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Remote Ischemic Preconditioning Provides Early and Late Protection Against Endothelial Ischemia-Reperfusion Injury in Humans

Role of the Autonomic Nervous System

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OBJECTIVES The aim of this study was to characterize the time course and neuronal mechanism of remote ischemic preconditioning (RIPC) of the vasculature in humans.

BACKGROUND Non-lethal ischemia of internal organs induces local (ischemic preconditioning) and systemic (RIPC) resistance to lethal ischemia-reperfusion (IR) injury. Experimental RIPC has two temporal components, is neuronally mediated, is induced by limb ischemia, and reduces infarct size. In humans, RIPC prevents IR-induced vascular injury. Determining the time course and mechanism is a prelude to clinical outcome studies of RIPC.

METHODS Endothelial IR injury was induced by arm ischemia (20 min) and reperfusion, and measured by flow-mediated dilation. To establish if there are early and late phases, RIPC (three 5-min cycles of ischemia of the contralateral arm) was applied immediately, 4, 24, and 48 h before IR. To determine neuronal involvement, trimetaphan (autonomic ganglion blocker; 1 to 6 mg/min intravenous) was infused during the application of the RIPC stimulus.

RESULTS Flow-mediated dilation was reduced by IR (8.7 ± 1.1% before IR, 4.9 ± 1.2% after IR; $p < 0.001$), but not when preceded by RIPC (8.0 ± 0.8% after IR; p = NS); RIPC did not protect after 4 h (4.9 ± 1.1% after IR; $p < 0.001$), but protected at 24 (8.7 ± 1.1% after IR; p = NS) and 48 h (8.8 ± 1.4% after IR; p = NS). Trimetaphan attenuated early (8.3 ± 1.1% before IR, 4.2 ± 0.9% after IR; $p < 0.05$) and delayed (7.3 ± 1.0% before IR, 2.3 ± 0.6% after IR, $p < 0.001$) RIPC.

CONCLUSIONS Remote ischemic preconditioning in humans has two phases of protection against endothelial IR injury; an early (short) and late (prolonged) phase, both of which are neuronally mediated. The potential for late phase RIPC to provide prolonged protection during clinical IR syndromes merits investigation. (J Am Coll Cardiol 2005;46:450–6) © 2005 by the American College of Cardiology Foundation

Ischemic preconditioning (IPC) is an innate mechanism that protects tissues from injury during ischemia and subsequent reperfusion (ischemia-reperfusion [IR] injury) (1). Preconditioning is initiated by brief, nonlethal periods of ischemia, and provides local tissue protection from the effects of further prolonged episodes of ischemia. In the heart, IPC reduces experimental infarct size by up to 75% in many different species (2–6). The effects of IPC are immediate, triggered by release of several mediators (including adenosine and bradykinin) (7,8), and dependent on the activation of complex second messenger systems (1). Immediate protection lasts just a few hours, but is followed 24 h later by a "second window" of protection (9), lasting for 48 to 72 h (10) and dependent on the induction of protective proteins (11). Although the magnitude of organ protection in experimental models of IPC is substantial, this approach has not yet led to clinical exploitation of preconditioning. This is, in part, because of the logistical difficulties of using ischemia to precondition vital organs, either in advance of clinical IR injury that is predictable (such as primary angioplasty, coronary artery bypass surgery, or transplantation), or to maintain a persistent state of preconditioning in readiness for spontaneous cardiovascular events in high-risk patients. Although much is known about the cellular mechanisms of both phases of IPC, remaining mechanistic uncertainties have so far precluded a successful pharmacologic approach to preconditioning in humans (1).

It is now clear that IPC has systemic effects that result in protection from IR injury of tissues remote from those undergoing preconditioning (12). This aspect of preconditioning, termed remote ischemic preconditioning (RIPC), was first described in the heart, where IPC initiated in the vascular distribution of one coronary artery caused protection throughout the myocardium (13). It is now apparent that protection from IPC spreads from distant organs to the heart (14,15) possibly via activation of the autonomic nervous system (14,16–18), and/or humoral factors (19,20). Mechanistically, RIPC resembles IPC and depends on similar triggers (17,18,21) and second messen-

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Abbreviations and Acronyms

FMD = flow-mediated dilation
GTN = glyceryl trinitrate
IPC = ischemic preconditioning
IR = ischemia-reperfusion
RIPC = remote ischemic preconditioning

gers (14,22,23) and in some studies provides protection for up to 24 h (24).

