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Transient Limb Ischemia Induces Remote Ischemic Preconditioning In Vivo

R.K. Kharbanda, MRCP; U.M. Mortensen, MD; P.A. White, PhD; S.B. Kristiansen, MD; M.R. Schmidt, MD; J.A. Hoschtitzky, MSc, MRCSEd; M. Vogel, MD, PhD; K. Sorensen, MD; A.N. Redington, MD, FRCP; R. MacAllister, MD, FRCP

Background—Ischemic preconditioning reduces local tissue injury caused by subsequent ischemia-reperfusion (IR), but may also have a salutary effect on IR injury of tissues remote from those undergoing preconditioning. We tested the hypothesis that limb ischemia induces remote preconditioning, reduces endothelial IR injury in humans, and reduces experimental myocardial infarct size.

Methods and Results—Endothelial IR injury of the human forearm was induced by 20 minutes of upper limb ischemia (inflation of a blood pressure cuff to 200 mm Hg) followed by reperfusion. Remote preconditioning was induced by three 5-minute cycles of ischemia of the contralateral limb. Venous occlusion plethysmography was used to assess forearm blood flow in response to acetylcholine at baseline and 15 minutes after reperfusion. Experimental myocardial infarction was achieved by 40 minutes of balloon occlusion of the left anterior descending artery in 15-kg pigs. Remote preconditioning was induced by four 5-minute cycles of lower limb ischemia. Triphenyltetrazolium staining was used to assess the extent of myocardial infarction. In the human study, the response to acetylcholine was significantly attenuated in the control group after 15 minutes' reperfusion, but remote preconditioning prevented this reduction. Limb ischemia caused a significant reduction in the extent of myocardial infarction relative to the area at risk compared with control ($26\pm 9\%$ versus $53\pm 8\%$, $P<0.05$).

Conclusion—Remote ischemic preconditioning prevents IR-induced endothelial dysfunction in humans and reduces the extent of myocardial infarction in experimental animals. Transient limb ischemia is a simple preconditioning stimulus with important potential clinical applications. (*Circulation*. 2002;106:2881-2883.)

Key Words: endothelium ■ ischemia ■ reperfusion ■ ischemic preconditioning, remote

Ischemia-reperfusion (IR) complicates myocardial infarction and stroke and contributes to the associated tissue injury and mortality; reducing IR injury may improve the outcome of reperfusion therapy for these conditions.¹ One successful approach in the experimental setting is ischemic preconditioning (IPC), whereby prior sublethal ischemia induces a state of protection against subsequent prolonged IR.² Although animal studies have shown that protection occurs locally in the tissue being preconditioned, systemic effects of localized IPC have been observed.³ This raises the possibility that regional ischemia of accessible nonvital tissues might protect remote vital organs undergoing IR, and some data support this in humans.⁴ In the present study we tested the hypothesis that short periods of limb ischemia induce remote preconditioning and reduce IR injury in vivo. We used a human model of endothelial IR injury to test whether remote limb ischemia induces systemic preconditioning in humans. Furthermore, we studied an experimental

model of myocardial infarction to characterize whether limb ischemia reduced myocardial IR injury.

Methods

Study 1: Remote Preconditioning of Human Endothelium by Contralateral Limb Ischemia

Subjects and Study Design

Fourteen healthy volunteers, with a mean age of 33 (range, 26 to 52) years, gave informed signed consent and were randomized to remote preconditioning and control groups. Studies were approved by the local Research Ethics Committee and performed in a temperature-controlled laboratory (24 to 26°C).

Induction of IR and Remote IPC

The nondominant forearm was made ischemic by inflating a 12-cm-wide blood pressure cuff placed around the upper arm to a pressure of 200 mm Hg for 20 minutes, as previously described.⁵ Remote IPC before the ischemic insult was induced by 3 cycles of ischemia (5 minutes of cuff inflation and deflation) of the contralateral arm. Endothelial function was assessed at baseline and at 15 minutes after IR.

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From University College (R.K.K., J.A.H., M.V., R.M.), London, UK; Aarhus University Hospital (U.M.M., S.B.K., M.R.S., K.S.), Aarhus, Denmark; Papworth Hospital (P.A.W.), Cambridge, UK; and the Hospital for Sick Children (A.N.R.), Toronto, Canada.

Correspondence to R. Kharbanda, Cambridge University, ACCI, Level 6, Box 110, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK. E-mail rk285@cam.ac.uk

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Assessment of Resistance Vessel Endothelial Function

Strain-gauge plethysmography was used to measure forearm blood flow responses to the endothelium-dependent dilator acetylcholine (ACh; 25, 50, and 100 nmol/min; each dose for 3 minutes; Clinalfa), as described previously.⁵

Study 2: Effect of Remote Preconditioning on Myocardial Infarction

Animals and Study Design

Seventeen 15-kg Danish Landrace pigs (Paaskehoejgaardcentret, Denmark) were randomized to either remote preconditioning or sham procedure (control) before myocardial infarction. The remote preconditioning stimulus was tourniquet occlusion of blood flow to one hindlimb, with four cycles of 5 minutes' occlusion followed by 5 minutes' rest, immediately before occlusion of the left anterior descending (LAD) artery. Circulatory arrest in the limb was confirmed by vascular Doppler. The animals were treated according to the principles stated in Danish law on animal experiments.

