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Ischaemia–reperfusion injury impairs tissue plasminogen activator release in man

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Aims Ischaemia–reperfusion (IR) injury causes endothelium-dependent vasomotor dysfunction that can be prevented by ischaemic preconditioning. The effects of IR injury and preconditioning on endothelium-dependent tissue plasminogen activator (t-PA) release, an important mediator of endogenous fibrinolysis, remain unknown.

Methods and results Ischaemia–reperfusion injury (limb occlusion at 200 mmHg for 20 min) was induced in 22 healthy subjects. In 12 subjects, IR injury was preceded by local or remote ischaemic preconditioning (three 5 min episodes of ipsilateral or contralateral limb occlusion, respectively) or sham in a randomized, cross-over trial. Forearm blood flow (FBF) and endothelial t-PA release were assessed using venous occlusion plethysmography and venous blood sampling during intra-arterial infusion of acetylcholine (5–20 μg/min) or substance P (2–8 pmol/min). Acetylcholine and substance P caused dose-dependent increases in FBF ($P < 0.05$ for all). Substance P caused a dose-dependent increase in t-PA release ($P < 0.05$ for all). Acetylcholine and substance P-mediated vasodilatation and substance P-mediated t-PA release were impaired following IR injury ($P < 0.05$ for all). Neither local nor remote ischaemic preconditioning protected against the impairment of substance P-mediated vasodilatation or t-PA release.

Conclusion Ischaemia–reperfusion injury induced substance P-mediated, endothelium-dependent vasomotor and fibrinolytic dysfunction in man that could not be prevented by ischaemic preconditioning.
Clinical Trial Registration Information: Reference number: NCT00789243, URL: http://clinicaltrials.gov/ct2/show/NCT00789243?term=NCT00789243&rank=1

Keywords Endogenous fibrinolysis • Endothelium • Ischaemia–reperfusion • Preconditioning • Substance P

Introduction

Acute arterial occlusion can lead to end-organ ischaemia and, ultimately, infarction. Although treatment is usually directed at prompt restoration of flow in the occluded artery, reperfusion itself may trigger additional injury beyond that induced by ischaemia alone, although the mechanism is poorly understood. Impaired endothelium-dependent vasomotor function following ischaemia and reperfusion has been demonstrated in experimental models[1] and *in vivo* in man,[2] and can be prevented by prior exposure to intermittent sublethal ischaemia—ischaemic preconditioning—induced either locally in the vascular bed immediately downstream of the preconditioning stimulus, or remotely in an organ anatomically distant from the preconditioning stimulus.[2,3] The effects of ischaemia–reperfusion (IR) injury and ischaemic preconditioning on other important aspects of endothelial function remain unknown.

In addition to the regulation of vascular tone, the endothelium is intimately involved in the prevention of intravascular thrombosis through the endogenous fibrinolytic pathway.[4] The activation of plasminogen is a critical step in endogenous fibrinolysis, with tissue plasminogen activator (t-PA) being the main plasminogen activator in man. However, only a relatively small proportion of the t-PA present in plasma is functionally active, largely due

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to binding and inhibition by the serpin, plasminogen activator inhibitor type 1 (PAI-1). In plasma, PAI-1 is present in molar excess over t-PA, hence for active unbound t-PA to reach a thrombus, rapid local release is vital, fibrinolysis being much more effective if t-PA is incorporated during, rather than after, thrombus formation. Thus, the ability of the endothelium to release t-PA rapidly plays a key role in determining local endogenous fibrinolytic activity, with a reduction in local endothelial t-PA release favouring thrombus formation and propagation and, ultimately, vascular obstruction.[5]

Using forearm venous occlusion plethysmography and the endothelium-dependent agonist, substance P, we have demonstrated that substance P-induced t-PA release is impaired in the forearm circulations of smokers[6] and in patients with coronary artery disease—the degree of impairment of t-PA release correlating with the risk of future cardiovascular events.[7] Similarly, endothelial t-PA release is impaired in the coronary circulation of patients with atherosclerotic coronary disease, the extent of impairment being inversely proportional to plaque burden.[8]

Preclinical models report an increase in local t-PA concentrations immediately following IR injury, which return to basal levels within 10 min although these findings may be confounded by intravascular pooling of t-PA.[9–13] In man, the acute effects of IR injury on endogenous fibrinolysis are less clear. Plasma t-PA concentrations are increased in patients with critical limb ischaemia,[14] but reduced in patients with intermittent claudication.[15]

We hypothesized that, in keeping with endothelium-dependent vasodilatation to acetylcholine, IR injury would impair endothelium-dependent endogenous fibrinolysis and that ischaemic preconditioning would protect against this. To address this, we examined the effect of IR injury and ischaemic preconditioning on substance P-mediated vasodilatation and t-PA release *in vivo* in man.

