

Angiotensin-Converting Enzyme Inhibition Enhances a Subthreshold Stimulus to Elicit Delayed Preconditioning in Pig Myocardium

M. T. Jaberansari, MD,* Gary F. Baxter, PhD, FIBIOL,† Cecile A. Muller, PhD,‡ Sean E. Latouf, MD,‡ E. Röth, MD, DSc,* Lionel H. Opie, MD, DPHIL, FACC,‡ Derek M. Yellon, DSc, FACC†

Pécs, Hungary; London, United Kingdom; and Cape Town, South Africa

OBJECTIVES	We assessed the effect of angiotensin-converting enzyme (ACE) inhibition in combination with a subthreshold preconditioning (PC) stimulus to elicit delayed preconditioning against infarction in pig myocardium.
BACKGROUND	Bradykinin triggers early PC. Angiotensin-converting enzyme inhibitors increase local bradykinin levels via inhibition of kinin breakdown and have been shown in experimental studies to augment early protection afforded by PC. A role for bradykinin in eliciting delayed PC has not so far been identified.
METHODS	We used a two-day protocol. On day 1 (closed chest), pigs were either sham-operated (group 1) or preconditioned, using balloon catheter inflation of the left anterior descending (LAD) coronary artery, with either a full (4 × 5 min PC, group 2) or subthreshold PC stimulus (2 × 2 min PC, group 3). Additional groups were pre-treated with perindoprilat (0.06 mg/kg i.v.) before sham (group 4) or subthreshold PC (group 5). On day 2 (open chest), all pigs were subjected to 40 min occlusion of the LAD followed by 3 h of reperfusion. Infarct size was determined by tetrazolium staining.
RESULTS	Group 1 had a mean infarct size of 42.8 ± 3.2% of the risk zone. Preconditioning with 4 × 5 min reduced the infarct size to 19.5 ± 3.9% (p < 0.05). Groups 3 and 4 had infarct sizes not statistically different from group 1. However, combining perindoprilat with subthreshold PC resulted in a significant limitation of the infarction (18.4 ± 3.1% p < 0.05), comparable with group 2.
CONCLUSIONS	This is the first study to show that ACE inhibition can augment a mild ischemic stimulus to induce a protected state 24 h later. (J Am Coll Cardiol 2001;37:1996–2001) © 2001 by the American College of Cardiology

Ischemic preconditioning (PC) is a powerful adaptive response whereby brief periods of ischemia confer increased myocardial tolerance to a subsequent episode of lethal ischemia. This protective response appears in two phases. Protection is seen immediately after PC and is lost within 2 to 3 h. This early phase of protection, known as “classic” PC, is followed 24 to 72 h later by a recurrence of protection called “second window” PC or delayed PC (1,2). During brief ischemia, numerous diffusible mediators are generated, including bradykinin, adenosine, catecholamines and opioid peptides, all of which are known to trigger classic PC (3–7).

Bradykinin is thought to play a particularly important role in triggering classic PC. There is evidence that myocardial ischemia leads to increased production of tissue bradykinin (8). The cardioprotective effects of bradykinin were first investigated in animal models in which angiotensin-converting enzyme (ACE) inhibitors afforded protection

during episodes of ischemia and subsequent reperfusion (4,9–11). It was hypothesized that the protective action of ACE inhibitors was associated with inhibition of bradykinin breakdown (11). This hypothesis has been confirmed with the use of HOE140 (icatibant), a bradykinin B₂ receptor antagonist that was shown to abolish such protection in animal models and in human myocardium (9,12,13).

At present, the mediators of delayed PC are incompletely characterized. There is persuasive evidence that adenosine and nitric oxide (NO) both contribute to trigger the adaptation that results in delayed protection (14,15). We have observed that exogenously administered bradykinin can elicit protection against infarction 24 h later in rat myocardium (16). Because it is now widely accepted that the ACE inhibitors exert their effect in PC through increased bradykinin levels, we developed a model to investigate the influence on delayed PC of an ACE inhibitor, perindoprilat. Accordingly, we studied the delayed PC effect in pigs using brief percutaneous transluminal coronary angioplasty (PTCA) balloon inflation before a sustained coronary occlusion insult 24 h later. We hypothesized that an ACE inhibitor, when combined with a subthreshold PC stimulus (2 × 2 min periods of ischemia), could augment myocardial bradykinin levels sufficiently to trigger a significant delayed cardioprotection in the pig myocardium.

