Repeated ischaemic preconditioning:

A novel therapeutic intervention and potential underlying mechanisms

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NEW FINDINGS

What is the topic of this review?

This review discusses the effects of repeatedly exposing tissue to ischaemic preconditioning on cardiovascular function, the attendant adaptations and their potential clinical relevance.

What advances does it highlight?

We discuss the effects of episodic exposure to IPC to prevent and/or attenuate ischaemic injury, and summarise evidence pertaining to improvements in cardiovascular function and structure. Discussion is provided regarding the potential mechanisms that contribute to both local and systemic adaptation. Findings suggest that clinical benefits result from both the prevention of ischaemic events and attenuation of their consequences.

ABSTRACT

Ischaemic preconditioning (IPC) refers to the phenomenon that short periods of cyclical tissue ischaemia confer subsequent protection against ischaemia-induced injury. As a consequence, IPC can ameliorate the myocardial damage following infarction and reduce infarct size. The ability of IPC to confer remote protection makes IPC a potentially feasible cardioprotective strategy. In this review, we discuss the concept that repeatedly exposing tissue to IPC may increase the "dose" of protection, and subsequently lead to enhanced protection against ischaemia-induced myocardial injury. This may be relevant for clinical populations, who demonstrate attenuated efficacy of IPC to prevent or attenuate ischaemic injury (and therefore myocardial infarct size). Furthermore, episodic IPC facilitates repeated exposure to local (e.g. shear stress) and systemic (e.g. hormones, cytokines, blood-borne substances) stimuli, which may induce improvement in vascular function and health. Such adaptation may contribute to prevention of cardio- and cerebro-vascular events. The clinical benefits of repeated IPC may, therefore, result from both the prevention of ischaemic events and attenuation of their consequences. We provide an overview of the literature pertaining to the impact of repeated IPC on cardiovascular function, related to both local and or remote adaptation, as well as potential clinical implications.

Ischaemic preconditioning (IPC) refers to the phenomenon whereby 3-4 brief periods of ischaemia, followed by tissue reperfusion, confers subsequent protection against the magnitude of tissue injury following ischaemia. This concept was introduced 30 years ago in a study which demonstrated that cycles of ischaemia and reperfusion of coronary arteries are able to protect the myocardium from subsequent prolonged ischaemia and reperfusion, leading to a reduction in infarct size (Murry *et al.*, 1986). A follow-up study, by Przyklenk and co-workers (Przyklenk *et al.*, 1993), demonstrated that cycles of coronary ischaemia and reperfusion also protect remote cardiac tissue not directly exposed to the ischaemia-reperfusion cycles. This study stimulated substantial research that resulted in the clinical application of IPC of a limb to protect *remote* tissue and/or organs, such as the heart, against the magnitude of tissue loss consequent to an ischaemic event (Pickard *et al.*, 2015). Reduction of myocardial damage by remote IPC, including improvement in clinical outcomes, has been demonstrated when applied in patients prior to cardiac surgery (Thielmann *et al.*, 2013) and in patients with suspected myocardial infarction treated with IPC in the ambulance (Botker *et al.*, 2010). Subsequently, studies have explored the impact of increasing the 'dose' of the traditional IPC-protocol (i.e. 3-4 cycles of 5-minutes ischaemia interspaced with 5 mins of reperfusion). Given the potent effects of a single dose of IPC, repeated episodes of IPC may, in theory, provide longer or more potent reduction in ischaemic myocardial damage (Whittaker & Przyklenk, 2014).

A second potential benefit of IPC has emerged from studies that have explored the effects of repeated IPC (i.e. daily episodes of the 4 bouts of ischemia and reperfusion) on systemic vascular function and health. Improvement in vascular function as a consequence of repeated IPC may contribute to a reduction in the risk of developing ischaemic events. Hence, IPC may be of direct benefit in terms of reducing the impact of infarction on affected cardiac

muscle, but also reducing the likelihood of atherothrombotic events occurring in the first instance by virtue of this impact on endothelial and vascular function.

The purpose of this review is to summarise research work that has investigated the potential impact of repeated (remote) IPC on both the ability to reduce ischaemic (myocardial) damage, and the capacity to improve vascular function. We also summarise proposed underlying mechanisms contributing to these adaptations of repeated (remote) IPC.

