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Ischemic Preconditioning Prevents Endothelial Injury and Systemic Neutrophil Activation During Ischemia-Reperfusion in Humans In Vivo

Rajesh K. Kharbanda, BSc, MRCP; Mark Peters, PhD, MRCP; Benjamin Walton, MRCP; Mia Kattenhorn, BSc; Michael Mullen, MRCP; Nigel Klein, FRCP; Patrick Vallance, MD, FRCP, FMedSci; John Deanfield, FRCP; Raymond MacAllister, MD, MRCP

Background—Endothelial dysfunction leading to neutrophil infiltration of tissues has been implicated in tissue injury caused by ischemia-reperfusion (IR). Tissue injury during IR can be reduced by prior ischemic preconditioning (IPC). In humans, it is unclear whether endothelial dysfunction occurs during IR or whether IPC offers protection against endothelial dysfunction and inflammatory cell activation. We studied the effects of experimental IR on endothelial and neutrophil function in the human forearm in vivo and examined the protection afforded by IPC.

Method and Results—The forearm was made ischemic for 20 minutes by inflating a blood pressure cuff to 200 mm Hg. We assessed endothelial function of conduit (radial artery flow-mediated dilation) and resistance vessels (blood flow responses to intra-arterial infusion of the endothelium-dependent dilator acetylcholine) in healthy volunteers before and after IR. IR reduced flow-mediated dilation of the radial artery at 15 minutes of reperfusion ($7.7 \pm 1.5\%$ to $3.5 \pm 0.9\%$) and the dilator response of resistance vessels to acetylcholine at 15, 30, and 60 minutes of reperfusion. IR did not reduce the dilator response of the radial artery to glyceryltrinitrate and only caused a small reduction of glyceryltrinitrate-induced dilation of resistance vessels at 60 minutes of reperfusion. IR caused an increase in neutrophil CD11b expression and platelet-neutrophil complexes in the circulating blood. IPC (three 5-minute episodes of ischemia) before IR prevented endothelial dysfunction and neutrophil activation.

Conclusions—A clinically relevant period of ischemia-reperfusion causes profound and sustained endothelial dysfunction and systemic neutrophil activation. IPC attenuates both of these effects in humans. (*Circulation*. 2001;103:1624-1630.)

Key Words: ischemia ■ endothelium ■ reperfusion ■ leukocytes

Reestablishing blood flow to ischemic organs is vital to prevent tissue death after arterial obstruction. However, reperfusion itself causes local injury secondary to an acute inflammatory response that involves tissue infiltration by activated neutrophils and platelets.¹ The endothelium has dilator, antiplatelet, and antineutrophil properties through the release of local mediators, including nitric oxide (NO) and prostacyclin. Endothelial dysfunction is a ubiquitous finding after ischemia-reperfusion (IR) of diverse tissues in a variety of species.² Reduced bioavailability of endothelial mediators might prolong vasoconstriction after reperfusion injury and amplify the expression of adhesion molecules and production of inflammatory cytokines. This could promote the recruitment of inflammatory cells in tissues, with local release of inflammatory mediators causing further endothelial dysfunction and tissue damage.^{3,4}

Animal studies indicate that IR injury is reduced by preceding brief periods of ischemia, so-called ischemic pre-

conditioning (IPC).⁵⁻⁷ IPC has direct effects on tissues, making them resistant to ischemic damage but also preventing endothelial dysfunction and inflammatory cell activation associated with IR. However, despite convincing data from studies in animals, in humans it remains unclear if endothelial dysfunction occurs during IR injury and whether IPC offers protection against endothelial dysfunction and inflammatory cell activation. The aim of this study was to determine the effects of experimental IR on endothelial and circulating blood cell function in humans in vivo and to examine the protection afforded by IPC.

Methods

Thirty-one healthy volunteers (17 men, 14 women; mean age, 33 years; range, 26 to 52) who gave informed, signed consent were recruited. Studies were approved by the local research ethics committee and performed in a temperature-controlled laboratory (24° to 26°C).

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From the Centre for Clinical Pharmacology and Therapeutics, University College London (R.K.K., P.V., R.M.), and the Vascular Physiology Unit (B.W., M.K., M.M., J.D.), Portex Department of Anaesthesia, Intensive Therapy and Respiratory Medicine (M.P.), and the Immunobiology Unit (M.P., N.K.), Institute of Child Health, University College London, London, UK.