From the *Department of Experimental Surgery, PTE, Pécs, Hungary; †The Hatter Institute, UCL Medical School, London, United Kingdom; and ‡Cape Heart Centre, University of Cape Town, South Africa. G. F. Baxter was supported by a British Heart Foundation fellowship (FS/97001). We thank the Hatter Foundation for continued support.

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Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
ANOVA	=	analysis of variance
BP	=	blood pressure
i.v.	=	intravenously
LAD	=	left anterior descending coronary artery
LCA	=	left coronary artery
NO	=	nitric oxide
PC	=	ischemic preconditioning
PTCA	=	percutaneous transluminal coronary angioplasty
VF	=	ventricular fibrillation
VT	=	ventricular tachycardia

METHODS

Pigs (Large White crossed with Landrace) of either gender, weighing 27 to 30 kg, were used for this study. All animals were cared for according to the recommendations from the Declaration of Helsinki and the Guiding Principles in the care and use of animals.

Anesthesia. The experimental procedure encompassed two days. On day 1, animals underwent coronary catheterization for either control or a PC protocol. On day 2, 22 to 24 h later, open-chest animals were subjected to acute myocardial infarction. For each procedure, pigs were pre-medicated with ketamine (10 mg/kg, intramuscularly) and diazepam (1 mg/kg). Surgical anesthesia was induced with thiopentone sodium 10 mg/kg intravenously (i.v.) and maintained using a gaseous mixture of nitrous oxide/oxygen (ratio 2:3), with 1.0% to 1.5% isoflurane. Standard limb electrocardiograms and systemic blood pressure (BP) were monitored continuously and recorded at regular intervals using a Siemens Sirecust hemodynamic monitor. Arterial pO₂ and pH were maintained within the physiologic range (120 to 160 mm Hg, 7.43 to 7.48 respectively) by adjusting gaseous mixture ratio and the frequency of ventilation.

Coronary catheterization procedure (day 1). The right carotid artery was isolated and cannulated with a 7F arterial sheath for PTCA. Under angiographic guidance, a 6F amplatz left 1 guiding catheter was inserted into the artery and advanced down the carotid artery and the arch of aorta and guided into the left coronary artery (LCA). Left coronary angiography was then performed in oblique view to delineate the LCA and its main branches. A 0.014-inch floppy guide wire was pushed down the guiding catheter and maneuvered such that it was positioned in the distal LAD. An inflatable balloon catheter was inserted over the floppy guide wire and positioned halfway to two-thirds down the LAD as defined by the angiogram. Control measurements were recorded and heparin was administered (1,000 IU i.v.). Animals were randomly allocated to sham or preconditioned groups. In the case of sham-operated animals, the balloon was not inflated. A sketch of the image was made, marking the site of intended ligature as well as the branches proximal and distal to the ligature. Precondi-

tioned animals underwent serial inflations (three bars) with intermittent 10-min reperfusions (see Treatment Protocols). A second angiogram ensured complete occlusion of the LAD distal to the balloon. Electrocardiograms and BPs were recorded before and after every balloon inflation. After completion of the procedure, catheters were removed, tissue and skin incisions were sutured, and the animal was extubated upon establishing a regular breathing pattern. Each pig received ampicillin 5,000 IU i.v., as well as heparin-calcium 5,000 IU subcutaneously before being returned to the pen.

Infarction procedure (day 2). The animal was pre-medicated and anesthetized as described earlier. After surgical anesthesia was established, a mid-line thoracotomy was performed. The animal was placed on the operating table in the same oblique position as on the previous day. Using the sketch we were easily able to determine the location of the balloon site bordered by the proximal and distal branches. The LAD was then prepared and isolated from the surrounding tissue at the same region as on day 1. A 3-0 silk suture was passed around the isolated portion of the LAD, and the ends of the thread were passed through a small vinyl tube to form a snare. The right femoral artery was cannulated for BP measurement and blood sampling. Before the snare was tightened, the pig received heparin 1,000 IU i.v. and thereafter every hour throughout the experiment. By tightening the snare, the LAD was occluded for 40 min followed by 180 min of reperfusion. When ventricular fibrillation (VF) occurred, direct-current cardioversion was applied immediately. If defibrillation could not be accomplished within 60 s, the experiment was terminated. Ventricular tachycardia (VT) was defined as more than four consecutive ventricular premature beats. Runs of VF or VT were considered terminated if they were followed by three or more normally conducted sinus beats.