We have recently demonstrated, in an in vivo model of myocardial infarction, that short periods of limb ischemia induce RIPC and reduce experimental infarct size by 50% (25). In addition, using a human model of IR, we demonstrated that IPC of one arm protects the contralateral arm from endothelial IR injury, consistent with a remote preconditioning effect (25). These studies establish the principle of using limb ischemia to induce RIPC, and indicate a way in which this technique might be investigated in the clinical setting. The aim of the present study was to characterize in humans the time course of protection from RIPC and, in particular, to establish if there are two separate phases of protection. In addition, we investigated the mechanism of spread of protection to remote tissues. Defining these fundamental characteristics of human RIPC is a prerequisite for the optimal design of future clinical studies.

METHODS

Subjects. A total of 115 studies were performed on 16 healthy volunteers (12 men, 4 women; mean age ± SD 28.9 ± 7.7 years; range 21 to 48 years) who gave informed consent. Studies were approved by the local research ethics committee and performed in a temperature-controlled laboratory (24°C to 26°C). All studies repeated in the same volunteers were at least seven days apart.

Induction of IR. The nondominant forearm was made ischemic by inflating a 9-cm-wide blood pressure cuff placed around the upper arm to a pressure of 200 mm Hg for 20 min, as described previously (26).

Induction of RIPC. Remote IPC was induced by inflating a 9-cm-wide blood pressure cuff placed around the upper part of the contralateral arm. The cuff was inflated to 200 mm Hg for 5 min (ischemia), followed by a 5-min deflation. The inflation/deflation cycle was performed three times.

Assessment of conduit vessel function. Endothelial function of the brachial artery was assessed by flow-mediated dilation (FMD) of the brachial artery in the nondominant arm, as previously described (27). A B-mode scan of the brachial artery was obtained in longitudinal section between 5 and 10 cm above the antecubital fossa using a 7.0-MHz linear array transducer (spatial resolution of 0.1 mm [28]) and a standard Acuson XP10 system (Acuson, Mountain View, California). Longitudinal, electrocardiogram-gated, end-diastolic images were acquired every 3 s for offline analysis. Arterial diameter over a 1- to 2-cm segment was determined for each image with the use of automatic edge-detection software (Brachial Tools, Iowa City, Iowa). Blood flow was manipulated in the brachial artery by a 7-cm-wide blood pressure cuff placed around the forearm immediately below the antecubital fossa. After 1 min of baseline flow, the cuff was inflated to 300 mm Hg for 5 min and released, resulting in a brief episode of reactive hyperemia. Brachial artery diameter changes in response to blood flow were assessed for a further 5 min. Blood flow velocity was continuously monitored by pulsed-wave Doppler. The dilator response of the brachial artery to glyceryl trinitrate (GTN) (25 μg sublingually) was used to assess endothelium-independent dilation.

Experimental protocols. EFFECT OF IR ON VASCULAR DILATOR FUNCTION. In order to determine the effect of IR on endothelial function, FMD was assessed before ischemia and at 20 min after reperfusion (n = 13) (Fig. 1A). Similarly, the effect of IR on smooth muscle function was determined in separate studies, by assessing the dilation of the brachial artery in response to sublingual GTN (25 μg) before and after IR (n = 7) (Fig. 1B). Pilot studies indicated that GTN had a direct action to reduce IR injury if administered immediately before, but not when administered 24 h before, IR. Therefore, to assess whether IR altered the dilator response to GTN, the control dilation to GTN was measured 24 h before IR and compared with the dilation to GTN immediately after IR.