1. Application of IPC in (pre)clinical work

1.1 Historical overview IPC

Murry *et al.* introduced the potential cardioprotective benefits of an episode of IPC (Murry *et al.*, 1986). In this study, the IPC protocol involved occlusion of left anterior descending artery of dogs 4 times (for 5-minutes per occlusion), alternated with 5-minutes of reperfusion. This was followed by 40-minute ischaemia of the same artery. A 75% smaller infarction size was evident after IPC, compared to control animals that underwent a sham-intervention. This finding provided experimental support for clinical observations in the mid-1980s, which suggested that post-myocardial infarction patients with a prior history of angina (i.e. myocardial ischaemia) demonstrated better ejection fraction (Matsuda *et al.*, 1984). These data contributed to the concept that exposure to (non-lethal) cardiac ischaemia in the period preceding coronary ischaemia may protect against the impact of reperfusion of the occluded artery on the magnitude of myocardial damage. Subsequent studies provided further clinical evidence that "pre-conditioning" of the myocardium before an acute myocardial infarction, for example through prodromal angina, leads to a smaller infarct size (Ottani *et al.*, 1995) and improves (in-hospital) outcome (Kloner *et al.*, 1995; Nakagawa *et al.*, 1995).

Despite these intriguing observations, direct clinical application of IPC is challenging and associated with some limitations. Compared to control groups undergoing traditional CABG, smaller post-surgery release of cardiac troponins was observed after IPC applied to human coronary arteries preceding coronary artery bypass surgery (CABG) (Jenkins *et al.*, 1997), along with attenuated impairment in cardiac function (Wu *et al.*, 2000). Nevertheless, the direct application of IPC to coronary vessels can only be applied to planned ischaemic injury or surgery and is obviously impractical in humans.

1.2 Effect remote IPC

Przyklenk and colleagues performed a landmark study in 1993, in which they demonstrated that cyclical ischaemia and reperfusion of the circumflex coronary artery was associated with protection of cardiac territory supplied by the left anterior descending artery (i.e. an area remote from the distribution of the circumflex coronary artery) (Przyklenk *et al.*, 1993). They provided support for the notion that IPC can afford infarct-sparing protection for distinct areas within the heart; and initiated several investigations to explore the potential effects of *remote* IPC (RIPC). Preclinical studies exploring the effects of RIPC have typically collected perfusate from ischaemic preconditioned tissue/animals and subsequently perfused naïve hearts using a Langendorff preparation. Interestingly, several studies have demonstrated that infarct sizes were significantly smaller in both donor hearts subjected to IPC and the naïve recipient hearts that received the perfusate from a pre-conditioned donor (Dickson *et al.*, 1999; Huffman *et al.*, 2008) (Figure 1). This demonstrates the ability of IPC to reduce damage upon ischaemic injury in remote areas, possibly through a blood-borne pathway. Furthermore, evidence is present for between-species protection of RIPC, since rabbit hearts demonstrated protection against prolonged ischaemia when perfused with human

preconditioned serum (Shimizu *et al.*, 2009; Michelsen *et al.*, 2012). This suggests a similarity in the factor/s conferring protection across species, and that such agent/s remain conserved during such procedures, allowing binding to the recipient receptors.

Experimental observations on the impact of RIPC have been supported by clinical studies demonstrating the potential of RIPC to prevent or attenuate (ischaemia-induced) tissue damage in the heart and various other organs (e.g. liver, brain, vascular endothelium and skeletal muscle (Pang *et al.*, 1995; Yoshizumi *et al.*, 1998; Stenzel-Poore *et al.*, 2003; Jabs *et al.*, 2010)). Kharbanda *et al.* used a swine model of RIPC of the limb (using a blood pressure cuff) and evoked reduction in the magnitude of myocardial damage against prolonged ischaemia (Kharbanda *et al.*, 2002). Kharbanda *et al.* extended their findings by examining endothelial ischaemia-reperfusion injury in humans and demonstrated that RIPC induced by forearm ischaemia protected the contra-lateral forearm against endothelial ischaemia-reperfusion injury. Other studies in humans followed-up on these findings and introduced RIPC (using a blood pressure cuff around a limb) as a simple strategy to evoke protective effects against subsequent ischaemia in remote territories.