Infarct size assessment. After 180 min of reperfusion, the LAD was ligated at the same location. To identify the risk zone, 5 ml patent blue (7.5%) solution (May & Baker, United Kingdom) in saline was injected into the left atrium. The heart was then excised, total left ventricular mass was recorded, and the risk zone (not stained) was isolated layer by layer. To ensure anatomical consistency of the ischemic risk zone, a predetermined exclusion criterion was a risk zone <15% or >30% of LV. The risk zone was sectioned at 3-mm thickness from apex to base. Slices were incubated in 1% triphenyltetrazolium chloride at 37°C for 15 min and fixed in 10% formalin for 24 to 48 h to enhance the contrast between viable and infarcted myocardium. The boundaries of the infarcted regions were traced onto acetate sheets and assessed by computerized planimetry (Summa Sketch II, Summa Graphics). The volumes of infarcted and risk zones were calculated by multiplication of each area with the thickness of the slice.

Treatment protocols. Treatment protocols are illustrated in Figure 1. There were five experimental groups. On day 2, all animals were anesthetized and subjected to 40 min

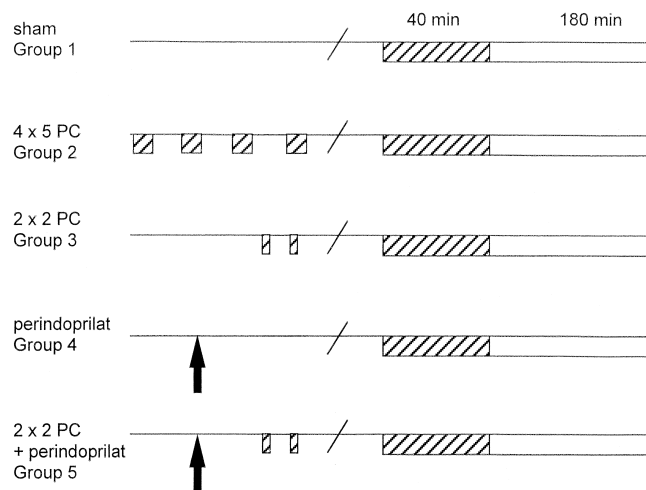


Figure 1. Experimental protocols. For details, see text. Perindoprilat 0.06 mg/kg i.v. was given in groups 4 and 5 (arrow).

coronary artery occlusion and 180 min reperfusion. Pre-treatment on day 1 was as indicated.

GROUP 1: CONTROL. Animals were anesthetized on day 1 and instrumented for coronary catheterization but were not preconditioned.

GROUP 2: DELAYED PC. Animals underwent coronary catheterization and a full PC stimulus consisting of four 5-min balloon inflations with 10 min intermittent deflation (4 × 5 PC).

GROUP 3: SUBTHRESHOLD PC. On day 1, pigs were subjected to two cycles of 2-min balloon inflation (ischemia) with an intermittent deflation given as the subthreshold stimulus (2 × 2 PC).

GROUP 4: PERINDOPRILAT. Pigs were subjected to the control procedure as in group 1 but received perindoprilat 0.06 mg/kg i.v., a dose that we previously demonstrated would decrease plasma ACE levels in pigs by >95% (17).

GROUP 5: PERINDOPRILAT + SUBTHRESHOLD PC. On day 1, pigs were subjected to two cycles of 2-min balloon inflation (ischemia) with an intermittent deflation given as the subthreshold stimulus (2 × 2 PC); 20 min before the first balloon inflation, pigs were given perindoprilat 0.06 mg/kg i.v.

Statistical analysis. All results are expressed as mean values ± SEM. Infarct-size data were analyzed with one-way analysis of variance (ANOVA) followed by unpaired *t* test with Bonferroni's correction for multiple comparisons. Hemodynamic data were analyzed using repeated-measures ANOVA. The null hypothesis was rejected when $p < 0.05$.