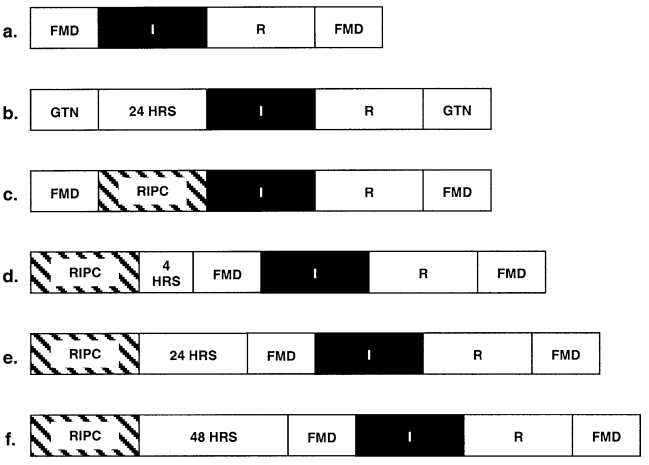

Figure 1. Protocol of studies to determine the time course of remote ischemic preconditioning (RIPC). Flow-mediated dilation (FMD) of the brachial artery was assessed before 20 min of arm ischemia (I) and at 20 min of reperfusion (R) **(a)**. The effect of IR on brachial artery smooth muscle function was determined by measuring dilation in response to sublingual glyceryl trinitrate (GTN) (25 μg) administered before and after IR **(b)**. Because pilot studies had shown that GTN prevented endothelial IR injury when administered immediately but not 24 h before, the control dilator response to GTN was determined 24 h before IR. The effect of RIPC of the contralateral arm on endothelial IR injury was determined by applying the RIPC stimulus immediately before IR **(c)**. To determine the time course of protection by RIPC, the RIPC stimulus was applied 4 h **(d)**, 24 h **(e)**, and 48 h **(f)** before IR.

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EFFECT OF RIPC ON IR INJURY TO THE ENDOTHELIUM. Flow-mediated dilation was assessed before and after IR preceded by RIPC (n = 13) (Fig. 1C). In control studies brachial FMD was measured before and after RIPC alone to determine whether RIPC had a direct effect on endothelial function (n = 7).

TIME COURSE OF THE PROTECTIVE EFFECT OF RIPC. Remote IPC was applied 4 h (n = 10) (Fig. 1D), 24 h (n = 12) (Fig. 1E), and 48 h (n = 8) (Fig. 1F) before IR; FMD was measured before and after IR.

MECHANISM OF SPREAD OF THE RIPC STIMULUS: ROLE OF THE AUTONOMIC NERVOUS SYSTEM. A venous cannula was placed in a forearm vein in the left arm under local anesthesia (1% lignocaine), and the N_N-cholinergic antagonist trimetaphan camsylate (Cambridge Laboratories, Wallsend, United Kingdom) was infused at 1 to 6 mg/min, with 1-mg/min dose increments at 5-min intervals. The dose was increased until the heart rate response to a Valsalva maneuver was abolished.

Eight volunteers underwent repeat assessment of the effects of IR alone (Fig. 1A), IR preceded by RIPC immediately (Fig. 1C), or 24 h before (Fig. 1E) on endothelial function. To determine the effect of autonomic blockade on RIPC, the same volunteers received trimetaphan by infusion during the application of the RIPC stimulus immediately before IR (Fig. 2A) or 24 h before IR (n = 7) (Fig. 2B). The effect of trimetaphan on baseline FMD (n = 4) (Fig. 2C) and the endothelial response to IR (n = 4) (Fig. 2D) was determined to exclude any direct effects on these measurements.

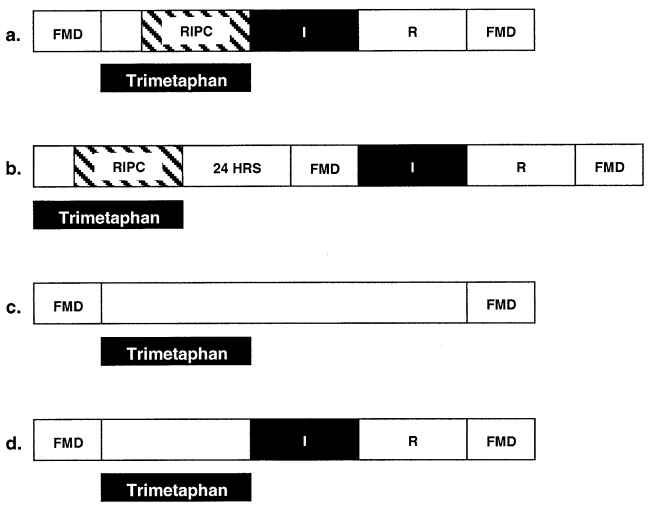

Figure 2. Protocol of studies to determine the effect of autonomic blockade on remote ischemic preconditioning (RIPC). Trimetaphan (1 to 6 mg/min) was administered by continuous intravenous infusion to cause autonomic nervous system blockade. To determine the effect of trimetaphan on early and late protection by RIPC, trimetaphan was infused during the RIPC stimulus that was applied immediately **(a)** and 24 h before ischemia and reperfusion (IR) **(b)**. Protocols **(c)** and **(d)** were designed in order to determine whether trimetaphan had direct effects on flow-mediated dilation (FMD) or the endothelial response to IR, respectively.