Methods

The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Lothian Research Ethics Committee approved the study and all subjects gave written informed consent.

Subjects

Twenty-two healthy male volunteers were recruited. All subjects were non-smoking, taking no regular medications and clinically well. Subjects abstained from vasoactive drugs for 7 days, caffeine-containing drinks and alcohol for 24 h, and fasted for at least 4 h before each visit. Subjects attended either for one study visit (Protocol 1) or for three study visits (Protocol 2), with at least 2 weeks between each visit.

Study protocols

Protocol 1

Ischaemia–reperfusion injury was induced by cuff inflation around the non-dominant upper arm to 200 mmHg for 20 min in 10 healthy subjects (Protocol 1, *Figure 1A*). Forearm blood flow (FBF) responses to intra-arterial acetylcholine (5–20 μg/min) were assessed at baseline and at 15 and 45 min after IR injury.

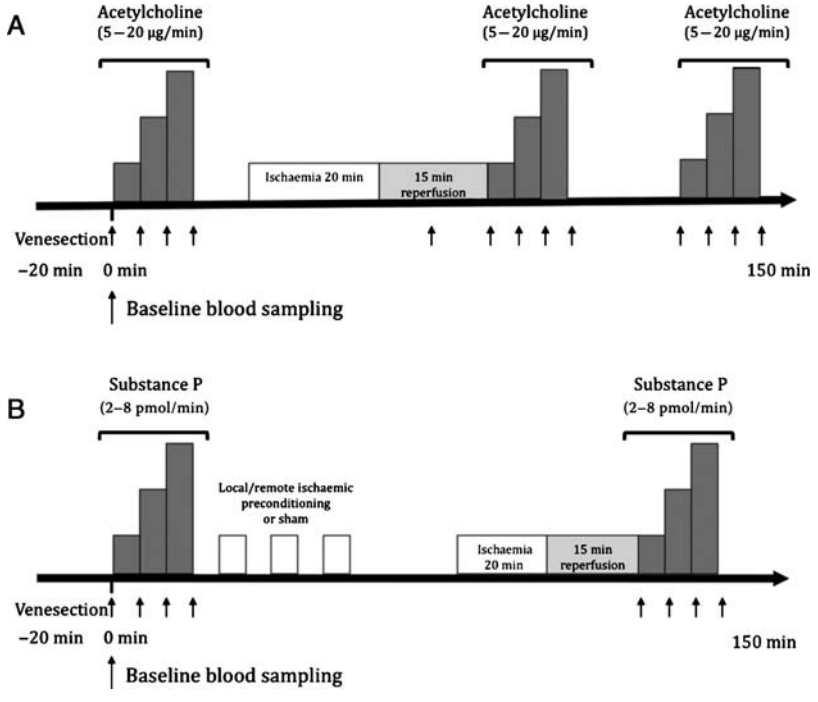

Figure 1 Study protocols.

Protocol 2

Local or remote ischaemic preconditioning, or sham, was performed prior to the induction of IR injury in the non-dominant arm in 12 subjects in a randomized, blinded, cross-over fashion (Protocol 2, *Figure 1B*). Local or remote ischaemic preconditioning was induced by cuff inflation around the non-dominant or dominant upper arm, respectively, to 200 mmHg for 5 min on three occasions 5 min apart. During sham procedures, the upper arm cuff was inflated to 10 mmHg for a similar time period. Forearm blood flow and plasma concentrations of t-PA and PAI-1 antigen and activity during intra-arterial infusion of substance P (2–8 pmol/min) were assessed at baseline and 15 min after IR injury.