RESULTS

Exclusions. A total of 54 pigs were used in this study. Fifteen pigs were excluded from final infarct analysis for the following reasons. Two animals were excluded because of air embolism in the coronary arteries during PTCA. Four

animals were excluded as a result of VF lasting >60 s. Five animals were excluded after opening the chest on day 2, when they were found to have evidence of old or recent infarction. One pig was excluded because of the presence of pericarditis on day 2. Three hearts were excluded because the risk zone was <15% of LV. The final numbers in each group were as follows: group 1, $n = 9$; group 2, $n = 8$; group 3, $n = 7$; group 4, $n = 8$; group 5, $n = 7$.

Hemodynamic data. There were no significant differences in heart rate, systemic BPs (not shown) or rate/pressure product among the five groups. Table 1 summarizes changes in heart rate and rate pressure products, an index of myocardial oxygen consumption, at selected intervals during both day 1 and day 2 of the experiments.

Risk zone and infarct size. Ischemic risk zone volume and infarct size are shown in Figure 2. Risk zone size was expressed as a percentage of total left ventricular mass. Figure 2a shows that the ischemic risk zone size was similar in all experimental groups at around 21% to 24% of LV mass. Infarct size as a percentage of the risk zone is shown in panel b. Sham-operated control pigs (group 1) had a mean infarct size of $42.8 \pm 3.2\%$ of the risk zone. Preconditioning with four 5-min coronary occlusion episodes limited the infarction to $19.5 \pm 3.9\%$ ($p < 0.05$). The milder PC stimulus (group 3) did not lead to statistically significant infarct limitation ($33.4 \pm 3.9\%$). Pre-treatment of pigs on day 1 with perindoprilat (group 4) resulted in a small but non-significant reduction in infarct size ($31.2 \pm 2.3\%$). This may be a reflection of the long biological half-life of perindoprilat. In group 5, when we combined subthreshold PC stimulus of 2 × 2 min balloon inflations in the presence of the ACE inhibitor perindoprilat, significant cardioprotection was observed ($18.4 \pm 3.1\%$), comparable with that observed in the 4 × 5 PC protocol ($19.5 \pm 3.9\%$). Thus, protection against infarction was observed 24 h after a full PC stimulus (group 2) or after a subthreshold PC stimulus augmented by perindoprilat (group 5). This protection was clearly independent of variations in risk-zone size. Further, because we and others have shown that the pig has a poorly developed native coronary collateral circulation (18,19), it is unlikely that infarct limitation is due to altered collateral flow.

Arrhythmias. Arrhythmias during the PC procedure on day 1 were rare and minor in nature. Figure 3 summarizes the incidence of VF during the 40-min coronary-occlusion period and 180-min reperfusion period on day 2. Although the majority of animals in all groups experienced some form of ventricular premature beats and/or VT (data not shown), their extent was highly variable, and no specific pattern could be determined. Fewer animals had VF during the ischemia-reperfusion period. Overall, there were no statistically significant differences in VT or VF across the groups, although there was a tendency to less reperfusion VF in group 2 and group 4.

Table 1. Hemodynamic Data

	Sham-operated (group 1)		4 × 5 PC (group 2)		2 × 2 PC (group 3)		Perind. (group 4)		2 × 2 + Perind. (group 5)	
	HR (beats/min)	RPP × 10 ³ (mm Hg-beats/min)	HR (beats/min)	RPP × 10 ³ (mm Hg-beats/min)	HR (beats/min)	RPP × 10 ³ (mm Hg-beats/min)	HR (beats/min)	RPP × 10 ³ (mm Hg-beats/min)	HR (beats/min)	RPP × 10 ³ (mm Hg-beats/min)
Day 1 control	112 ± 9	13.1 ± 1.3	127 ± 2	15.7 ± 1.0	116 ± 4	13.8 ± 0.8	119 ± 5	13.5 ± 0.9	118 ± 5	13.0 ± 0.6
PC 1	114 ± 6	13.8 ± 1.3	130 ± 5	15.2 ± 1.2	115 ± 6	13.5 ± 0.9	118 ± 5	13.3 ± 0.9	115 ± 4	12.8 ± 0.6
PC 2	113 ± 7	14.2 ± 1.4	127 ± 6	15.9 ± 1.5	115 ± 6	13.0 ± 1.1	120 ± 5	13.7 ± 0.7	113 ± 4	12.8 ± 0.6
Day 2 control	99 ± 8	11.4 ± 2.3	108 ± 10	12.1 ± 1.6*	100 ± 4	11.0 ± 0.9	98 ± 6*	9.1 ± 0.7*	99 ± 6	9.0 ± 0.9*
Lig. 20'	108 ± 8	12.1 ± 2.2	135 ± 16	13.6 ± 1.2	96 ± 3*	10.9 ± 0.3	98 ± 5*	9.4 ± 0.7*	96 ± 7*	9.0 ± 1.0*
Rep. 60'	109 ± 5	12.7 ± 1.6	129 ± 9	13.6 ± 1.0	110 ± 8	12.2 ± 1.0	141 ± 9*	12.4 ± 1.0	123 ± 8	12.7 ± 1.4