Induction of Myocardial Infarction

Animals were preanesthetized with midazolam and pentobarbital, intubated and ventilated at 4.5 L/min with a 50/50 mixture of atmospheric air and oxygen. Anesthesia was maintained with an infusion of pentobarbital. Alterations to the ventilation or electrolytes to maintain physiological levels of oxygenation, electrolytes, and ventilation were guided by hourly blood gas measurements. Temperature was kept between 36.5°C and 38.0°C with the use of a heating blanket. Heparin was administered to maintain anticoagulation.

A standard 6F angioplasty guide catheter was used to introduce a guidewire into the LAD. A 2-mm angioplasty balloon was positioned immediately distal to the first diagonal and inflated to achieve vessel occlusion for 40 minutes. Angiography was used to confirm LAD occlusion, distal to the first diagonal. After 40 minutes, the balloon was deflated and reflow confirmed by repeat angiography. Reperfusion was for 120 minutes, after which median sternotomy was performed. A suture was applied immediately distal to the first diagonal, and the heart was perfusion-stained with intra-atrial injection of sodium fluorescein before euthanization and excision of the heart. During ischemia and reperfusion, animals were paced atrially at 10 bpm greater than the resting heart rate. Blood pressure was maintained at above 80 mm Hg with inotropic support with adrenaline as needed (2 animals in the control group and 1 animal in the preconditioning group). Ventricular fibrillation was treated with DC cardioversion.

Assessment of Ventricular Function

Real-time left ventricular (LV) pressure–volume loops were generated by the use of 8 polar conductance catheters with integrated micromanometer (5F, Millar Houston) as previously described.⁶ Preload was varied by transient balloon occlusion of the inferior vena cava. Volume and pressure data were analyzed offline with custom-designed software. Systolic and diastolic load independent indices of left ventricular performance were measured at baseline, at 10, 20, and 35 minutes during ischemia and at 15, 45, 60, 90, and 120 minutes after reperfusion.

Assessment of Myocardial Infarction

The LV was cut into 5-mm slices, perpendicular to the septum from the apex to the base. All slices were weighed, and the area at risk was marked under a Wood's lamp. Viable myocardium was stained by incubating the slices in 1% 2,3,5-triphenyltetrazolium chloride (Sigma) (pH 7.4) at 37°C for 15 minutes. All slices were photographed before and after staining, and area at risk, area not at risk, and infarct size were assessed by computer planimetry. The ratio of mass of the area at risk to the LV mass and the ratio of infarct size to the area at risk were calculated. An investigator blinded to the treatment groups performed all analyses.

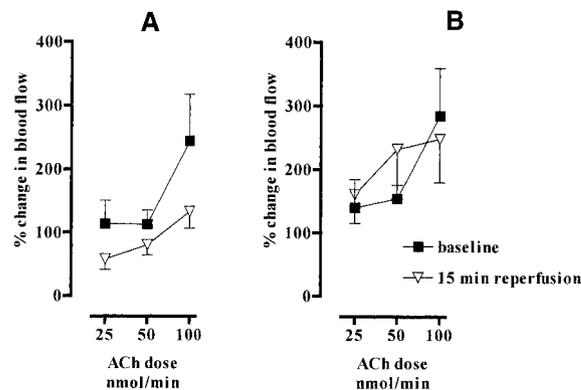


Figure 1. A, IR injury causes endothelial dysfunction at 15 minutes of reperfusion ($n=7$, $P<0.05$, repeated measures ANOVA). B, Remote preconditioning prevents endothelial dysfunction after 15 minutes' reperfusion ($n=7$, $P<0.05$, repeated measures ANOVA).

Calculations and Statistics

Forearm blood flow was measured in mL/100 mL forearm volume per minute and the mean ratio of flow in the infused/noninfused (control) arm was calculated for the 2-minute period before drug infusion and used as baseline flow. Vasodilator responses were expressed as the percentage increase in the ratio of forearm blood flow (infused/noninfused arm) relative to this baseline. Dose-response curves were constructed at each time point and comparisons made by 2-way analysis of variance (ANOVA). Indices of LV performance were expressed as a ratio relative to baseline for each subject. The time course during ischemia and reperfusion was compared between groups by 2-way ANOVA. The extent of myocardial infarction was corrected for area at risk and expressed as a percentage. All data are expressed as mean (SEM) unless otherwise stated. Parametric data were compared using Student's *t* test. In all cases, $P<0.05$ was considered statistically significant.

