JACC: BASIC TO TRANSLATIONAL SCIENCE
VOL. 1, NO. 6, 2016
ISSN 2452-302X
http://dx.doi.org/10.1016/j.jacbts.2016.06.002

PRECLINICAL RESEARCH

Adenosine Receptor Activation in the "Trigger" Limb of Remote Pre-Conditioning Mediates Human Endothelial Conditioning and Release of Circulating Cardioprotective Factor(s)

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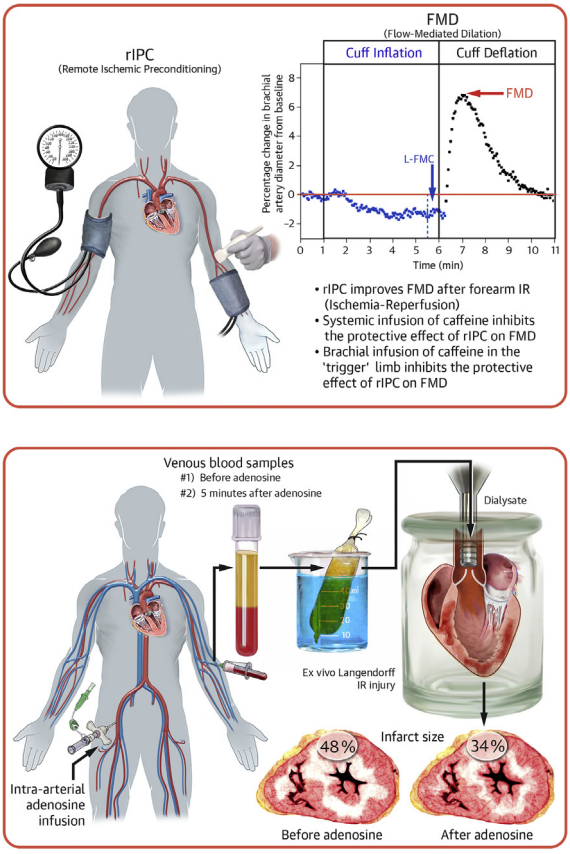
VISUAL ABSTRACT



Contractor, H. et al. J Am Coll Cardiol Basic Trans Science. 2016;1(6):461–71.

HIGHLIGHTS

- **Pre-conditioning has emerged as a potentially powerful means of reducing ischemia-reperfusion injury.**
- **Several animal models have implicated adenosine in pre-conditioning pathways, but its role in human physiology is unknown.**
- **In human volunteers, the authors demonstrate that adenosine receptor activation in "trigger" tissue is an important step in initiating a pre-conditioning signal, but adenosine receptor blockade in "target" tissue does not block the protection afforded by pre-conditioning.**
- **The authors also demonstrate that pre-conditioning elaborates a transferrable cardioprotective factor(s) into the serum. This elaboration is prevented by adenosine receptor blockade but can be mirrored by the infusion of exogenous adenosine.**
- **An improved understanding of the physiological effectors of pre-conditioning may allow for better targeted clinical studies of pre-conditioning and pre-conditioning mimetics in the future.**

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 1, NO. 6, 2016
OCTOBER 2016:461–71

ABBREVIATIONS AND ACRONYMS

Ach = acetylcholine

ANOVA = analysis of variance

FMD = flow-mediated dilation

GTN = glyceryltrinitrate

IR = ischemia-reperfusion

LV = left ventricular

NMD = nitrate-mediated dilation

rIPC = remote ischemic pre-conditioning

SUMMARY

Remote ischemic pre-conditioning (rIPC) has emerged as a potential mechanism to reduce ischemia-reperfusion injury. Clinical data, however, have been mixed, and its physiological basis remains unclear, although it appears to involve release of circulating factor(s) and/or neural pathways. Here, the authors demonstrate that adenosine receptor activation is an important step in initiating human pre-conditioning; that pre-conditioning liberates circulating cardioprotective factor(s); and that exogenous adenosine infusion is able to recapitulate release of this factor. However, blockade of adenosine receptors in ischemic tissue does not block the protection afforded by pre-conditioning. These data have important implications for defining the physiology of human pre-conditioning and its translation to future clinical trials. (J Am Coll Cardiol Basic Trans Science 2016;1:461–71)

Remote ischemic pre-conditioning (rIPC) induces protection of central (target) organs from ischemia-reperfusion (IR) by brief ischemia of peripheral (trigger) tissue (1,2). Limb ischemia achieves rIPC in humans, and has been tested with positive results in clinical studies (3-10). However, other recent clinical studies also show neutral effects (11-15), and better understanding of the mechanisms of rIPC in humans may lead to maximization of its benefits. The signaling pathway from the trigger limb to target organs is poorly defined, but involves release of circulating factor(s) (16-23) and neural pathways (19,24-26).

In preclinical animal models, adenosine antagonists inhibit cardiac rIPC induced by renal and mesenteric ischemia (27,28). Furthermore, femoral arterial (but not femoral vein) adenosine infusion pre-conditioned rat hearts and was inhibited by femoral nerve transection. In a rabbit model, femoral artery (but not femoral vein) infusion of adenosine released humoral cardioprotective factor(s) into the circulation, which reduced infarct size when transferred to a naive Langendorff heart (19). However, a more recent porcine study suggests that adenosine is not involved in rIPC (29). The role of the adenosine pathway in human rIPC remains unknown but clearly warrants further investigation.