Correspondence to Dr R. Kharbanda, Centre for Clinical Pharmacology and Therapeutics, 5 University St, London, UK WC1E 6 JJ. E-mail r.kharbanda@ucl.ac.uk

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IR and IPC of the Forearm Vascular Bed

The effects of IR on endothelial function of the radial artery and forearm resistance vessels were investigated. The nondominant forearm was made ischemic by inflating a 12-cm-wide cuff placed around the upper arm to a pressure of 200 mm Hg for 20 minutes. IPC was induced by three 5-minute periods of upper cuff inflation to 200 mm Hg separated by 5 minutes before IR.

Assessment of Resistance Vessel Endothelial Function

Mercury-in-silastic strain-gauge plethysmography was used to measure forearm blood flow in both arms, as described previously.⁸ Drugs were administered in saline (0.9% [wt/vol] sodium chloride) and infused at 0.5 mL/min through a 27-gauge needle inserted into the nondominant brachial artery (Cooper's Needle Works). During recording periods, the hands were excluded from the circulation by inflation of wrist cuffs to 200 mm Hg. Forearm blood flow responses were measured in response to infusion of the endothelium-dependent dilator acetylcholine (ACh; 25, 50, and 100 nmol/min; each dose for 3 minutes, Clinalfa) or the endothelium-independent dilator glyceryl-trinitrate (GTN; 4, 8, and 16 nmol/min; each dose for 3 minutes, DBL Laboratories).

Assessment of Conduit Vessel Endothelial Function

The effect of IR on flow-mediated dilation (FMD) of the radial artery was assessed. The use of this vessel avoided movement artifacts of

the brachial artery caused by inflation of an upper arm-occluding cuff. Vessel diameter in the nondominant arm was measured with high-resolution vascular ultrasound (Acuson Aspen, 7.0-MHz linear array transducer). Longitudinal, ECG-gated, end-diastolic images were acquired every 3 seconds with customized software, and arterial diameter over a 1- to 2-cm segment was determined for each image with the use of an automatic edge-detection algorithm (Information Integrity).⁹ Pulsed-wave Doppler was used to measure blood flow velocity expressed as the velocity time integral for a single cardiac cycle. The velocity time integral was multiplied by heart rate (bpm) and vessel cross-sectional area (cm²) to derive radial artery blood flow (mL/min). Radial artery diameter and blood flow were measured for 1 minute (baseline), during 5 minutes of reduced blood flow (induced by inflation to 300 mm Hg of a pneumatic cuff placed at the wrist, distal to the segment of artery being analyzed), and for 5 minutes during reactive hyperemia after release of the wrist cuff. The dilator response of the radial artery to administration of sublingual GTN (25 μ g) was used to assess endothelium-independent dilation.

Assessment of Neutrophil Activation and Platelet-Neutrophil Complexes

Neutrophil adhesion molecule expression and platelet-neutrophil complexes (PNC) were investigated as previously described.¹⁰ Venous blood was drawn from the antecubital veins, and 50 μ L (heparinized; 10 U/mL) was added to saturating concentrations of monoclonal antibodies. After 10 minutes at room temperature, 200 μ L of FACSlyse (Becton Dickinson) was added, and samples were