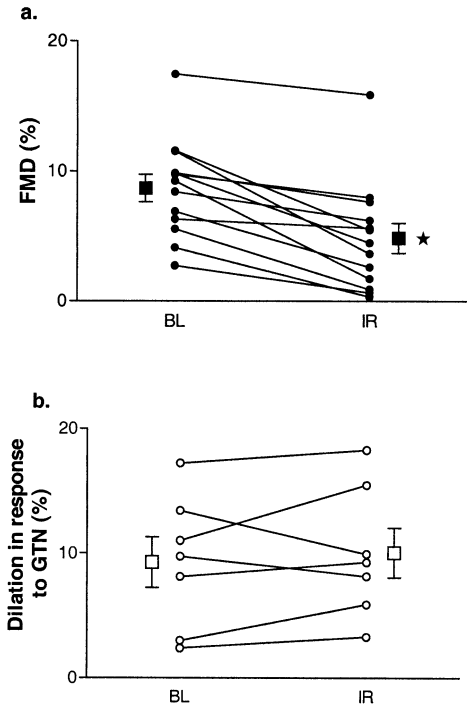

Figure 3. Effect of ischemia and reperfusion (IR) on endothelial and smooth muscle function. Flow-mediated dilation (FMD) was 8.7 ± 1.1% at baseline (BL) and was reduced by IR **(a)** (IR 4.9 ± 1.2%; *p < 0.001 vs. BL, analysis of variance; n = 13). Glyceryl trinitrate (GTN) dilation was 9.3 ± 2.0% at BL, and was unaffected by IR **(b)** (IR 10.0 ± 2.0%; p = NS, *t* test; n = 7).

Calculations and statistics. All data are expressed as mean ± SE unless otherwise stated. Brachial artery diameter was measured in millimeters and dilation expressed as percentage increase from baseline diameter. Data were compared using the Student paired *t* test or repeated measures analysis of variance (ANOVA), as appropriate. For multiple comparisons (five groups), p values by ANOVA were Bonferroni-adjusted. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

All subjects tolerated the procedures without any complications. There were no differences in the responses between men and women. The IR protocol had no effect on blood pressure, heart rate, or basal flow at 20 min of reperfusion (data not shown). Mean brachial artery diameter was 3.9 ± 0.1 mm.

Effect of IR on vascular dilator function. Ischemia-reperfusion reduced brachial artery FMD (8.7 ± 1.1% before IR vs. 4.9 ± 1.2% after IR, $p < 0.001$; n = 13) (Fig. 3A) but had no effect on blood flow during reactive hyperemia (peak to baseline volume flow ratio 8.8 ± 0.9 before IR vs. 11.2 ± 1.3 after IR, p = NS). Ischemia reperfusion had no effect on GTN dilation (9.3 ± 2.0% before IR vs. 10.0 ± 2.0%, p = NS; n = 7) (Fig. 3B).

Effect of RIPC on endothelial function. Remote IPC did not alter baseline blood flow or arterial diameter (data not

shown) and had no direct effect on brachial artery FMD (7.6 ± 0.8% before RIPC vs. 7.2 ± 0.9% after RIPC, p = NS; n = 7). Remote IPC immediately before IR prevented endothelial dysfunction (FMD 9.4 ± 0.7% before IR vs. 8.0 ± 0.8% after IR, p = NS; n = 13) Fig. 4A).

Time-course of protection by RIPC. Remote IPC did not prevent endothelial dysfunction when applied 4 h before IR (FMD 8.6 ± 1.1% before IR vs. 4.9 ± 1.1% after IR, p < 0.001; n = 10) (Fig. 4B). When RIPC was applied 24 h before IR, FMD was preserved (FMD 8.7 ± 1.1% before IR vs. 8.4 ± 1.2% after IR, p = NS; n = 12) (Fig. 4C). Similar findings were observed when RIPC was applied 48 h before IR (FMD 10.0 ± 0.9% before IR vs. 8.8 ± 1.4% after IR, p = NS; n = 8) (Fig. 4D).