Intra-arterial drug administration

All studies were performed with patients lying supine in a quiet, temperature-controlled (22–25°C) room. Under local anaesthesia, a 27-gauge needle (Cooper Needle Works Ltd) was inserted into the brachial artery of the non-dominant arm. The rate of intra-arterial drug infusion remained constant throughout at 1 mL/min.

Forearm blood flow and blood pressure

Bilateral arterial FBF was measured using venous occlusion plethysmography (Hokanson EC6 plethysmograph, DE Hokanson, Inc., USA, and Chart v5.0.1 software, ADInstruments Ltd, UK) as previously described.[16] Plethysmographic data were extracted from the Chart data files, and FBF calculated for individual venous occlusion cuff inflations. Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. Heart rate and blood pressure were recorded in the non-infused arm at intervals throughout the study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical, Inc., Japan).

Venous blood samples and assays

For Protocol 2, 17-gauge venous cannulae were inserted bilaterally into a large antecubital vein. Ten millilitres of blood was withdrawn simultaneously from each arm at baseline and in the last minute of each drug infusion period and collected into acidified, buffered citrate (Biopool Stabilyte, Umeå) for t-PA assays and citrate (Monovette, Sarstedt, Numbrecht) for PAI-1 assays. Estimated net release of t-PA antigen and activity were defined previously as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused FBF) and the concentration difference between the infused and non-infused arms.[16] Samples were kept on ice before being centrifuged at 2000*g* for 30 min at 4°C. Platelet-free supernatant was decanted and stored at −80°C before assay. Plasma t-PA (t-PA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1, Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined using enzyme-linked immunosorbent assays.

Data analysis and statistics

The software package GraphPad PRISM 5 was used for analysis (GraphPad Software, Inc., La Jolla, CA 92037, USA). All data are expressed as mean ± SEM unless otherwise stated. Data were analysed using repeated measures two-way ANOVA, one-way ANOVA, and paired Student's *t*-test, where appropriate. Statistical significance was defined as a two-sided *P*-value <0.05.

Results

There were no differences in the heart rate, blood pressure, haematocrit, FBF, or t-PA release at baseline between or during visits (*Table 1*; Supplementary material online, *Table S1* and *Figure S1*). Ischaemia–reperfusion injury and ischaemic preconditioning were well tolerated by all subjects, with no reported side effects.

Vasomotor function

Intra-arterial infusion of acetylcholine caused a dose-dependent increase in the FBF in the infused arm in all studies ($P < 0.05$ for all) that was attenuated by IR injury ($P = 0.007$; *Figure 2*).

Substance P caused a dose-dependent increase in FBF in the infused arm in all studies ($P < 0.05$ for all) that was impaired following IR injury ($P < 0.01$ for all; *Table 2*, *Figure 3*). Compared with sham, neither local nor remote ischaemic preconditioning altered the reduction in substance P-mediated vasodilatation following IR injury (*Table 2*, *Figure 3*).

Fibrinolytic function

Substance P caused a dose-dependent increase in absolute t-PA antigen ($P < 0.0005$ for all; *Table 3*) and activity ($P < 0.0001$ for all; *Table 3*), and net release of t-PA antigen and activity in the infused arm in all studies ($P < 0.01$ for all; *Figure 4*) that was attenuated by IR injury ($P < 0.05$ for all; *Figure 4*). Compared with sham, neither local nor remote ischaemic preconditioning altered

Table 1 Baseline characteristics

Parameter	Protocol 1	Protocol 2*		
	IR injury	Sham + IR	Local IPC + IR	Remote IPC + IR
Age, years	23.6 ± 1	22.3 ± 1	22.3 ± 1	22.3 ± 1
Body mass index, kg/m^2	23.8 ± 1	23.5 ± 0.5	23.5 ± 0.5	23.5 ± 0.5
Heart rate, b.p.m.	58.0 ± 3	62.7 ± 2	61.0 ± 2	64.8 ± 2
Mean arterial pressure, mmHg	92.4 ± 2	96.5 ± 2	96.3 ± 2	93.5 ± 3
Baseline haematocrit, %	43.6 ± 1	41.6 ± 1	41.7 ± 1	39.8 ± 2

IR, ischaemia–reperfusion injury; IPC, ischaemic preconditioning.
*$P > 0.05$ for all, paired Student's *t*-test; sham + IR vs. local IPC + IR and sham + IR vs. remote IPC + IR.

substance P-mediated release of t-PA antigen or activity following IR injury (*Table 3*, *Figure 4*).