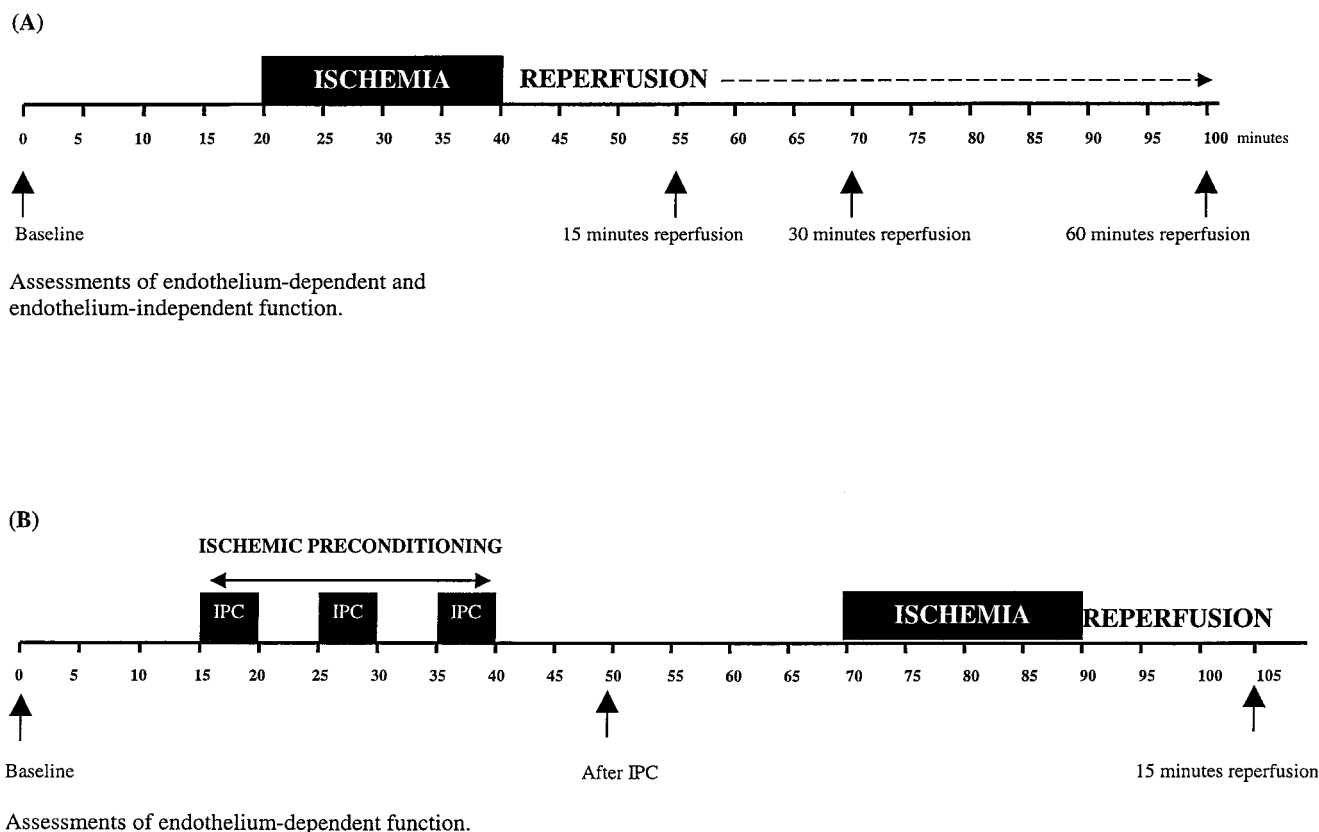


Figure 1. A, Protocol 1: Effect of IR on vessel function. IR in resistance vessels: Cumulative dose-response curves to ACh or GTN were constructed at baseline. After 10-minute recovery period, upper arm cuff was inflated for 20 minutes (ischemia) followed by reperfusion. After 15, 30, or 60 minutes of reperfusion, response to ACh or GTN was determined. IR in conduit vessels: FMD of radial artery was measured at baseline. After 10-minute recovery, upper arm cuff was inflated for 20 minutes (ischemia) followed by reperfusion. FMD was measured at 15 and 60 minutes of reperfusion. Endothelium-independent dilation was assessed by measuring dilation to GTN (25 μ g sublingual). B, Protocol 2: Effect of IPC on response of forearm vessels to IR. Endothelial function was assessed at baseline. This was followed by IPC (three 5-minute episodes of upper arm cuff inflation, each separated by 5 minutes) and further assessment of endothelial function. After 15-minute period of reperfusion, endothelial function was reassessed.

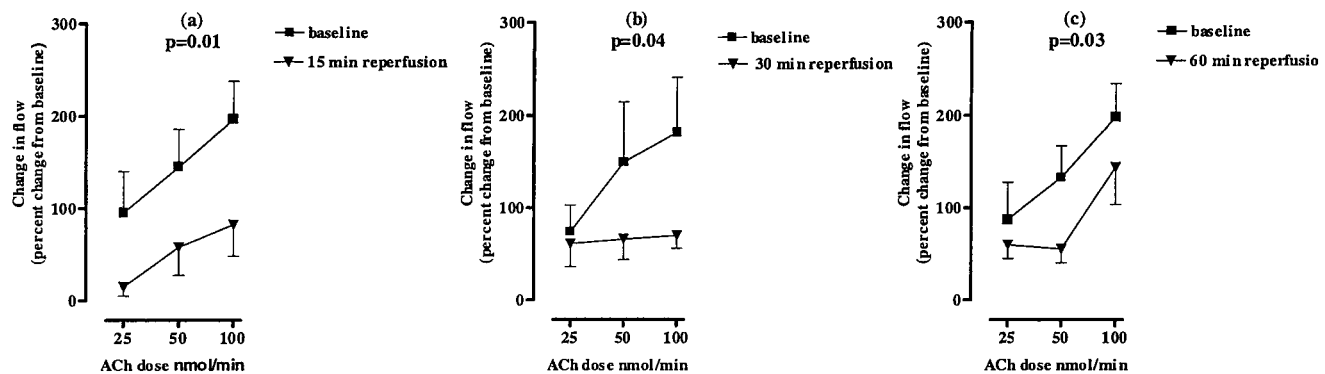


Figure 2. Effect of IR on resistance vessel endothelial function. Forearm blood flow responses to incremental doses of ACh at 15 (a), 30 (b), and 60 (c) minutes of reperfusion compared with baseline responses (probability value by repeated-measures ANOVA).