Effect of autonomic blockade on RIPC. Autonomic blockade by trimetaphan (5.5 ± 1.3 mg/min) reduced systolic blood pressure (117.3 ± 1.9 mm Hg at baseline vs. 100.8 ± 2.1 mm Hg after autonomic blockade, p < 0.0001), increased heart rate (65 ± 2 beats/min at baseline vs. 89 ± 2 beats/min after autonomic blockade, p < 0.0001), but had no effect on diastolic blood pressure (65.4 ± 2.0 mm Hg at baseline vs. 63.9 ± 1.9 mm Hg after autonomic blockade, p = NS). The increase in heart rate in response to the Valsalva maneuver was prevented by trimetaphan (18.6 ± 1.5 beats/min increase in heart rate at baseline vs. 2.2 ± 0.8 beats/min increase in heart rate after autonomic blockade, p < 0.0001). The hemodynamic effects of trimetaphan were only present while it was being infused during RIPC; after cessation of the infusion, blood pressure (114.7 ± 2.4 mm Hg, p = NS vs. baseline) and heart rate (62 ± 2 beats/min, p = NS vs. baseline) had returned to normal by the time FMD was repeated.

Trimetaphan did not directly affect baseline brachial artery FMD (6.7 ± 1.3% before trimetaphan vs. 6.8 ± 0.9% after trimetaphan, p = NS; n = 4); IR reduced FMD (7.3 ± 1.2% before IR vs. 2.6 ± 0.7% after IR, p < 0.01; n = 8) (Fig. 5A), and this was not significantly affected by trimetaphan (FMD 8.5 ± 1.2% before IR vs. 4.5 ± 0.7% after IR, p < 0.01; n = 4). Remote IPC prevented endothelial dysfunction when applied immediately before IR (FMD 8.2 ± 0.9% before IR vs. 7.0 ± 0.8% after IR, p = NS; n = 8) (Fig. 5B), but protection was diminished when RIPC was applied in the presence of systemic trimetaphan (FMD 8.3 ± 1.1% before IR vs. 4.2 ± 0.9% after IR, p < 0.05; n = 7) (Fig. 5C). Similarly, RIPC, applied 24 h before IR, prevented IR injury (FMD 8.0 ± 1.2% before IR vs. 7.9 ± 1.6% after IR, p = NS; n = 7) (Fig. 5D), but trimetaphan blocked the protective effect of RIPC at this time point (FMD 7.3 ± 1.0% before IR vs. 2.3 ± 0.6% after IR, p < 0.001; n = 7) (Fig. 5E).

DISCUSSION

This study demonstrates for the first time in humans in vivo that RIPC prevents endothelial IR injury in conduit vessels with two temporally distinct phases of protection. An early

