemia (7), or the reduced
activation of the sympathetic nervous system activity. In our model of ischemia, we observed a small but significant increase in production of GSH after 20 min of ischemia in the control subjects, while this rise was not observed in subjects submitted to RIPC. Erythrocyte production of GSH, an antioxidant released to defend against oxidative damage, would be expected to decrease during ischemia. Therefore, the response may reflect an increased production of ROS (12), perhaps as an adaptive response to oxidative challenge. Indeed, GSH has been shown to increase following acute myocardial infarction (12). Our data may indicate that RIPC prevented the production of ROS and hence the release of antioxidant defenses; however, we acknowledge that the changes were very small.

Whether the buffering of the sympathetic activation is due to better availability of NO induced by RIPC is uncertain because, in our IR model, sympathetic activation was transient, being only seen during the ischemic period, while the decrease in concentration of NO/NO2 as used as an index of NO availability seemed to occur only during the reperfusion period and was marginal. Experimental studies have also demonstrated that ROS may increase sympathetic nervous system activity through decreased production/availability of NO and interleukin-1 (2), suggesting that, during ischemia, these processes are most likely closely linked. Conversely, one could postulate that ischemia-induced increased sympathetic nervous system activity could also reduce NO availability and aggravate endothelial function, as a recent study indicated that the sympathetic nervous system regulates the mobilization and recruitment of endothelial progenitor cells in mice during hindlimb ischemia (13).

Limitations of the study include the assessment in young, healthy individuals who are unlikely to experience an ischemic event and the use of a mild level of ischemia compared with what patients may experience during an ischemic event. Hence, we cannot determine whether an older population at high cardiovascular risk may show comparable sympathetic buffering response to RIPC following an ischemic injury. This issue was raised in a previous study showing that older age is associated with an abolished effect of RIPC to protect against endothelial dysfunction after IR in the brachial artery (34). In addition, older age and increased cardiovascular risk are typically associated with altered sympathetic nervous function at rest and during physiological challenges (14). Sympathetic tone was measured in the skeletal muscle in the right leg, and, given that sympathetic tone is highly regionalized (3), this may not reflect the response in other vascular beds. An indirect index of endothelial function was measured, before inducing the sequences of RIPC and IR. This involved the induction of a 5-min ischemia in the forearm, which may, in itself, already induce a protective effect, although the protection mechanism is usually described as two or three cycles of IR (21). The Endopat device uses a reactive hyperemia peripheral arterial tonometry approach, which measures pulse volume changes at the fingertips after an occlusion of the brachial artery. The gold standard method to assess vascular endothelial function is FMD by means of brachial artery ultrasound scanning. The method we used was recently shown to have higher within-day variability compared with FMD (25); however, it has the advantage of being operator independent and shows a significant correlation with FMD (16).

**Conclusion and perspective.** This study indicates that, in a human model of IR, RIPC is able to reduce and delay the degree of sympathetic nervous activation and ameliorate RHI, an index of endothelial function in the ischemic tissue. Prevention of decreased NO bioavailability and increased generation of ROS may be among the mechanisms involved. This observation underlines that modulation of sympathetic activity may play a role in the protective effects of RIPC, at least in the short term. While observational data suggested that RIPC administered to patients undergoing cardiac surgery resulted in beneficial clinical outcomes (6, 30), two recent large-scale, prospective, randomized, sham-controlled trials of RIPC in patients undergoing cardiac surgery and requiring cardiopulmonary bypass were negative (5, 24). This raises the issue that RIPC may offer protection only in certain patient groups. It is possible that the protective effects of RIPC may be masked by factors, such as pharmacological agents, older age, or underlying pathology, that interfere with either neural or humoral mechanisms involved in RIPC processes. The pathways by which RIPC may offer protection and its possible limitations need to be further evaluated in a wider group of patients.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