incubated for a further 10 minutes before the addition of 250 μ L of 0.2% formaldehyde in PBS. Samples were analyzed by flow cytometry within 1 hour of collection on a Becton Dickinson FASCalibur, with FITC fluorescence at 515 nm and PE fluorescence at 580 nm measured. Neutrophils were distinguished from monocytes and lymphocytes by their typical physical characteristics, resulting in a distinct population that is readily identifiable on forward and side-scatter plot. We have previously confirmed that this plot population has <2% contamination with other cell types.¹⁰ A minimum of 5000 neutrophil events was counted on each sample.

Neutrophil activation was assessed by the level of expression of CD11b (α -chain of the integrin adhesion molecule CD11b/CD18, Mac-1) and measured by fluorescence intensity of FITC-conjugated IgG1 monoclonal antibody directed against CD11b (Serotec), expressed as the median fluorescence intensity (MFI) of the total neutrophil population. PNC were determined as the percentage of the total neutrophil population staining positive for CD42b (a component of the platelet von Willebrand factor receptor). Events staining positive for both neutrophil and platelet antigens (ie, CD11b and CD42b) were considered to represent PNC. Expression of CD42b was determined by staining with IgG2a R-phycoerythrin (PE)-conjugated CD42b antibody (Dako). Results were compared with isotype-matched antibody staining and considered positive if the fluorescence intensity exceeded that of 98% of the isotype-matched control antibodies (Becton Dickinson).

Experimental Protocols

Protocol 1: Effect of IR on Resistance and Conduit Vessel Function

Measurement of basal blood flow was made after a 25-minute rest period (Figure 1). Resistance vessel endothelial or smooth muscle function was assessed before ischemia and at 15, 30, or 60 minutes after reperfusion (in a single experiment, a maximum of 2 time points were studied). FMD of the radial artery or dilation of the radial artery in response to sublingual GTN was assessed in separate studies before ischemia and at 15 and 60 minutes after reperfusion.

Protocol 2: Effect of IPC on Response of Forearm Vessels to IR

Endothelial function in resistance and conduit vessels was assessed before and 10 minutes after IPC. Ten minutes later, the IR protocol was started, and endothelial function was assessed 15 minutes after reperfusion.

Protocol 3: Effects of IR and IPC on Neutrophil Activation

Expression of neutrophil activation markers and PNC in circulating blood were measured before ischemia and 15 and 30 minutes after reperfusion. Venous blood sampled from the ischemic arm reflected local effects of IR, and the control arm reflected systemic effects. During the IPC studies, neutrophil activation was measured at

baseline, after the preconditioning stimulus, and at 15 minutes after reperfusion following 20 minutes of ischemia.

Calculations and Statistics

Forearm blood flow was measured in milliliters per 100 milliliters 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. Radial artery diameter was measured in millimeters and dilation expressed as both absolute dilation from baseline (mm) and percentage increase from baseline. Absolute peak dilation, percentage peak dilation, and the area under the curve (AUC) of percentage dilation during the 5 minutes after wrist cuff release were used for analysis. Radial artery blood flow (expressed as mL/min) was measured at baseline and every 15 seconds for 2 minutes after cuff release. The AUC for the blood flow/time curve was used to quantify this stimulus.

All data are expressed as mean (SEM) unless otherwise stated. For resistance vessel studies, dose-response curves were constructed for drugs at each time point; comparisons with the dose-response curve before ischemia was made by 2-way ANOVA. For conduit vessel responses, maximum absolute FMD and GTN dilation, maximum percentage dilation, AUC for the percentage dilation over 5 minutes after cuff release, and AUC for blood flow/time profiles were compared by paired Student's *t* test. The effect of IPC on FMD at 15 minutes after IR was compared with the FMD response after IR alone by 1-way ANOVA with a post hoc Bonferroni test. Neutrophil activation and PNC data were compared by means of the Student's *t* test. In all cases, a value of $P < 0.05$ was considered statistically significant.

Results

All subjects tolerated the procedure without complications. There were no differences noted in the responses of men and women. The IR protocol had no effect on blood pressure, heart rate, or basal blood flow at 15 minutes of reperfusion (data not shown).