The aims of this study were to address: 1) whether adenosine receptor activation is involved in human endothelial rIPC-induced by limb ischemia; 2) whether adenosine receptor activation is involved in the "trigger" or "target" phases of rIPC; 3) its effects on release of circulating cardioprotective factor(s); and 4) whether arterial infusion of adenosine liberates release of a circulating cardioprotective factor(s) in humans.

METHODS

Protocols were approved by the local research ethics committee (refs 08/H0604/152, 09/H0604/118, 10/H0604/28).

EXPERIMENTAL METHODS. Venous occlusion plethysmography. Strain-gauge plethysmography was used to measure forearm blood flow as described previously (30). For each study, the brachial artery of the nondominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works, Birmingham, United Kingdom) under local anesthesia (3 ml of 1% lignocaine). Drugs or normal saline (sodium chloride 0.9% wt/vol) were infused continuously at 1 ml/min. During recording periods, the hands were excluded from the circulation by inflation of wrist cuffs to 200 mm Hg. Responses to both acetylcholine (Ach) (25, 50, and 100 nmol/min) and glyceryltrinitrate (GTN) (4, 8, and 16 nmol/min) were assessed before and after combined rIPC and IR. All recordings and analysis were made using LabChart v.6 (AD Instruments, Chalgrove, United Kingdom).

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Manuscript received May 2, 2016; revised manuscript received June 9, 2016, accepted June 16, 2016.

Flow-mediated dilation. The brachial artery was continuously imaged longitudinally just above the level of the antecubital fossa and flow-mediated dilation (FMD) calculated as the maximum percentage change in the vessel diameter from baseline following release of a distal pressure cuff after 5 min of inflation (200 mm Hg) as previously described (31). Vessel diameters were calculated using the automatic B-mode edge detection software Brachial Analyzer for Research (Medical Imaging Applications, Coralville, Iowa). Following each FMD, 25 μg of GTN was given sublingually and nitrate-mediated dilation (NMD) assessed as a measure of endothelium-independent vasorelaxation.

Induction of IR and rIPC. IR consisted of 20 min of ischemia followed by 15-min reperfusion, and rIPC consisted of 3 cycles of 5 min of ischemia and 5-min reperfusion of the contralateral arm, both achieved by inflation of a proximal pressure cuff to 200 mm Hg (3,32).

Mouse langendorff infarction bioassay. Mice received heparin (200 IU, i.p.; Sigma-Aldrich, St. Louis, Missouri) and were anesthetized with pentobarbital (60 mg/kg, i.p.; Ceva Sante Animale, Libourne, France). Isolated hearts were mounted on the Langendorff perfusion apparatus (Radnoti Technologies, Monrovia, California), and perfused under nonrecirculating conditions at a constant pressure of 80 mm Hg with 37°C Krebs-Henseleit buffer (consisting of the following in mmol/l: NaCl 120.0, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, EDTA 0.5, and glucose 15). The solution was continuously gassed with 95% O_2–5% CO_2 (pH 7.4). After 20-min stabilization, isolated hearts were perfused for 30 min using dialysate, washed out for 5 min, and then subjected to 30 min of no-flow global ischemia followed by 60 min of reperfusion. The left ventricular (LV) developed pressure was calculated as the difference between the systolic and end-diastolic LV pressures. After freezing, the heart was then sliced into 1-mm slices from the apex to the base, and slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4) (Sigma-Aldrich) at 37°C for 15 min. All slices were then scanned on a flat-bed digital scanner and weighed. Infarct size was determined by semiautomated computer planimetry (Image Pro Analyzer v7.0, Bethesda, Maryland). Because this was a model of global ischemia, the whole heart acts as the "area at risk", and infarct size is presented as a percentage of whole-heart mass.

Production of dialysate to assay for circulating cardioprotective factor(s). Heparinized whole blood was centrifuged at 4,640 *g* and the plasma fraction pipetted into a 15-kDa dialysis membrane and placed in 20× volume of a modified KH buffer containing (all in mmol/l) NaCl 118.5, KCL 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.19, and D-glucose 11.0. After 48 h, the dialysate solution was decanted and stored at −80°C. Before use, $NaHCO_3$ 25 mmol/l and $CaCl_2$ 2.4 mmol/l was added to the buffer and saturated with 95% O_2 and 5% CO_2.

EXPERIMENTAL PROTOCOLS. Protocol 1. Is adenosine receptor activation involved in human rIPC (**Figure 1**, upper panel)?

This was addressed in a randomized, parallel group, double-blind placebo-controlled study utilizing the forearm model of combined rIPC/IR in healthy volunteers. Systemic caffeine (4 mg/kg) (33) was used as a pharmacological inhibitor of adenosine. Twenty volunteers were randomized to infusion of caffeine (n = 10) or vehicle (normal [0.9%] saline) (n = 10). FMD was measured at baseline, after infusion of caffeine or vehicle, and 15 min after reperfusion following combined rIPC and forearm IR. Studies were analyzed blinded to study group allocation. In 5 subjects, a control study was conducted to test the effect of caffeine infusion on IR injury alone.