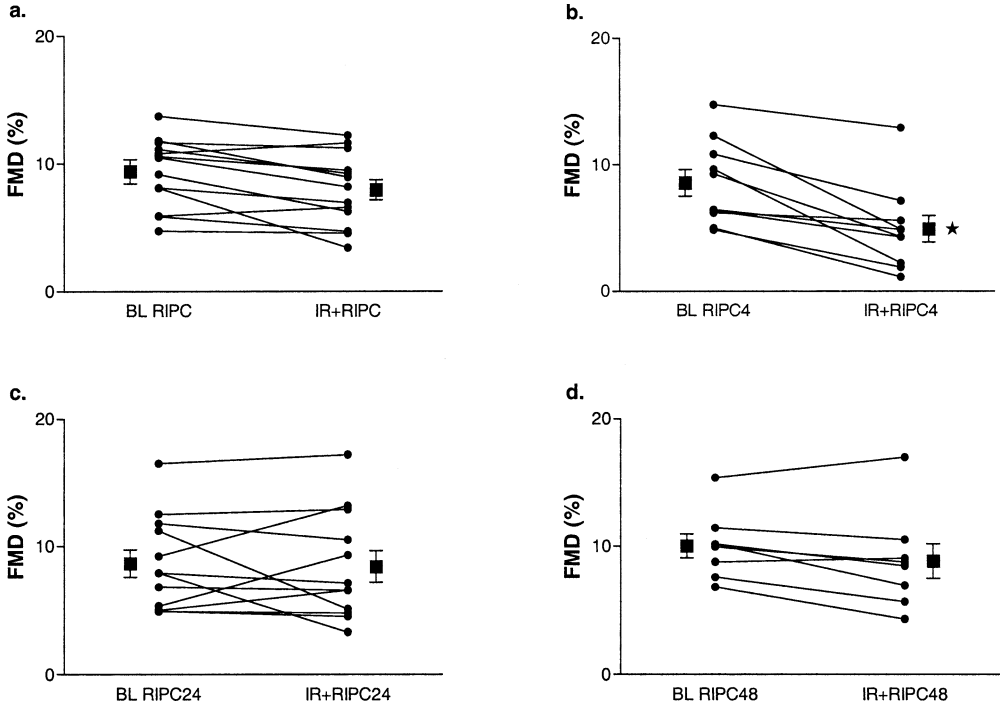

Figure 4. Time course of the protective effect of remote ischemic preconditioning (RIPC) on ischemia-reperfusion (IR)-induced endothelial dysfunction. Flow-mediated dilation (FMD) was 9.4 ± 0.7% at baseline (BL) and was unaffected by IR preceded immediately by RIPC **(a)** (IR+RIPC 8.0 ± 0.8%; p = NS vs. BL, analysis of variance [ANOVA]; n = 13). Ischemia-reperfusion reduced FMD when RIPC was applied 4 h before IR **(b)** (BL 8.6 ± 1.1% vs. IR+RIPC4 4.9 ± 1.1%; *p < 0.001, ANOVA; n = 10). However, the effect of IR to reduce FMD was prevented when RIPC was applied 24 h before IR **(c)** (BL 8.7 ± 1.1% vs. IR+RIPC24 8.4 ± 1.2%; p = NS, ANOVA; n = 12) and 48 h before IR **(d)** (BL 10.0 ± 0.9% vs. IR+RIPC48 8.8 ± 1.4%; p = NS, ANOVA; n = 8).