Effect of IR on Resistance and Conduit Vessel Function

ACh and GTN caused dose-dependent increases in forearm blood flow before IR. Compared with control, the response to ACh was significantly blunted at 15 minutes ($n=10$; $P=0.009$; Figure 2a), 30 minutes ($n=10$; $P=0.04$; Figure 2b), and 60 minutes ($n=12$; $P=0.03$; Figure 2c) after reperfusion. The response to GTN was unaffected by IR at 15

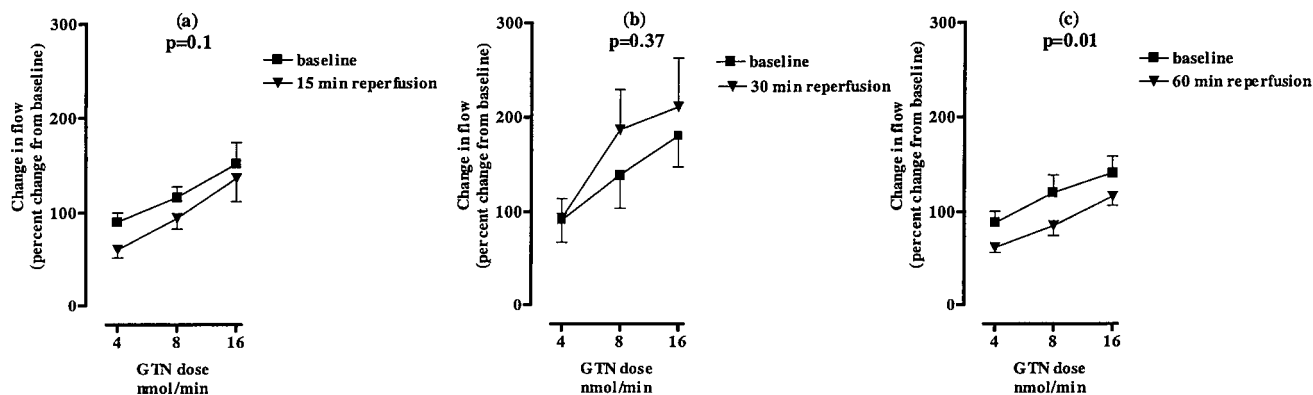


Figure 3. Effect of IR on resistance vessel smooth muscle function. Forearm blood flow responses to incremental doses of GTN at 15 (a), 30 (b), and 60 minutes (c) of reperfusion compared with baseline (probability value by repeated-measures ANOVA).

(n=10) and 30 (n=6) minutes, but a small reduction was observed at 60 (n=10) minutes after reperfusion (n=12; $P=0.02$; Figure 3, a through c).

Baseline radial artery diameter and blood flow did not change during the conduit vessel study. The reactive hyperemia flow stimulus after cuff release was unchanged after ischemia. However, peak FMD (% dilation; absolute dilation in mm) was reduced after 15 minutes of reperfusion ($7.7 \pm 1.5\%$, 0.181 ± 0.04 mm before and $3.5 \pm 0.9\%$, 0.083 ± 0.02 mm after reperfusion; n=10; $P=0.005$ for both) but had returned to baseline values by 60 minutes ($7.7 \pm 2.0\%$, 0.145 ± 0.03 mm; $P=0.98$ and $P=0.13$, respectively). Peak GTN dilation was unchanged ($10.4 \pm 1.6\%$, 0.27 ± 0.02 mm before and $13.0 \pm 1.9\%$, 0.35 ± 0.03 mm at 15 minutes and $10.8 \pm 0.9\%$, 0.3 ± 0.03 mm at 60 minutes; n=7; $P=0.1$ and $P=0.76$ for percentage dilation; $P=0.07$ and $P=0.46$ for absolute change relative to baseline; Figure 4). The AUC of the percent dilation/time curves after wrist cuff release was $953 \pm 251\%$ before and $334 \pm 117\%$ after 15 minutes of reperfusion (n=10; $P=0.04$; Figure 5).

Effect of IPC on Response of Forearm Vessels to IR

Preconditioning did not alter baseline blood flow or diameter (data not shown) and had no effect on the dilation of the resistance vasculature to ACh (n=7; $P=0.7$; Figure 6) or FMD of the radial artery (n=10; $8.5 \pm 1.5\%$, 0.21 ± 0.03 mm

before and $7.0 \pm 1.6\%$, 0.17 ± 0.03 mm after IPC; $P=0.1$ for both). In the preconditioned arm, IR did not attenuate the response to ACh (n=7; $P=0.7$; Figure 6) or FMD of the radial artery at 15 minutes of reperfusion (n=10; $7.8 \pm 0.8\%$, 0.2 ± 0.01 mm at 15 minutes of reperfusion; $P=0.5$) relative to baseline. FMD responses after IR alone were significantly different from baseline and from IR preceded by IPC (1-way ANOVA; $P<0.05$; Figure 6).