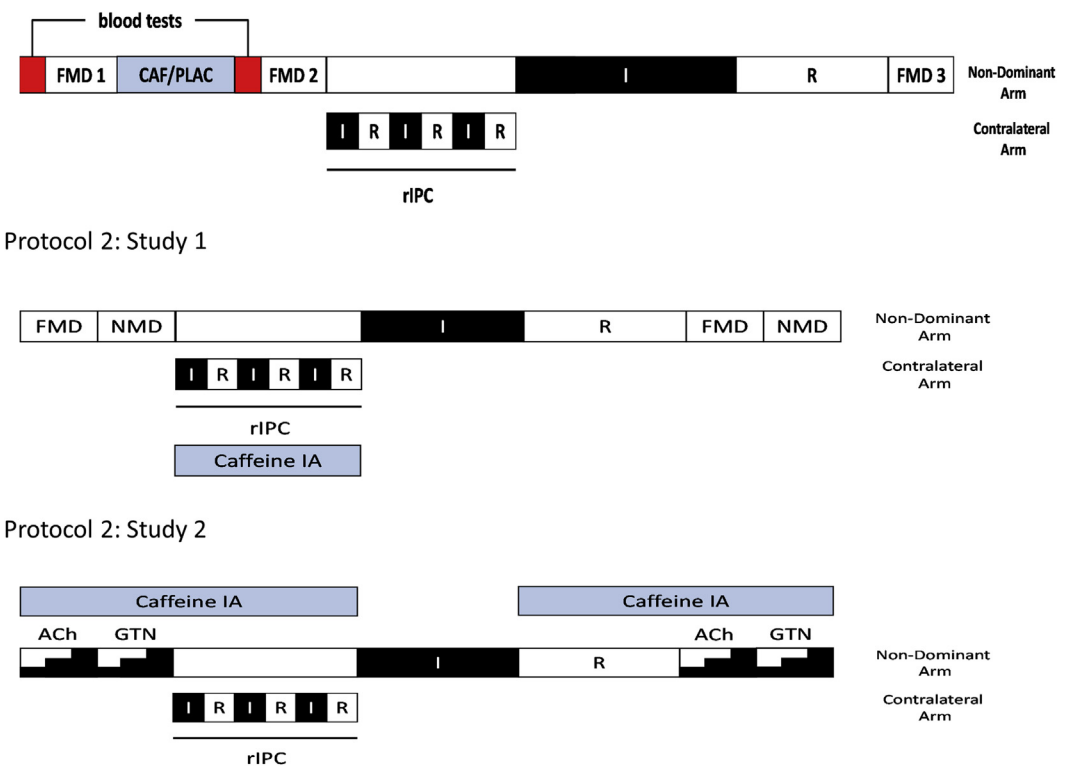
Protocol 2. Is adenosine receptor activation involved in the trigger and/or effector phase of human rIPC, and does this influence release of the circulating cardioprotective factor(s) (**Figure 1**, middle and lower panels)?

This was addressed in a crossover design study of 11 male volunteers who underwent 2 studies separated by 8 weeks. The human forearm model of rIPC/IR was used. In this study, the brachial artery of the upper limb being studied was directly infused with caffeine 90 $\mu g \cdot min^{-1}$ per 100-ml forearm volume to achieve a high local concentration of caffeine in the study limb (34).

Study 1. The trigger phase was studied. The limb used to generate rIPC was infused with caffeine. The contralateral limb was subjected to IR, with measurement of brachial artery FMD before ischemia and 15 min after reperfusion. In addition, venous blood was drawn for producing dialysate and testing for the presence of circulating cardioprotective factor(s) before and after rIPC.

Study 2. The target phase was studied. The limb used to generate the rIPC stimulus was not instrumented. The limb subjected to IR, was infused with caffeine, with measurement of blood flow responses to Ach and GTN before ischemia and 15 min after reperfusion. In addition, venous blood was drawn for producing dialysate and testing for the presence of a circulating cardioprotective factor(s) before and after rIPC.

FIGURE 1 Protocols 1 and 2 Schematics



(Top) Protocol 1: flow-mediated dilation (FMD) 1 measured at baseline, FMD 2 measured after infusion of caffeine (CAF) or placebo (PLAC). Remote ischemic pre-conditioning (rIPC) is then delivered, and FMD 3 is measured after ischemia-reperfusion (IR). **(Middle)** Protocol 2: Study 1. FMD 1 is measured before rIPC. Caffeine is infused into the trigger arm generating rIPC. FMD is measured after IR. **(Bottom)** Protocol 2: Study 2. The target arm is infused with caffeine, and the dose response to acetylcholine (Ach) is measured. rIPC is delivered. Dose response to ACh is measured after IR. GTN = glyceryltrinitrate; NMD = nitrate-mediated dilation.

Protocol 3. Does arterial infusion of adenosine liberate systemic release of a circulating cardioprotective factor(s) in humans?

This was addressed in a randomized dose-ranging study in 20 nondiabetic patients with suspected or known stable coronary disease undergoing coronary angiography. Following diagnostic coronary angiography, 75 ml of blood was withdrawn from a femoral venous sheath into heparinized containers. Patients were randomized in a 1:1 fashion to 1 of 2 doses of adenosine (0.25 mg/kg or 0.75 mg/kg). An adenosine solution (total volume 30 ml) was infused through the femoral arterial sheath over 1 min with central pressure monitoring and continuous electrocardiogram recording. Five minutes after the completion of infusion, a second venous blood sample was taken. Blood was used to produce dialysate, and cardioprotective efficacy was tested in the murine Langendorff model as described in the preceding text.