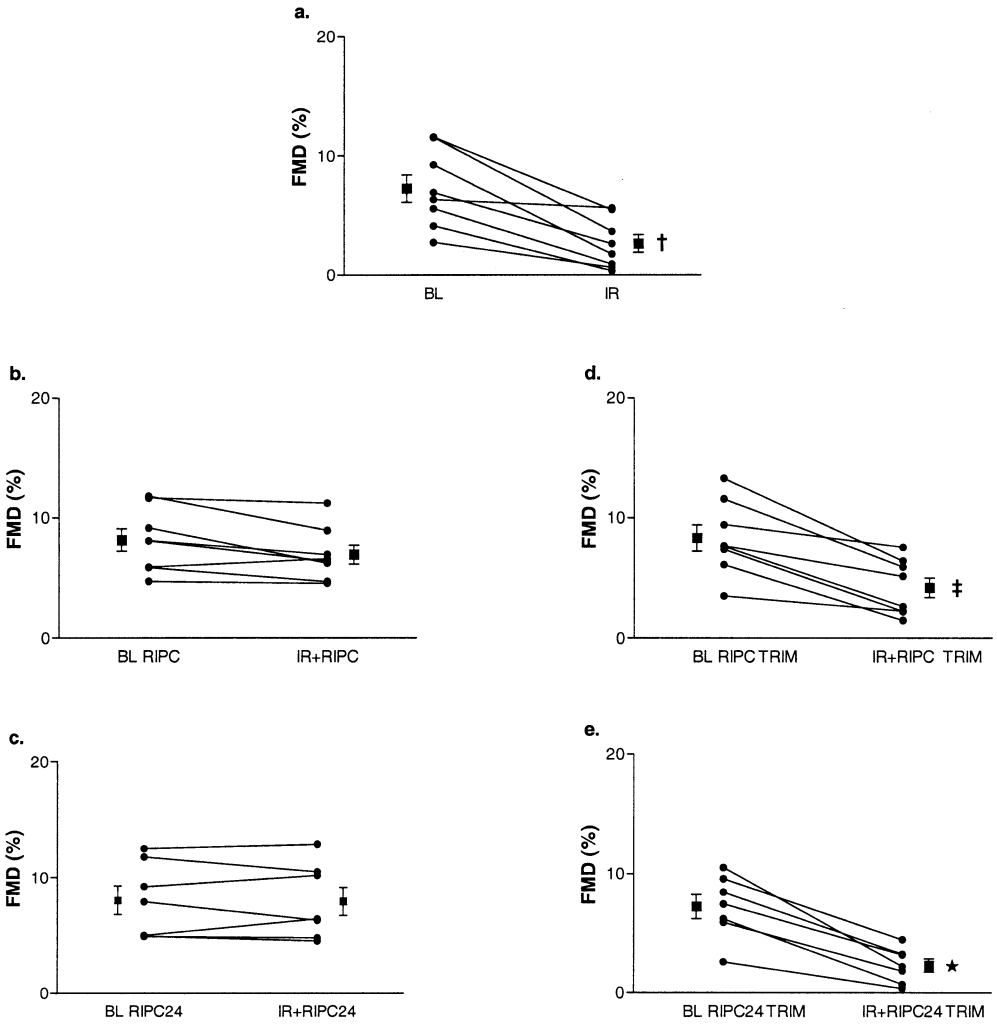

Figure 5. Effect of autonomic blockade on remote ischemic preconditioning (RIPC). Flow-mediated dilation (FMD) was 7.3 ± 1.2% at baseline (BL) and was reduced by ischemia reperfusion (IR) **(a)** (IR 2.6 ± 0.7%; †$p < 0.01$ vs. BL, analysis of variance [ANOVA]; n = 8). The effect of IR on FMD was prevented by RIPC immediately before **(b)** (BL 8.2 ± 0.9% vs. IR+RIPC 7.0 ± 0.8%; p = NS, ANOVA; n = 8) or 24 h before IR **(c)** (BL 8.0 ± 1.2% vs. IR+RIPC24 7.9 ± 1.2%; p = NS, ANOVA; n = 7). Protection by RIPC was blocked by administration of trimetaphan (TRIM) (1 to 6 mg/min intravenously) during RIPC immediately before IR **(d)** (BL 8.3 ± 1.1% vs. IR+RIPC TRIM 4.2 ± 0.9%; ‡$p < 0.05$, ANOVA; n = 7) and 24 h before IR **(e)** (BL 7.3 ± 1.0% vs. IR+RIPC24 TRIM 2.3 ± 0.6%; *$p < 0.001$, ANOVA; n = 7).

phase is activated immediately and disappears within 4 h; a second phase presents 24 h after the application of the RIPC stimulus and is sustained for at least 48 h. Both phases of RIPC are dependent on intact autonomic function. If such protection extends beyond the vasculature, then these data suggest ways in which preconditioning can be elicited in patients undergoing planned procedures complicated by IR injury.

Systemic protective effects of IPC. Ischemic preconditioning, elicited by brief ischemic episodes, reduces tissue damage occurring during prolonged ischemia (1), but the perception that it was necessary to induce IPC in the at-risk organ has limited exploitation of this phenomenon in clinical practice. The realization that preconditioning has systemic effects to induce protection in tissues remote from those undergoing preconditioning substantially increases the clinical applicability of preconditioning strategies (12). In previous work we demonstrated that RIPC could be elicited by ischemia of nonvital tissues; limb ischemia in an animal model reduced experimental myocardial infarct size and in humans prevented IR injury to the endothelium of the forearm resistance vasculature (25). Taken together these findings suggest that it may be possible to harness endogenous cardioprotection, triggered by ischemia of easily accessible tissues.

RIPC of the limb protects against endothelial IR injury. The present study demonstrates that RIPC prevents IR-induced endothelial dysfunction in conduit arteries, and is consistent with our previous data in the resistance vasculature (25). The vascular endothelium is implicated in the pathogenesis of IR injury; reduced endothelial dilator and anticoagulant function during IR injury may exacerbate vasospasm and encourage persistence of cellular aggregates and thrombus within conduit and resistance vessels. These aspects of endothelial dysfunction may directly affect the extent of tissue reperfusion (29,30), and some of the benefits

of preconditioning might be a consequence of preservation of dilator function (30), although not all studies have shown this (31,32). Changes in FMD were not explained by effects of IR injury or RIPC on blood flow increases during reactive hyperemia. Moreover, the protective effect of RIPC was not explained by changes in smooth muscle responsiveness, as RIPC did not alter the GTN-induced dilation.