Effects of IR and IPC on Neutrophil Activation

Effects of IR and IPC on Neutrophil Activation in Nonischemic (Control) Arm

At baseline, the MFI for CD11b expression was 25 ± 3 U in the control arm (n=6). Fifteen minutes after reperfusion, there was a significant increase in neutrophil CD11b expression (44 ± 5 U; $P=0.02$; Figure 7a), but by 30 minutes of reperfusion this was not significantly raised above baseline values (37 ± 7 ; $P=0.2$). PNC showed a similar pattern; at baseline, PNC were $21 \pm 2\%$ in the control arm (n=8). At 15 minutes after reperfusion, there was a significant increase in the PNC in venous blood from the control arm ($34 \pm 4\%$; $P=0.02$; Figure 7b), and this had returned to baseline values by 30 minutes ($22 \pm 3\%$; $P=0.5$).

The IPC stimulus itself did not change CD11b expression in venous blood from the control arm (25 ± 1 before and 25 ± 2 after IPC; n=8; $P=0.8$). Fifteen minutes after reper-

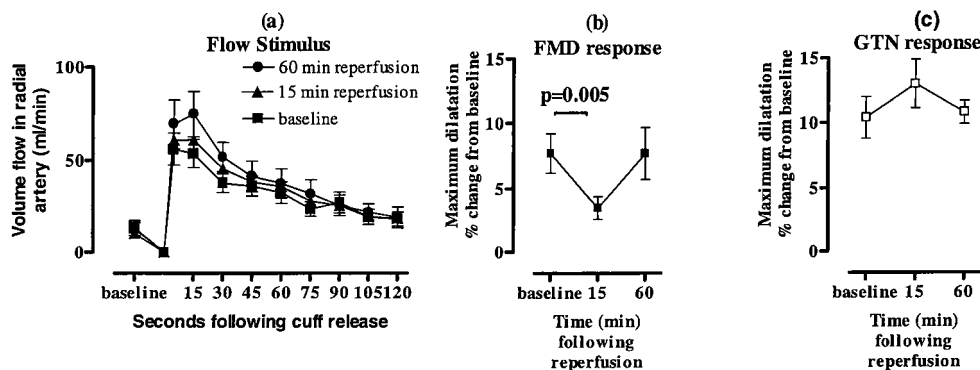


Figure 4. Effect of IR on conduit vessel flow stimulus (radial artery blood flow) after wrist cuff release (a), maximum percentage radial artery FMD (b), and maximum percentage radial artery dilation to GTN (c) at baseline and 15 and 60 minutes after reperfusion (mean \pm SEM).

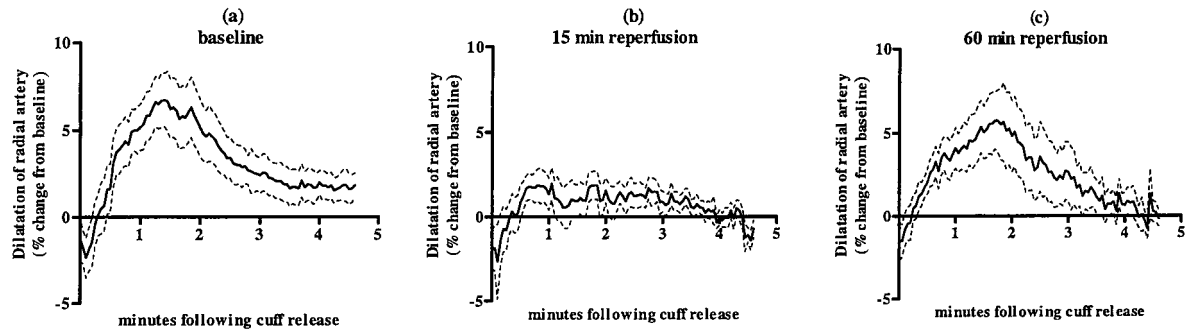


Figure 5. Profile of radial artery percentage dilation for 5 minutes during reactive hyperemia of hand at baseline (a) and 15 minutes (b) and 60 minutes (c) after reperfusion (mean in bold line and SEM in dashed line).

fusion, when there had been prior IPC, CD11b expression in blood from the control arm was similar to baseline values (28 ± 1 ; $P=0.15$) and significantly smaller than that observed without IPC (28 ± 1 versus 44 ± 5 , $P=0.01$; Figure 8a). However, the effects of IPC on PNC generation were less prominent: baseline PNC were $26 \pm 3\%$ in the control arm, and IPC had no effect on PNC in venous blood from the control arm ($24 \pm 2\%$, $P=0.65$). At 15 minutes after reperfusion, there was an increase in PNC in blood from the control arm that failed to reach statistical significance ($31 \pm 3\%$; $P=0.06$), but the level of PNC generated was similar to that observed without ischemic preconditioning ($31 \pm 3\%$ versus $34 \pm 5\%$, $P=0.6$; Figure 8b).