STATISTICAL ANALYSIS. Statistical testing was performed using GraphPad Prism v5.03 (GraphPad Software, La Jolla, California) or SAS version 9.2 (SAS Institute, Cary, North Carolina). In protocol 1, analysis of baseline, post-caffeine, and post-ischemia FMD was by repeated measures analysis of variance (ANOVA). In protocol 2 study 1, pre- and post-IR FMD comparison was by paired *t* test, whereas in study 2, pre- and post-ischemia dose-response curves in the plethysmography protocol were compared using 2-way ANOVA with a post hoc Bonferroni multiple comparison test. Analysis of infarct size (expressed as percentage of LV) in the mouse Langendorff model was by paired *t* test. Paired *t* tests were also used in analysis of pre- and post-infusion caffeine levels. All quoted values are the mean ± SEM. A p value of <0.05

was considered statistically significant. Sample sizes for all studies were calculated using data from previous studies assuming a power of 80% with an α of 0.05. For the FMD studies, assuming a reduction from 8.0 ± 4.0% to 3.5 ± 2.0% post-IR, a sample size of 7 subjects in each group was calculated, and for the plethysmography studies, assuming a reduction in blood flow response from 450 ± 190% to 220 ± 90% at the top dose of Ach after IR, a sample size of 5 in each group was calculated to show a difference.

RESULTS

PROTOCOL 1. Is adenosine receptor activation involved in human rIPC?

All studies were analyzed blinded to study group allocation. Demographic characteristics of groups were similar, and caffeine did not increase blood pressure, heart rate, or baseline brachial artery diameter (Table 1, Figure 2). Baseline caffeine measurements confirmed that participants had adhered to caffeine avoidance. Caffeine levels increased appropriately, confirmed correct group allocations after unblinding, and were comparable to previously described values using this protocol (1.7 μmol/l to 20.0 μmol/l in the caffeine group and 3.0 μmol/l to 2.2 μmol/l in the placebo group). The protocol was well tolerated by participants and was without sequelae.

Control experiment: effect of caffeine on FMD and IR. Five participants underwent control

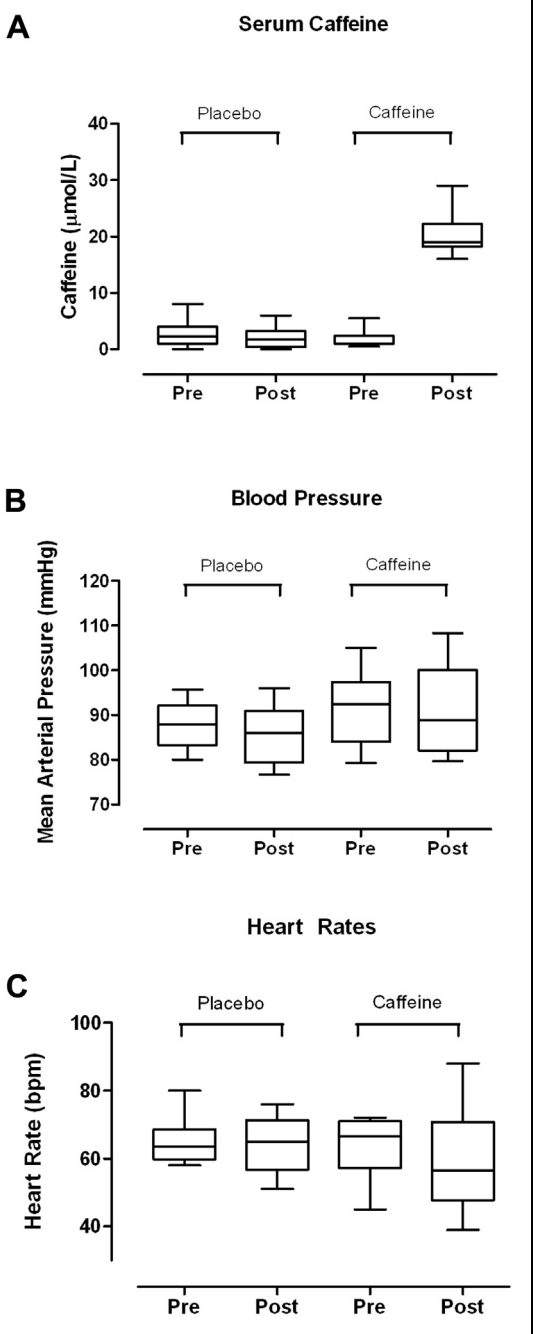
TABLE 1 Participant Demographics (Protocol 1)

	Caffeine Group (n = 10)	Placebo Group (n = 10)
Age (yrs)	26.5 ± 2.2	29.5 ± 1.8
Male	7	5
Estimated caffeine intake (mg/day)	210.0 ± 72.5	231.0 ± 80.4
Fasting glucose (mmol/l)	5.0 ± 0.2	5.2 ± 0.2
BMI (kg/m^2)	23.7 ± 0.9	25.0 ± 0.8
Baseline		
MAP (mm Hg)	92.9 ± 2.8	87.2 ± 2.7
HR (beats/min)	66.0 ± 4.3	67.0 ± 2.3
Post-infusion		
MAP (mm Hg)	91.8 ± 3.0	86.5 ± 2.7
HR (beats/min)	57.5 ± 3.7	56.0 ± 2.6
Brachial diameter (mm)	3.5 ± 0.1	3.5 ± 0.2
Caffeine pre (μmol/l)	1.7 ± 0.5	3.0 ± 0.7
Caffeine post (μmol/l)	20.0 ± 1.2	2.2 ± 0.6

Values are mean ± SEM. There were no significant differences in between group demographic characteristics. Caffeine demonstrated a highly significant rise in the caffeine infusion group as expected ($p < 0.0001$ by paired *t* test).