HR = heart rate; Lig. = LAD ligation; Perind. = perindoprilat; PC = preconditioning; Rep. = reperfusion; RPP = rate pressure product. *p < 0.05 versus day 1 control value in the same group. There were no significant differences among groups (repeated measures analysis of variance). Data are mean values ± SEM.

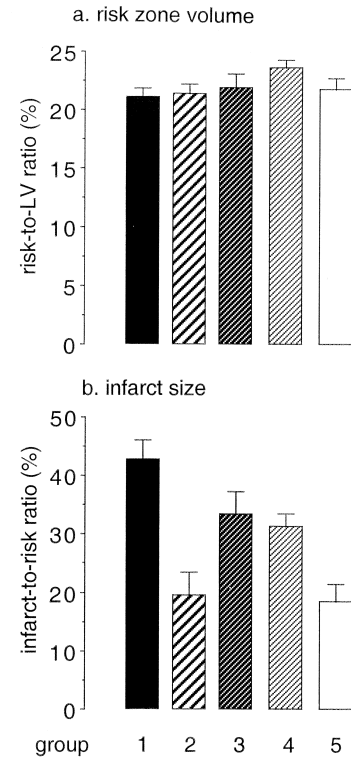


Figure 2. Infarct size. a: infarct-to-risk zone ratio; b: risk zone-to-LV ratio. *p < 0.01 versus group 1 (sham-operated control) (one-way analysis of variance).

DISCUSSION

In this study, we have observed a delayed ischemic PC effect in pig myocardium associated with limitation of infarct size 24 h after a PC event. Moreover, we found that a milder PC protocol, when combined with an ACE inhibitor, was as protective as a full PC stimulus.

Delayed PC against infarction in pig heart. Although the antistunning effects of delayed PC in pig myocardium are well characterized (15,20), this is the first study to show that delayed PC against infarction can be elicited in pig myocardium. Two previous studies did not observe a statistically significant limitation of infarction 24 h later (20,21). The extent of infarct-size limitation observed in the present study is similar to that seen in all other species in which delayed PC has been shown (22).

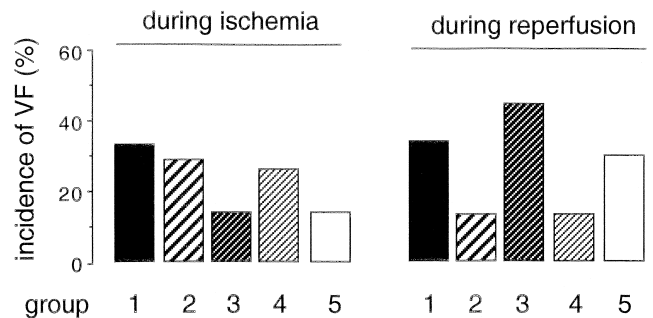


Figure 3. Incidence of ischemia and reperfusion-induced VF during the infarct protocol (day 2). Differences are not statistically significant.