Time course of protection by RIPC. Animal studies indicate that RIPC offers protection from IR injury that lasts for up to 24 h (24,33,34). However, it has not been established whether there are early and late phases of protection (as for IPC) rather than a single period of prolonged protection. Our data confirm that RIPC mirrors IPC with an early phase of protection lasting only a few hours after the RIPC stimulus, and followed 24 h later by a second window lasting for up to 48 h. The reappearance of protection and its prolonged time course is consistent with altered protein expression in the vessel wall, and it is possible that such changes may be similar to those identified in second window IPC. Further studies are needed to identify these molecular mechanisms.

Effect of autonomic blockade on RIPC. One the most intriguing questions regarding RIPC is how the transfer of the protective signal from the site of preconditioning to remote tissues occurs. There is evidence for humoral mediators that may include endogenous opioids (35,36). In addition, a neurogenic pathway has also been suggested, with evidence for involvement of the autonomic nervous system in the mechanism of the early phase (14,16–18), and sensory C fibers in the late phase (34,37,38). The present study clearly implicates the autonomic nervous system in the spread of protection during RIPC. The autonomic ganglion blocker trimetaphan was administered by intravenous infusion at a dose sufficient to cause autonomic block (confirmed by its effects on blood pressure, heart rate, and the Valsalva response). Because of its short-lived action, autonomic blockade was restricted to the RIPC phase of the protocols, and baseline hemodynamics were restored in advance of the repeat assessment of FMD. Time-control studies confirmed that trimetaphan had no direct effect on FMD, or the endothelial response to IR injury. However, when administered during RIPC, it blocked its early and late protective effects on endothelial IR injury. It is possible that release of local triggers of IPC (including bradykinin and adenosine) activate the autonomic nervous system either directly (17,18) or via sensory nerves (34,37,38), and transfers the signal to the myocardium or other remote tissues. How this leads to tissue protection is not clear at present, but animal data implicate a similar mechanism to that described for classical IPC, with activation of protein kinase C (14,22,23) and mitochondrial K_{ATP} channels (15,23,36,39). Our data do not indicate which component of autonomic function (muscarinic or adrenergic) is involved, and further studies are required to dissect these pathways.

Study limitations. In our model, IR-induced endothelial injury resolves spontaneously within 60 min of reperfusion (26), consistent with "endothelial stunning" rather than necrosis in response to 20 min of ischemia. This may explain the complete abrogation of endothelial dysfunction caused by RIPC that we observed. However, it also raises concerns that the protocol of limb RIPC that we used might be insufficient to protect from more substantial injury, as would occur in the clinical setting. We have shown in a previous study that a similar protocol of limb ischemia reduces infarct size in the pig after 40 min of coronary artery occlusion (25). Moreover, recent preliminary data in humans indicates that preconditioning using limb ischemia reduces troponin T release from the myocardium after cardiopulmonary bypass in children (40). These observations suggest that the limb is a suitable substrate to trigger systemic preconditioning, and that the present model of IR injury is suitable to examine the time course of protection.

One additional potential limitation is the lack of specificity of trimetaphan, which also has alpha-adrenoreceptor blocking properties and induces the release of histamine. Moreover, trimetaphan has direct vasodilator actions, although the mechanisms of this effect are not currently known. Although these additional actions are unlikely to account for the effects described in our study, unknown effects of the drug that alter the response of the vascular endothelium to RIPC cannot be excluded. One way of eliminating this potential source of error is to test whether RIPC can be induced in patients with autonomic nervous system dysfunction.

Conclusions. Irrespective of uncertainties regarding the details of mechanisms of IPC and RIPC, it is their similarity that is most striking, and strongly suggests that IPC and RIPC are two aspects of the same biological mechanism. When animal and human data are taken in their totality, this method of initiating IPC of vital tissues offers similar protection to that caused by direct IPC. The present study confirms that in humans RIPC offers enduring (up to 48 h) protection against endothelial IR injury. If this is applicable to other tissues, then our data suggest a simple way in which the effect of RIPC to reduce ischemic damage in clinical IR syndromes can be tested. Preconditioning could be triggered 24 h in advance of cardiopulmonary bypass surgery, angioplasty, or transplantation and provide up to 48 h of resistance to cardiac and noncardiac ischemia. Such investigations are likely to yield definitive information on the clinical utility of RIPC.

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