BMI = body mass index; HR = heart rate; MAP = mean arterial pressure.

FIGURE 2 Serum Caffeine and Hemodynamic Indices in Protocol 1



Serum caffeine levels and hemodynamic changes. An expected significant increase was seen in systemic caffeine levels following caffeine infusion from 1.7 ± 0.5 to 20.3 ± 1.2 **(A)** ($p < 0.0001$ by paired *t* test). This had no significant effect on the heart rate **(B)** or blood pressure **(C)** graph. Box and whiskers represent the median and 5th and 95th percentiles, respectively. Abbreviations as in Figure 1.

experiments to determine any effect of caffeine on FMD or IR injury alone. Baseline FMD was 4.9 ± 0.85%, and following caffeine infusion, was 5.1 ± 0.57% ($p > 0.05$ by repeated measures ANOVA). After IR, FMD was reduced to 0.8 ± 0.22%, demonstrating a significant reduction from both baseline and post-caffeine FMD ($p = 0.001$) (Figure 3, upper panel, left-hand graph). NMD was not significantly affected by either caffeine or IR injury (data not presented).

Placebo group (n = 10). Baseline FMD was 6.2 ± 1.3, and this did not change significantly after placebo infusion to 5.2 ± 0.4 ($p = 0.4$). After combined rIPC and IR, there was no significant change in FMD (5.4 ± 1.2) in comparison with either the baseline or post-infusion FMD, demonstrating the protective effect of rIPC against IR injury ($p = 0.6$) (Figure 3, upper panel, middle graph).

Caffeine infusion (n = 10). In the group receiving caffeine ($n = 10$) mean baseline FMD was 7.5 ± 1.4, and this did not change after caffeine (7.9 ± 1.0; $p = 0.80$). However, after combined rIPC and IR, FMD decreased significantly to 3.5 ± 1.4 ($p = 0.001$), demonstrating loss of the protective effect of rIPC by adenosine receptor blockade ($p = 0.0009$ repeated measures ANOVA) (Figure 3, upper panel, right-hand graph).

PROTOCOL 2. Is adenosine receptor activation involved in the trigger and/or effector phase of human rIPC, and does this influence release of the circulating cardioprotective factor(s)?

Participant demographic characteristics are listed in Table 2. There was no significant change in blood pressure, heart rate, or baseline brachial artery diameter with the infusion of caffeine into the brachial artery. After infusion of caffeine, there was a small, but statistically significant, rise in systemic caffeine levels (1.2 ± 0.3 μmol/l to 2.6 ± 0.3 μmol/l; $p = 0.001$), but with levels approximately 10-fold lower than those observed following intravenous caffeine infusion as in Protocol 1.

Study 1: Infusion of caffeine into the trigger arm. FMD was 4.9 ± 0.4 at baseline and reduced to 1.5 ± 0.7 after combined rIPC and IR, demonstrating a statistically significant decrease in FMD in the presence of caffeine infusion in the arm triggering rIPC (paired *t* test, $p = 0.01$) (Figure 3, middle panel, left graph), consistent with inhibition of the protective effect of rIPC. There was no significant change in NMD throughout the study.

The effects of dialysate (produced from systemic blood) on myocardial infarction demonstrated no significant difference in infarct size between dialysate produced before or after rIPC (37.3 ± 1.9 before and 34.25 ± 2.0 after rIPC, n = 10, paired *t* test, $p = 0.31$) (Figure 3, middle panel, right graph), consistent with adenosine receptor blockade inhibiting the release of the circulating cardioprotective factor(s).

Study 2: Infusion of caffeine into the target arm. There was no significant reduction in forearm blood flow in response to Ach after combined rIPC and IR, consistent with ongoing protection by rIPC, despite infusion of caffeine in the target organ ($n = 10$, 2-way ANOVA, $p = 0.22$) (Figure 3, lower panel, left graph). There was no significant change in NMD throughout the study.

The effects of dialysate on myocardial infarction demonstrated that there was a significant difference in infarct size between dialysate produced before or after rIPC (47.8 ± 2.4% whole heart before rIPC and 26.5 ± 2.0% whole heart after rIPC, n = 10, paired *t* test, $p = 0.0001$) (Figure 3, lower panel, right graph), consistent with release of the circulating preconditioning factor(s).

PROTOCOL 3. Does arterial infusion of adenosine liberate systemic release of a circulating cardioprotective factor(s) in humans?