Effects of IR and IPC on Neutrophil Activation in Ischemic (Study) Arm

At baseline, the MFI for CD11b expression was 26 ± 2 U in neutrophils from venous effluent in the ischemic arm ($n=6$). At 15 and 30 minutes after reperfusion, there was no significant change in CD11b expression in neutrophils from the ischemic arm ($34 \pm 6\%$ and $24 \pm 2\%$; $P=0.29$ and $P=0.7$; Figure 7a). The pattern for PNC was similar; at baseline, the PNC were $24 \pm 2\%$ in the ischemic arm and at 15 and 30 minutes of reperfusion, there was no change in PNC compared with baseline ($29 \pm 3\%$ and $23 \pm 3\%$; $P=0.17$ and $P=0.7$; Figure 7b). There were no changes in CD11b or PNC levels in venous blood from the ischemic arm during the IPC protocol.

Discussion

We have shown that a brief and clinically relevant period of ischemia followed by reperfusion causes a profound reduction in endothelium-dependent dilation of human conduit and resistance vessels in vivo. In addition, there is an increase in the number of activated neutrophils and PNC in the systemic circulation after IR injury but no increase in the numbers of activated neutrophils or PNC in the venous blood draining the reperfused arm. Our findings indicate that IPC of the endothelium occurs in humans in vivo because brief periods of forearm ischemia that preceded IR prevented both endothelial dysfunction and activation of neutrophils in the circulating blood.

Endothelial dysfunction has previously been characterized in animal models of IR. In our clinical study, both resistance and conduit vessel NO-dependent endothelial function was impaired, whereas smooth muscle responses were largely unchanged. Dilation to agonist (ACh) and physical (blood flow) stimuli was reduced, indicating that the endothelial defect after IR is not specific for the muscarinic receptors, and demonstrates that the mechanisms of flow-mediated dilation in the blood vessel wall are also impaired by IR. In the later stages of reperfusion there was a small reduction in the dilator response to GTN in resistance but not conduit vessels. Although this might reflect the relatively small sample size, similar findings have been reported in one animal model,¹¹ suggesting that IR injury of small arteries might not be restricted to the endothelium.

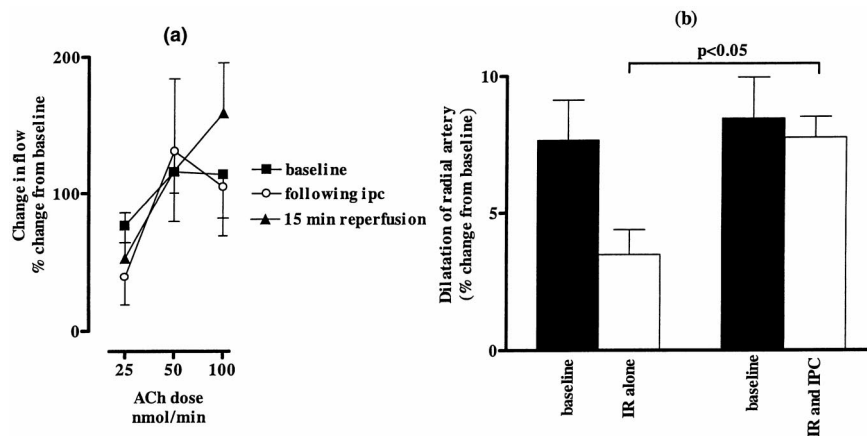


Figure 6. Effect of IPC on response of forearm vessels to IR. a, Forearm blood flow in response to ACh at baseline, after IPC, and 15 minutes after reperfusion. Neither IPC nor IR after IPC altered response to ACh. b, Peak radial artery FMD at baseline, 15 minutes after IR alone (data taken from Figure 4), and 15 minutes after IR preceded by IPC. FMD after IR alone was significantly smaller compared with IR preceded by IPC (by 1-way ANOVA and post hoc Bonferroni test).

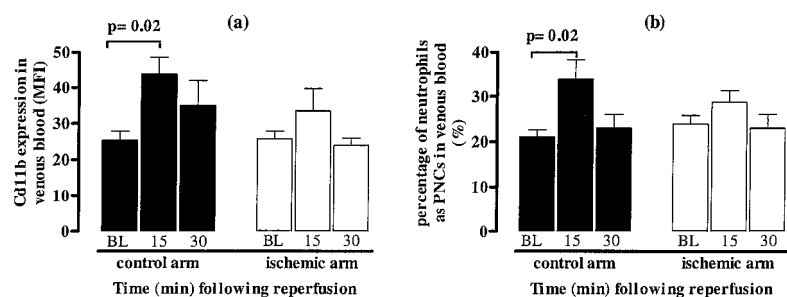


Figure 7. Effect of IR on neutrophil CD11b expression (a) and PNC (b) in venous blood from control and ischemic arms at baseline (BL) and 15 and 30 minutes after reperfusion.