ACE inhibition and classic PC. A number of reports point to the efficacy of bradykinin in mediating acute cardioprotective effects against various end points of injury in several other animal models, including rabbits, rats and dogs (23–30). Taken together, these studies strongly suggest that bradykinin, generated during the brief periods of PC ischemia, plays a major role in triggering the cellular events that ultimately result in short-term protection of the ischemic myocardium in some species, that is, classic PC. However, a number of autocrine and paracrine substances, including adenosine, bradykinin, catecholamines and opioid peptides, act in concert to induce the final cardioprotective effects of PC. There is sufficient redundancy in the system to allow for cardioprotection by other mechanisms when any individual trigger is inhibited. This “threshold” hypothesis proposed by Downey’s group (25) was based on a study in which pre-treatment with the bradykinin B₂-receptor antagonist HOE140 abolished protection from ischemic PC with 5 min of ischemia and 10 min of reperfusion, but not when the PC stimulus was four cycles of 5-min ischemia/10-min reperfusion. The hypothesis was tested in human myocardium (13), where we demonstrated that a subthreshold PC stimulus was able to precondition human myocardium in the presence of ACE inhibition and that the protective effects observed were directly due to bradykinin.

ACE inhibition and delayed PC. With regard to delayed PC, three principal diffusible molecular triggers have been identified: adenosine, NO and reactive oxygen species (22). In rabbit studies, blockade of adenosine receptors with a pharmacological inhibitor during PC resulted in a loss of protection 24 h later. Conversely, administration of a short-acting selective adenosine A₁ agonist led to infarct-size limitation 24 h later (14). A similar trigger role for NO has been identified through a similar investigative approach (15). It has been proposed that NO and superoxide anion generated in response to PC ischemia combine to form peroxynitrite anion, which is a key signaling intermediate leading to the activation of protein kinase C and downstream kinases. The ability of bradykinin to modulate NO production is well established, and it is possible that bradykinin release may be an important early event preceding the generation of NO. Recently, we have found that exogenously administered bradykinin triggers a delayed PC effect in rat heart (16). This sub-acute action of bradykinin was abolished by NO synthase inhibition. The results of the present study are consistent with a role for bradykinin in triggering delayed PC. However, in the absence of any direct measurements of bradykinin concentration in tissue or coronary sinus blood, or of studies with bradykinin receptor antagonists, we cannot conclusively implicate bradykinin at this stage. Angiotensin-converting enzyme (kininase II) is known to cleave many substrate peptides other than angiotensin I and kinins. Other substrates include enkephalins and the partially processed peptide hormone pro-enkephalin, as well as substance P (31). Inhibition of ACE could therefore augment those peptide mediators that

might contribute to triggering the PC effect. This limitation of the present study should be addressed in further studies.

Implications for ACE-inhibitor therapy. Angiotensin-converting enzyme inhibitors were introduced as antihypertensive agents. However, they have been shown convincingly to decrease mortality in patients with congestive heart failure by reducing pre-load, after-load and systolic wall stress, thus resulting in increased cardiac output without an increase in heart rate (32–35). Large clinical trials have also indicated that ACE inhibitors reduce mortality and reduce ventricular dysfunction after acute myocardial infarction (36,37), limiting left ventricular remodeling as well as myocardial ischemic events. More recently, the HOPE study clearly demonstrated that the ACE inhibitor ramipril significantly lowered the risk of major cardiovascular outcomes by 25% to 30% in a large cohort of high-risk patients (38). Interestingly, the risk reduction for cardiovascular events was greater than would be expected from the observed mean difference in BP between groups, which suggests that the effects of ACE inhibition in this study were greater than could be attributed to its effect on lowering BP. We hypothesize that the cardioprotective effects of ACE inhibitors could, at least in part, be a consequence of the ability of endogenous bradykinin to initiate both classic and delayed PC effects. Moreover, some ACE inhibitors have been shown to exert direct infarct-limiting effects in experimental models, independent of their ability to augment PC (10,12,39,40). Indeed, in this study, treatment with perindoprilat, which has a long biological half-life, 24 h before infarction resulted in a small, although non-significant, protective effect. The infarct-limiting effects of short-term administration of perindoprilat during the infarct-associated coronary occlusion may warrant further investigation.

In conclusion, an ACE inhibitor, in conjunction with a mild ischemic stimulus, served as an effective trigger of delayed PC against subsequent infarction in pig myocardium. This finding may reflect a contributory mechanism by which ACE inhibition exhibits beneficial effects in patients with ischemic heart disease.

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Reprint requests and correspondence: Prof. Derek M. Yellon, Director of Institute and Head of Centre for Cardiology, The Hatter Institute for Cardiovascular Studies, Centre for Cardiology, UCL Hospitals and Medical School, Grafton Way, London WC1E 6DB, United Kingdom. E-mail: hatter-institute@ucl.ac.uk.

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