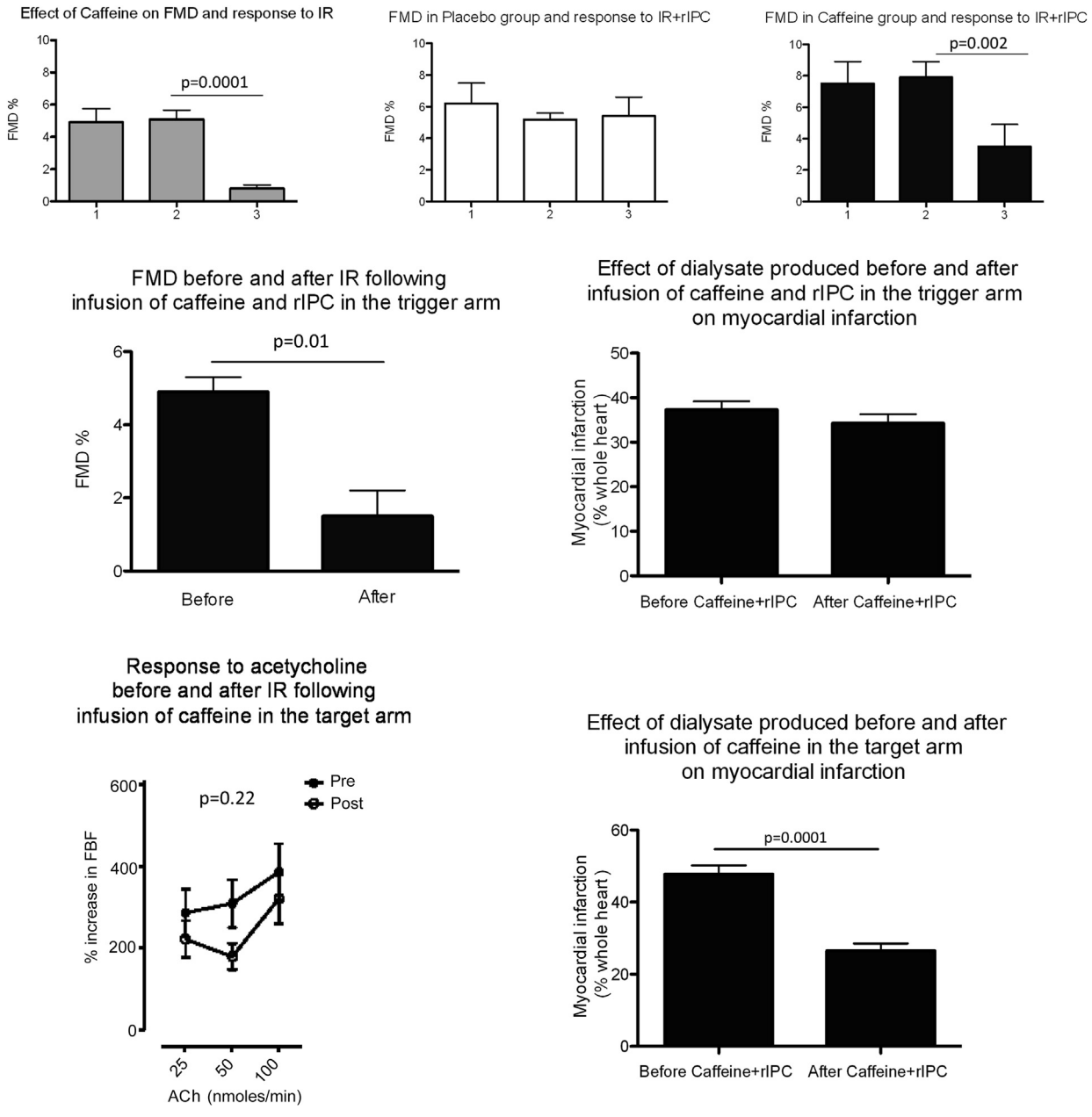
A total of 20 studies were conducted with 10 patients randomized to each group. Participant demographic characteristics are listed in Table 3.

Arrhythmia and hemodynamic data. ***Adenosine dose 0.25 mg/kg.*** There were no episodes of arrhythmia noted. Mean arterial pressure dropped from 96.8 ± 6.6 mm Hg to 90.6 ± 5.2 mm Hg at 30 s and recovered rapidly to baseline values. Heart rate increased from 66.8 ± 2.8 beats/min to 80.4 ± 4.4 beats/min at 60 s but decreased to 78.4 ± 3.2 beats/min at 80 s and had returned to baseline at 5 min. Adenosine infusion was well tolerated, with no clinically significant side effects reported.

Adenosine dose 0.75 mg/kg. One patient had pre-existing first-degree heart block, which became prolonged (without symptoms) before returning to baseline. Episodes of 2:1 block were transient and required no intervention. Mean arterial pressure dropped from a mean of 92.9 ± 3.0 mm Hg to 85.6 mm Hg at 60 s but recovered rapidly, with a mean arterial pressure of 90.7 ± 5.9 mm Hg recorded at 80 s. Heart rate increased from 62.2 ± 2.3 beats/min to 75.1 ± 4.0 beats/min at 60 s and continued to increase to 82.7 ± 3.7 beats/min at 80 s before returning to baseline at 5 min. Once more, the adenosine infusion was well tolerated, with no clinically significant side effects reported.