The mechanisms of endothelial dysfunction and reduced NO bioavailability in IR injury are unclear. Data from studies in animals suggest that there might be reduced substrate or cofactor for NO synthesis.^{12,13} Alternatively, increased free radicals (including superoxide) generated by neutrophils might inactivate NO. Irrespective of the mechanism, reduced bioavailability of NO might augment the local inflammatory response through loss of its anti-inflammatory properties.¹⁴ Endothelial dysfunction might itself predispose to vasoconstriction, platelet adherence, and aggregation, leading to microvascular obstruction and thrombosis in larger vessels. This would limit the extent of reperfusion after ischemia and contribute to the “no-reflow” phenomenon, best characterized in humans after acute coronary revascularization by angioplasty.

Activation of neutrophils and platelets has been implicated in the endothelial dysfunction and tissue damage associated with IR, as evidenced by the protective effects of neutrophil depletion or specific blockade of neutrophil or platelet adhesion molecules in animal models of IR.^{15–17} In addition, release of proinflammatory cytokines (IL-6, IL-8, IL-10) from tissues injured by IR causes systemic activation of neutrophils^{18–20} and might predispose to distant organ dysfunction after IR. In the present study, there was evidence for activation of peripheral neutrophils (assessed by surface CD11b expression) after reperfusion, consistent with a systemic response. Moreover, expression of activation markers was associated with increased neutrophil adhesiveness, shown by the increase in PNC. Despite systemic activation of

circulating cells (in the control arm), there was no increase in the number of activated neutrophils or PNC in venous effluent from the ischemic arm. These findings are most likely explained by sequestration of activated cells after reperfusion, consistent with adherence of inflammatory cells and aggregates of inflammatory cells to the vascular endothelium, a precursor to tissue infiltration. The data also suggest that the systemic changes were not mediated simply by an excess of neutrophils draining the affected arm.

The involvement of the endothelium in the pathogenesis of IR injury has led to the investigation of strategies to prevent endothelial dysfunction. In this study, the preconditioning stimulus itself did not directly alter endothelial function but prevented endothelial dysfunction in both conduit and resistance vessels in response to IR. Furthermore, IPC reduced expression of CD11b on neutrophils in the systemic vasculature after IR. We were unable to demonstrate such a marked effect of IPC on PNC generation. Although we could show an attenuation of the increase in PNC formed, these were not significantly different from levels that were generated without prior IPC. Whether this reflects differential sensitivity of platelets or neutrophil/platelet adhesive mechanisms to IPC remains to be determined.

These data support the hypothesis that strategies to preserve endothelial function may protect local tissue function and limit reperfusion injury, perhaps by modulating inflammatory cell recruitment. Clinical observational data support a role for IPC as a determinant of the outcome of arterial occlusion in humans. Reduced cardiac infarct size and improved reperfusion after coronary thrombolysis are linked with episodes of unstable angina before myocardial infarction.^{21,22} The ability to reproduce this phenomenon in an experimental model in humans opens the possibility of identifying underlying mechanisms and testing pharmacological approaches to preventing IR injury. This would enable the powerful protective mechanism of endothelial preconditioning to be harnessed and potentially improve the management of conditions in which arterial flow is restored after ischemia.

Acknowledgments

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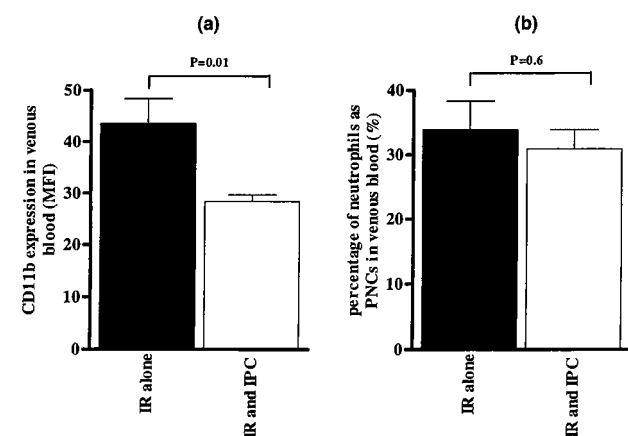


Figure 8. Effect of IPC on neutrophil activation (a) and PNC (b) after IR. CD11b expression 15 minutes after reperfusion was significantly reduced by prior IPC. PNC were not significantly reduced by IPC.

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