There were no differences in plasma PAI-1 concentrations at baseline or following IR injury (Supplementary material online, online data table).

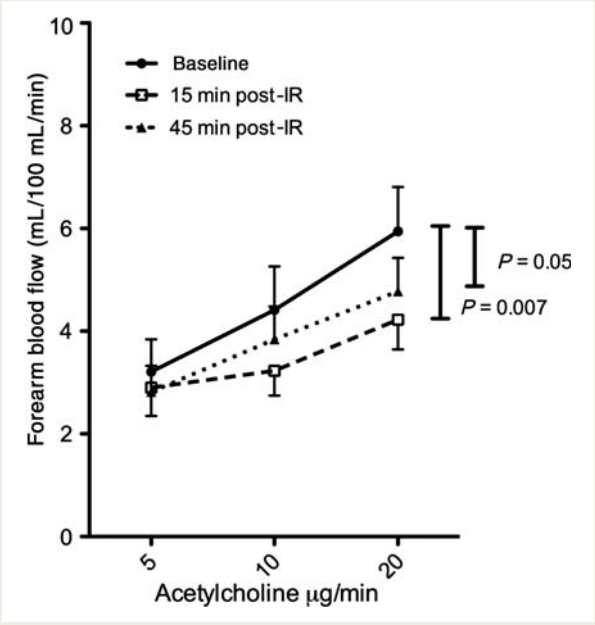

Figure 2 Effect of ischaemia–reperfusion injury on acetylcholine-mediated vasodilatation. IR indicates ischaemia–reperfusion (Protocol 1). Data analysed using two-way ANOVA with repeated measures.

Discussion

To our knowledge, this is the first study to examine the effect of IR injury and ischaemic preconditioning on endogenous fibrinolysis in man. Having first confirmed the validity of the forearm model using acetylcholine, we have demonstrated that substance P-induced vasodilatation and endothelial t-PA release are impaired following IR injury. Neither local nor remote ischaemic preconditioning protected against the impairment of substance P-mediated vasodilatation or t-PA release induced by IR injury.

A major finding of this study is that IR injury attenuates the dynamic release of t-PA from the endothelium in the human peripheral vasculature *in vivo* in man. Preclinical data, predominantly from *ex vivo* models, suggest that short periods of IR injury are associated with increased local t-PA concentrations immediately after the onset of reperfusion.[9–13] In these experimental studies, t-PA concentrations peaked 2–5 min after the onset of reperfusion and returned to baseline within 10 min of reperfusion. However, the capacity of the endothelium to release t-PA appeared to decline with repeated episodes of vascular occlusion.[11] The apparent conflict between preclinical data and the current study may be explained, at least in part, by temporal differences in the assessment of t-PA release relative to the onset of reperfusion. We assessed the potential for t-PA release 15 min after the ischaemic insult because we wished to avoid the potential confounding of pooling of t-PA and the effects of the direct ischaemic stimulus itself. These effects underlie the principles of why t-PA concentrations rise during the venous occlusion test.[4]

In contrast to the effects of ischaemic preconditioning on acetylcholine-induced vasodilatation, we found no effect of ischaemic preconditioning on substance P-induced vasodilatation or endothelial t-PA release following IR injury. The reason for these

Table 2 Effect of ischaemia–reperfusion injury alone or ischaemia–reperfusion injury preceded by local or remote ischaemic preconditioning on substance P-mediated vasodilatation in the infused and non-infused arms