Murine Langendorff bioassay data. ***Adenosine 0.25 mg/kg.*** Mean infarct size was 48.3 ± 2.9% with pre-infusion dialysate, which significantly reduced

FIGURE 3 **Adenosine Receptor Inhibition Abrogates rIPC Signal Generation**



(Top) Protocol 1 results. **Left** shows the results from control studies showing that caffeine does not itself alter the response to rIPC/IR injury. **Middle** shows no significant change between FMD 1 and FMD 2. FMD 3 was not reduced, confirming rIPC was effective in the placebo group. **Right** shows no significant change between FMD 1 and FMD 2, confirming that caffeine itself did not affect FMD. FMD 3 was significantly reduced confirming rIPC was inhibited in the caffeine group. **(Middle)** Protocol 2: study 1 results. **Left** shows the significant reduction in FMD responses after rIPC/IR with infusion of caffeine in the trigger arm. This confirms inhibition of the protective effect of rIPC. **Right** shows the effect of dialysate produced on myocardial infarction (MI) showing that there is no reduction in MI, suggesting that the observed inhibition of rIPC is in part through reduction of release of a circulating factor(s). **(Bottom)** Protocol 2: study 2 results. **Left** shows no significant reduction in response to acetylcholine after rIPC and IR with infusion of caffeine in the target arm. This confirms a protective effect of rIPC. **Right** shows the effect of dialysate produced on MI, showing that there is a significant reduction in MI, suggesting preserved release of circulating factor(s). Abbreviations as in Figure 1.

TABLE 2 Participant Demographics (Protocol 2)

	Study 1	Study 2
Age (yrs)	23.8 ± 1.8	
Est caff intake (mg/day)	186 ± 54.4	
Fasting Glc (mmol/l)	5.2 ± 0.1	5.2 ± 0.2
BMI (kg/m^2)	23.4 ± 0.7	23.6 ± 0.6
Forearm vol. (cm^3)	1,162 ± 17.8	
Baseline		
MAP (mm Hg)	102.0 ± 2.6	104.0 ± 2.3
HR (beats/min)	58 ± 2.8	56 ± 2.4
Post-infusion		
MAP (mm Hg)	105.8 ± 3.5	104.8 ± 1.6
HR (beats/min)	65.0 ± 3.9	54.0 ± 1.5
Caffeine pre (μmol/l)	1.3 ± 0.3	1.3 ± 0.4
Caffeine post (μmol/l)	2.2 ± 0.3	2.6 ± 0.3
Hemoglobin (g/dl)	15.8 ± 0.4	15.9 ± 0.3

Values are mean ± SEM. In this crossover study, there were no significant changes seen in the 8 weeks between visits.
Abbreviations as in Table 1.

to 34.0 ± 2.6% when hearts were perfused with post-infusion dialysate (n = 10 hearts, paired *t* test, p = 0.006) (Figure 4, left graph).

Adenosine 0.75 mg/kg. Mean infarct size was 47.1 ± 1.9% with pre-infusion dialysate, which significantly reduced to 35.7 ± 2.1% when hearts were perfused with post-infusion dialysate (n = 10, paired *t* test, p = 0.001) (Figure 4, right graph).

TABLE 3 Participant Demographics (Protocol 3)

	0.25 mg/kg (n = 10)	0.75 mg/kg (n = 10)
Age (yrs)	60.4 ± 4.1	61.9 ± 2.4
Male	9	9
BMI (kg/m^2)	28.0 ± 1.1	25.9 ± 1.1
Resting		
MAP (mm Hg)	96.8 ± 6.5	92.9 ± 3.0
HR (beats/min)	66.8 ± 2.8	62.2 ± 2.3
Smoking status		
Current or recent	3 (30)	1 (10)
Ex-smoker	3 (30)	4 (40)
Never	4 (40)	5 (50)
Hypertension	3 (30)	4 (40)
Family history	2 (20)	4 (40)
Previous MI	1 (10)	4 (40)
Statin use	6 (60)	9 (90)
ACE inhibitor use	4 (40)	4 (40)
Aspirin use	9 (90)	10 (100)
Coronary disease		
No significance (%)	4 (40)	1 (10)
1-vessel (%)	3 (30)	3 (30)
2-vessel (%)	2 (20)	4 (40)
3-vessel (%)	1 (10)	2 (20)

Values are mean ± SEM or n (%). There were no significant between-group differences.
ACE = angiotensin converting enzyme; MI = myocardial infarction; other abbreviations as in Table 1.

DISCUSSION

The novel findings from this study are as follows: 1) adenosine receptor activation is a mediator of human remote pre-conditioning; 2) adenosine receptors are involved in the trigger phase of signaling; 3) adenosine receptors mediate rIPC in part through release of circulating cardioprotective factor(s); and 4) pharmacological stimulation of arterial adenosine receptors liberates a circulating cardioprotective factor(s). These findings have implications for the design and interpretation of ongoing clinical studies in rIPC. Importantly, we have identified a novel pathway that liberates circulating cardioprotective factor(s), which can be pharmacologically stimulated in humans.

In protocol 1, we delivered caffeine systemically and this inhibited the protective effect of rIPC. In protocol 2, we delivered the caffeine into the brachial artery to achieve inhibition of adenosine receptors in either the trigger or target limb. We demonstrate that adenosine receptor inhibition in the trigger limb blocks the induction of systemic rIPC and inhibits release of circulating cardioprotective factor(s). However, rIPC is preserved when caffeine is delivered into the target organ, and release of the circulating factor(s) after rIPC is also maintained. For methodological reasons, we used FMD to assess endothelial responses of the contralateral arm when we infused into the trigger arm, because we did not deem it appropriate to cannulate both brachial arteries. We used venous occlusion plethysmography when we infused into the target tissue, because FMD cannot be practically performed in this situation due to the proximity of the ultrasound probe to the arterial cannula and its impingement upon it. Caffeine has been used to investigate pre-conditioning pathways in humans previously (33).