Results

Study 1: Remote Preconditioning of Human Endothelium by Contralateral Limb Ischemia

The IR protocol had no effect on blood pressure, heart rate, or basal blood flow at 15 minutes' reperfusion. ACh caused a dose-dependent increase in forearm blood flow before IR. Compared with baseline, the response to ACh was significantly blunted at 15 minutes after reperfusion in the control group ($n=7$; $P=0.03$, Figure 1). In the remote preconditioning group there was no reduction in the response to ACh ($P=0.66$, Figure 1).

Study 2: Effect of Remote Preconditioning on Myocardial Infarction

Assessment of Ventricular Function

Indices of ventricular performance were compared during the ischemia and reperfusion phase. There was a reduction in ejection fraction (EF) during ischemia, which was maintained during reperfusion in the control group. The reduction in EF was significantly greater in the control group than the remote preconditioning group ($P=0.02$, Figure 2A). There was no significant difference in systolic load-independent indices. However, the time constant of ventricular relaxation (τ) increased during ischemia, but was significantly less in the remote preconditioning group ($P=0.02$, Figure 2B). At reper-

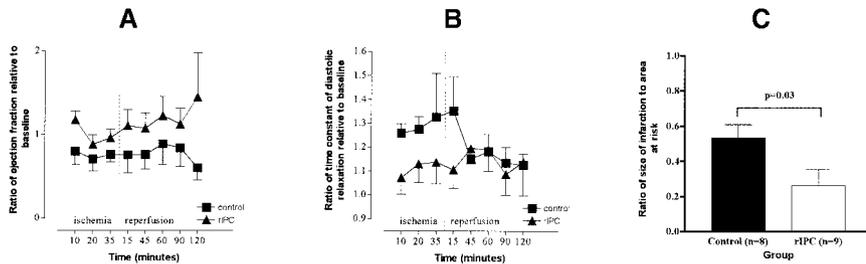


Figure 2. Remote preconditioning, induced by limb ischemia, causes a significant difference between intervention and control groups in (A) ratio of ejection fraction during ischemia and reperfusion relative to baseline, (B) ratio of the time constant of diastolic relaxation during ischemia relative to baseline, and (C) ratio of the area of myocardial infarction relative to the area at risk.

fusion, this difference was abolished, but τ remained elevated.

Assessment of Size of Myocardial Infarction

There were no significant differences in left ventricular mass, mass of area at risk, or their ratio between the groups. In control subjects, transmural myocardial infarction occurred in $53 \pm 8\%$ of the area at risk ($n=8$). However, in the remote preconditioning group there was a significant reduction in myocardial infarction, with $26 \pm 9\%$ of the area at risk showing infarction ($n=9$, $P<0.05$, unpaired t test, Figure 2C). Calculated mass of myocardial infarction was significantly lower in the remote preconditioning group compared with the control group (3.5 g versus 7.5 g, $P<0.05$).

Discussion

The present study shows that a brief and clinically practical period of limb ischemia induced remote preconditioning of human arterial vessels *in vivo*. Moreover in an animal model, hindlimb ischemia induced remote IPC, preserving ventricular function during prolonged ischemia and substantially reducing myocardial infarct size. These results suggest that this simple technique might have potential to reduce tissue damage in vital organs at risk of clinical IR injury.

We have previously shown that IPC by the ipsilateral arm prevents endothelial dysfunction in the human forearm after IR. In the present study, ischemia of the contralateral arm prevented IR-induced endothelial dysfunction. This implies that in humans, IPC has systemic effects to protect distant organs undergoing IR injury. To determine if remote preconditioning might protect the myocardium during IR injury, we used an *in vivo* experimental model of myocardial infarction. Remote preconditioning by the hindlimb reduced the magnitude of ventricular dysfunction during ischemia and subsequent infarct size after reperfusion.

Previous studies in animals have indicated that remote preconditioning seems to involve release of adenosine, bradykinin, or norepinephrine and activation of K_{ATP} channels and bears mechanistic resemblance to local preconditioning.⁷ In addition to humoral factors, remote preconditioning may also involve the autonomic nervous system or modulate the functions of circulating cells such as platelets.^{8,9} There is increasing evidence that endothelial dysfunction and reduced NO bioavailability are important components of IR injury, possibly limiting the extent of reperfusion after ischemia and

amplifying IR injury. Remote IPC might therefore reduce tissue injury by limiting endothelial damage during IR as was seen in the present study. In addition, we observed that ventricular function during ischemia was improved in the preconditioned animals, suggesting a direct effect on the myocardium, possibly modifying the metabolic response to ischemia. Whether endothelial preconditioning contributes directly to the reduction in myocardial IR injury remains to be determined, but the results of the present study indicate that remote preconditioning prevents tissue injury in animal and human models.

Our experimental model suggests that remote preconditioning may produce clinical benefits. Unlike previous animal models, the stimulus we used may easily be transferred into the clinical arena. These data provide support for further clinical studies using limb ischemia to induce a preconditioned state in the heart and other organs.

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