Parameter	Baseline				Post-IR			
	Baseline	Substance P (pmol/min)			Baseline	Substance P (pmol/min)		
		2	4	8		2	4	8
Sham-IPC + IR								
FBF, mL/100 mL/min								
Infused	1.8 ± 0.2	6.2 ± 0.6	7.3 ± 0.7	8.9 ± 0.8*, **	1.9 ± 0.2	4.7 ± 0.7	5.3 ± 0.8	6.6 ± 0.9*, **
Non-infused	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.9 ± 0.2	1.7 ± 0.3	1.8 ± 0.4	1.8 ± 0.5	1.8 ± 0.4
Local IPC + IR								
FBF, mL/100 mL/min								
Infused	2.3 ± 0.3	6.2 ± 0.5	7.7 ± 0.6	9.7 ± 0.8*, ***	2.2 ± 0.3	5.0 ± 0.7	5.9 ± 0.8	7.2 ± 1.0*, ***
Non-infused	2.0 ± 0.2	2.0 ± 0.2	1.9 ± 0.2	2.2 ± 0.3	2.0 ± 0.3	1.9 ± 0.2	1.9 ± 0.3	2.0 ± 0.3
Remote IPC + IR								
FBF, mL/100 mL/min								
Infused	2.4 ± 0.4	6.4 ± 0.6	7.8 ± 1.0	10.1 ± 1.3*, ****	2.3 ± 0.3	5.4 ± 0.5	6.4 ± 0.6	7.8 ± 0.9*, ****
Non-infused	2.0 ± 0.3	1.8 ± 0.3	1.8 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	1.8 ± 0.3	1.7 ± 0.2	1.8 ± 0.3

FBF, forearm blood flow; IPC, ischaemic preconditioning; IR, ischaemia–reperfusion injury.
ANOVA dose response *$P < 0.0001$ for all; ANOVA baseline vs. post-IR **$P < 0.0001$, ***$P = 0.012$, ****$P = 0.014$.

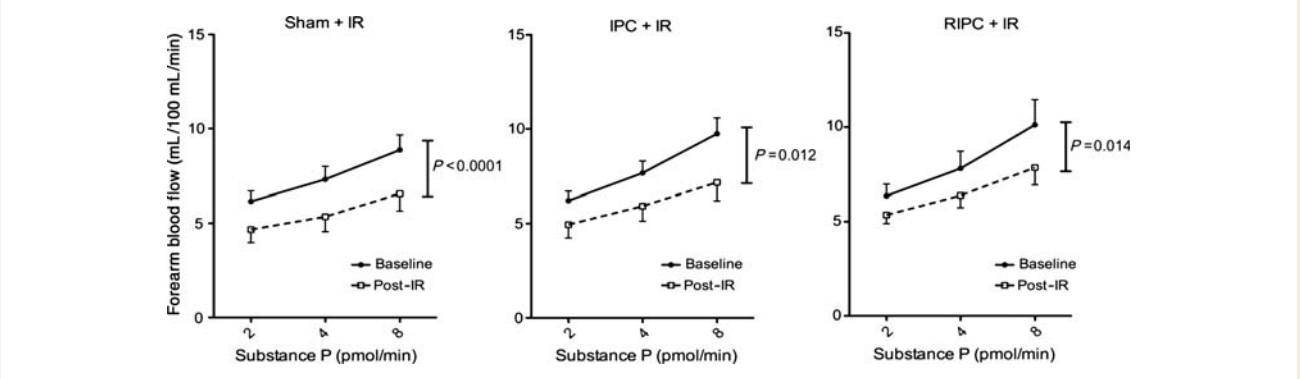

Figure 3 Effect of ischaemia–reperfusion injury alone and ischaemia–reperfusion injury preceded by local or remote ischaemic preconditioning on substance P-induced vasodilatation (Protocol 2). IR indicates ischaemia–reperfusion; IPC, local ischaemic preconditioning; and RIPC, remote ischaemic preconditioning. Data analysed using two-way ANOVA with repeated measures.

Table 3 Effect of ischaemia–reperfusion injury alone or ischaemia–reperfusion injury preceded by local or remote ischaemic preconditioning on substance P-mediated tissue plasminogen activator antigen and activity in the infused and non-infused arms