Four nonhuman studies investigating kidney or intestinal ischemia have reported that adenosine receptor antagonists inhibit cardiac rIPC (27,35-37). One study has shown no effect of adenosine antagonist on renal rIPC (38). The explanation for these observations may relate to differential expression of adenosine receptor subtypes in different tissues, activation of differing signaling pathways such as neurogenic or circulating factor(s) and/or species differences. There are no data available regarding human rIPC and adenosine, but our findings are consistent with animal data showing that adenosine receptor activation in the trigger organ is important in rIPC. Whether adenosine receptor activation is

FIGURE 4 **Exogenous Adenosine Releases a Circulating Cardioprotective Factor**

Protocol 3 results. **Left** shows the effect of dialysate produced before and after 0.25 mg/kg adenosine on myocardial infarction (MI), showing that there is a significant reduction in MI, suggesting release of a circulating factor(s). **Right** shows similar results after infusion of 0.75 mg/kg adenosine.

important in the target organ may depend upon the target tissue, but in our study, it does not appear to be important for human endothelial rIPC.

The nature of the circulating factor(s) that contribute to rIPC is not known. Our group has shown that rIPC liberates <15-kDa, hydrophobic, cross-species factor(s) that can be dialyzed. In animals, it can be released by peripheral nerve activation, arterial adenosine infusion, or capsaicin, and its release is inhibited in patients with diabetic neuropathy (19,39,40). We now show that activation of arterial adenosine receptors liberates a circulating cross-species cardioprotective factor(s) in humans. Whether this factor(s) is itself important in mediating the clinical effects of rIPC or a surrogate is not known, but our findings raise the possibility to investigate the biological and clinical effects of pharmacological enhancement of the rIPC phenotype. Arterial adenosine has been used in one surgical study as a post-conditioning stimulus. Thirty patients undergoing valve replacement received 1.5 mg/kg adenosine through the arterial aortic catheter at the end of the operation (41). This was associated with a transient nearly 30 mm Hg reduction in blood pressure, but reduced troponin I release, lessened inotrope requirement, and reduced intensive care stay. With this study in mind, our experimental protocol was designed to try and replicate the activation of a conditioning pathway using lower doses of adenosine. If these cardioprotective effects could be duplicated by lower doses with lesser side effects in a peripheral artery, then this approach may have more widespread applicability. Our data suggest that lower doses in a peripheral artery are better tolerated and may achieve biologically effective humoral cardioprotection. Indeed, this has relevance for future work in the field of rIPC and may eventually lead to translation into clinical studies with testing during cardiac surgery or even primary angioplasty for myocardial infarction. Before this step, however, further preclinical work is warranted to optimize factors such as timing, dosage, and delivery route, as well as to gain further data on potential clinical effectiveness and safety.

STUDY LIMITATIONS. As with all studies using competitive inhibitors, it is possible that off-target effects of caffeine influence our results, but there are currently no available adenosine receptor subtype-specific antagonists for human use. It is also true that our model is specific to human endothelial rIPC rather than human cardiac rIPC, and although we term the liberated factor(s) cardioprotective, it has only been demonstrated to truly protect myocardium in experimental animal models, and its effect on human myocardium in vivo is unknown. However, further work is necessary in this regard.

It should also be noted that the volunteers in our studies in protocols 1 and 2 were young and free of chronic health problems, and so as such, were not representative of the more elderly individuals, with multiple comorbidities and on medications, who more typically present with coronary disease syndromes, and in whom this intervention is most relevant. Indeed, it may be that pre-conditioning in these

individuals is modified by both their disease processes (42) and their drug therapies (43,44). However, the volunteers in protocol 3 were individuals with proven or suspected coronary disease undergoing angiography, and we were nevertheless able to demonstrate release of cardioprotective factor(s) into their serum after adenosine infusion, suggesting that further investigation is warranted.

Despite these limitations, however, not only are our studies internally consistent in showing that inhibition of rIPC by caffeine in the trigger limb is associated with lack of circulating factor(s), but we also show that activation of adenosine receptors can release such factor(s).

CONCLUSIONS

Adenosine receptors activation in the trigger limb signals rIPC in part through release of circulating cardioprotective factor(s). This can be reproduced by arterial infusion of adenosine. The clinical implications of these findings to potentially mimic rIPC need further investigation.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: IR injury is a common cause of cardiovascular morbidity and mortality. Effective means to reduce IR may lead to large clinical gains with activation of innate pre-conditioning mechanisms potentially able to reduce infarct size by 50%. Currently, our understanding of the physiology of pre-conditioning is incomplete, and clinical data are mixed. Here, we demonstrate a key role for adenosine receptors in activating human pre-conditioning and demonstrate the liberation of circulating pre-conditioning factor(s) by exogenous adenosine.

TRANSLATIONAL OUTLOOK: Future translational studies should examine the effects of specific adenosine receptor subtypes to further clarify the physiology of human pre-conditioning and to select potential drug candidates and doses for future trials. Studies should also be conducted to ascertain whether adenosine receptor activation may induce additive effects to those of mechanical ischemic pre-conditioning, and if positive, clinically powered randomized controlled trials should be considered in patients undergoing cardiac surgery or presenting with acute myocardial infarction.

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KEY WORDS adenosine, endothelium, ischemia, pre-conditioning