Parameter	Baseline				Post-IR			
	Baseline	Substance P (pmol/min)			Baseline	Substance (P pmol/min)		
		2	4	8		2	4	8
Sham-IPC + IR								
t-PA antigen, ng/mL								
Infused	3.2 ± 0.7	3.8 ± 0.7	3.8 ± 0.6	5.2 ± 0.7*	2.9 ± 0.7	3.0 ± 0.6	3.4 ± 0.7	4.7 ± 0.8*
Non-infused	3.0 ± 0.7	3.3 ± 0.7	3.6 ± 0.7	3.4 ± 0.6	2.8 ± 0.6	3.2 ± 0.6	3.3 ± 0.7	3.1 ± 0.6
t-PA activity, U/mL								
Infused	0.6 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.7 ± 0.3*	0.6 ± 0.05	0.8 ± 0.1	1.1 ± 0.1	1.5 ± 0.3*
Non-infused	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.05	0.6 ± 0.05	0.6 ± 0.05
Local IPC + IR								
t-PA antigen, ng/mL								
Infused	3.1 ± 0.6	3.4 ± 0.7	3.8 ± 0.8	4.8 ± 1.1*	3.0 ± 0.7	3.2 ± 0.7	3.6 ± 0.9	4.7 ± 1.4**
Non-infused	3.1 ± 0.6	3.1 ± 0.6	3.2 ± 0.7	3.2 ± 0.6	2.9 ± 0.6	3.0 ± 0.6	3.2 ± 0.6	3.0 ± 0.6
t-PA activity, U/mL								
Infused	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.4 ± 0.2*	0.6 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	1.4 ± 0.2*
Non-infused	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.05	0.6 ± 0.05	0.6 ± 0.05	0.7 ± 0.05
Remote IPC + IR								
t-PA antigen, ng/mL								
Infused	3.5 ± 0.6	3.9 ± 0.6	4.5 ± 0.9	6.2 ± 1.2*	3.0 ± 0.6	3.3 ± 0.7	3.6 ± 0.6	4.3 ± 0.9**
Non-infused	3.2 ± 0.6	3.3 ± 0.6	3.9 ± 0.7	3.4 ± 0.6	3.3 ± 0.7	3.0 ± 0.6	3.1 ± 0.7	3.2 ± 0.6
t-PA activity, U/mL								
Infused	0.6 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	1.8 ± 0.3*	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	1.1 ± 0.3*
Non-infused	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1

t-PA, tissue-plasminogen activator; IPC, ischaemic preconditioning; IR, ischaemia–reperfusion injury.
ANOVA dose response *$P < 0.0001$ for all, **$P = 0.0004$.

apparent differences is unclear but is likely to reflect differences in intracellular signalling. Previous work has implicated the mitochondrial ATP-sensitive potassium channel in the protection of acetylcholine-mediated vasodilatation afforded by ischaemic preconditioning.[17,18] In contrast, thrombin-stimulated release of t-PA from endothelial cells appears to occur independently of both

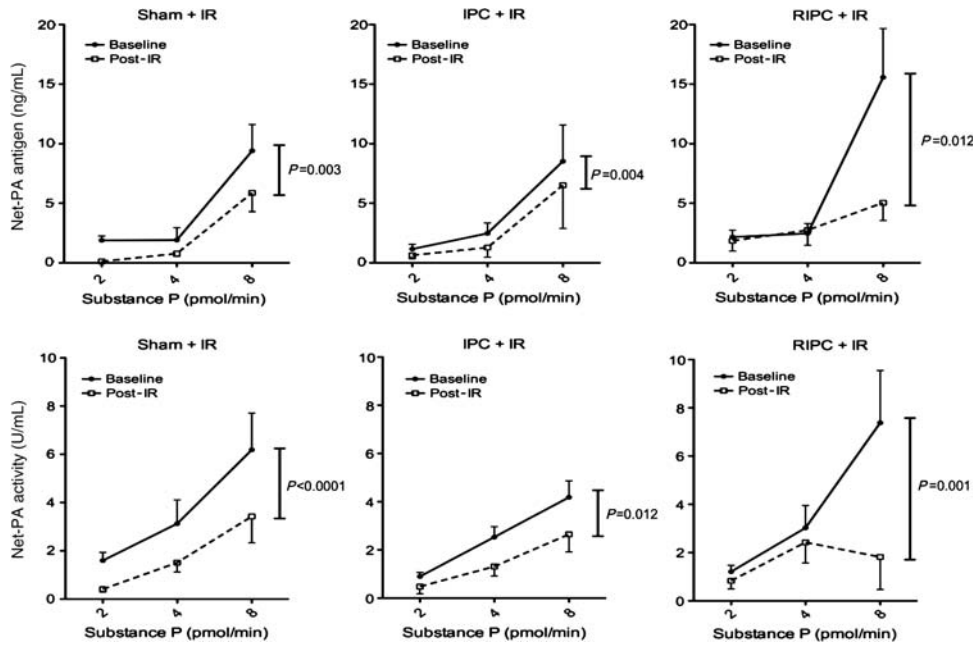

Figure 4 Effect of ischaemia–reperfusion injury alone and ischaemia–reperfusion injury preceded by local or remote ischaemic preconditioning on substance P-induced net release of tissue plasminogen activator antigen and activity (Protocol 2). IR indicates ischaemia–reperfusion; IPC, local ischaemic preconditioning; RIPC, remote ischaemic preconditioning; and t-PA, tissue-plasminogen activator. Data analysed using two-way ANOVA with repeated measures.

nitric oxide and K^+ channels *in vitro*,[19] and if anything, in contrast to acetylcholine-mediated vasodilatation, nitric oxide inhibition augments thrombin-induced t-PA release *in vivo* in man.[20]

Limited preclinical data have suggested a potential role for substance P in the mechanism of ischaemic preconditioning.[21] We have previously demonstrated that forearm vascular responses to substance P are highly reproducible at 30 min and 7 days.[22] Taken together, our results do not support the hypothesis that substance P-induced signalling protects against endothelial dysfunction induced by IR injury, although we acknowledge that the current study was not designed to specifically address this issue.

Clinical relevance

Our findings have potentially significant clinical implications. Despite optimal antithrombotic therapy, early arterial reocclusion and microvascular obstruction remain recognized complications following reperfusion therapy for acute myocardial infarction and are associated with adverse clinical outcomes.[23–26] A number of mechanisms have been implicated in the pathophysiology of microvascular obstruction, or 'no reflow', including vasomotor dysfunction and vasospasm, localized oedema and inflammation,[27] coronary micro-embolization,[28] and *in situ* thrombus formation.[23,29] Our findings suggest a novel mechanism, namely an inducible defect in local endogenous fibrinolytic activity, favouring local thrombus formation in the pathophysiology of microvascular obstruction and re-infarction. In support of this hypothesis, streptokinase reduces myocardial congestion and improves microvascular perfusion in a canine model of IR injury[30] and acutely improves indices of coronary microvascular perfusion when administered immediately following primary percutaneous coronary intervention in patients with ST-segment elevation myocardial infarction.[31]

Direct assessment of coronary endothelial function *in vivo* in man necessitates complex invasive studies with obvious limitations. The accessibility of the forearm vascular bed makes it an attractive model by which to assess vascular function *in vivo*. Although less susceptible to atherosclerosis and thrombosis, consistent findings between the peripheral[2,6,32] and coronary circulations[8,33,34] provide support for the forearm model as a surrogate for the coronary circulation.[35]

Limitations

We employed a robust, randomized, cross-over study design. Although study investigators were blinded to the intervention performed at each visit (IR injury, ischaemic preconditioning, or sham), for obvious reasons it was not possible to ensure blinding of the study subjects. Forearm venous sampling during local infusion of agonists is a well established technique in the assessment of endogenous t-PA release.[4,6,7,36] To minimize the potential for regional variation in venous effluent, venous sampling is always performed from a large vein located in the antecubital fossa.

Intravascular thrombus formation is dependent on a number of factors, including the endothelial function, the coagulation cascade, and platelets.[5] We have previously demonstrated that IR injury increases markers of platelet activation and that remote ischaemic preconditioning protects against this platelet activation.[37] The current study was designed to examine the effects of IR injury

and ischaemic preconditioning on a specific aspect of endothelial function, t-PA release, intimately involved in thrombus formation. In addition to t-PA antigen, we assessed enzymatic activity using a standard immunological technique. We acknowledge that this model may be relatively simplistic in the context of the complex *in vivo* response to thrombus formation. There are, however, obvious methodological difficulties in directly assessing the effect of endogenous t-PA release on arterial thrombosis *in vivo* in man. Further work is required to examine the effects of ischaemic preconditioning and IR injury on the coagulation cascade and markers of inflammation *in vivo* in man.

In summary, we have demonstrated that substance P-induced endothelium-dependent vasodilatation and t-PA release are impaired following IR injury in the human forearm. In contrast to acetylcholine, neither local nor remote ischaemic preconditioning protected against the impairment of substance P-mediated vasomotor and fibrinolytic function induced by IR injury. Our findings support an inducible defect in endogenous fibrinolysis as a novel mechanism in the pathophysiology of IR injury in man.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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