Studies have typically explored clinical effects by applying an episode of cyclical RIPC on a limb, before (planned) prolonged myocardial ischaemia. Several studies have examined the impact of RIPC in patients undergoing impending CABG or percutaneous coronary interventions (PCI), as these strategies induce global myocardial ischaemia (and subsequently cardiac damage) as reflected by a post-surgery elevation in cardiac troponins. Accordingly, strategies that can attenuate the global myocardial ischaemia have clinical relevance. Most of these studies reported lower peri-/post-operative levels of troponins in patients undergoing

CABG and elective PCI (Heusch, 2013), findings reflected by meta-analyses (Brevoord *et al.*, 2012; D'Ascenzo *et al.*, 2012). More importantly, RIPC may reduce peri-operative myocardial infarction (Brevoord *et al.*, 2012; Thielmann *et al.*, 2013) as well as post-operative atrial fibrillation (Candilio *et al.*, 2015). For example, Thielmann and co-workers demonstrated in 329 patients undergoing CABG that preceding RIPC reduced post-CABG troponin levels, and also lowered all-cause mortality following 1.5-years follow-up (Thielmann *et al.*, 2013). RIPC may possess potential long-term clinical benefit in humans.

Instituting RIPC *before* prolonged ischaemia does not seem to be a pre-requisite for cardioprotection, since RIPC, applied to the lower limbs of pigs *during* cardiac ischaemia, was also associated with reduced severity of myocardial infarction and improved indices of cardiac function (Schmidt *et al.*, 2007). In 2010, Bøtker and colleagues explored this concept in humans by randomising patients with suspected acute myocardial infarction to RIPC or a control intervention during transport to the hospital for primary PCI (Botker *et al.*, 2010). They found that RIPC before hospital admission was associated with better myocardial salvage, measured by myocardial perfusion imaging. A follow-up study of this patient population indicated that, after a median follow-up of 3.8 years, the RIPC-treated patients experienced fewer major adverse cardiac and cerebrovascular events (Sloth *et al.*, 2014) (Figure 2). The potential of RIPC to improve outcomes in patients with acute myocardial infarction undergoing primary PCI was supported by subsequent studies (Munk *et al.*, 2010; Rentoukas *et al.*, 2010).

1.3 Potential problems with IPC

A consensus has emerged that RIPC confers beneficial cardioprotective effects, reduces myocardial damage and confers fewer cardiovascular events post-surgery/event. These

effects seem largely independent of species, timing of RIPC, and/or the protocol of RIPC. Nevertheless, a number of potential concerns have been raised pertaining to this body of data. The majority of pre-clinical work is based on healthy animals, whilst ischaemic heart disease in humans is a complex disorder that may interfere with the efficacy of RIPC (Ferdinandy *et al.*, 2007). The ageing process (Boengler *et al.*, 2009) or anaesthetics/drug intake (Ferdinandy *et al.*, 2014) may also interact with pathways normally associated with beneficial IPC effects.

Tissue damage is mediated by prolonged ischaemia *per se*, but also through reperfusion of the ischaemic tissue, commonly referred to as ischaemia-reperfusion injury. Endothelial cells are particularly sensitive to ischaemia-reperfusion injury, leading to endothelial injury and swelling. Damage to the endothelium can contribute to further ischaemia by impeding blood flow upon reperfusion, which has been termed the 'no-reflow phenomenon' and is present in the myocardium (Chan *et al.*, 2012) as well as the brain (Asiedu-Gyekye & Vaktorovich, 2003). Impaired endothelial function is therefore clinically relevant, since the no-reflow phenomenon may lead to larger tissue damage and is associated with worse clinical outcome and increased mortality in patients undergoing PCI (Chan *et al.*, 2012). Studies in humans often utilise brachial artery endothelial function (e.g. using flow-mediated dilation) as a valid surrogate for coronary function, before and after upper limb ischaemia, followed by reperfusion. In a series of recent studies, we explored the impact of older age and heart failure on the efficacy of IPC in humans to prevent endothelial ischaemia-reperfusion (van den Munckhof *et al.*, 2013; Seeger *et al.*, 2014a). We found that IPC prevented the decline in endothelial function after ischaemia-reperfusion in young healthy, but not older individuals (van den Munckhof *et al.*, 2013). In line with these observations, heart failure patients demonstrated reduced efficacy of IPC to prevent endothelial ischaemia-reperfusion injury (Seeger *et al.*, 2014a). The latter study also revealed that heart failure patients demonstrate an

exaggerated decline in endothelial function after ischaemia-reperfusion injury, compared to age-matched healthy controls.

The data outlined in this sub-section support the concept that cardiovascular comorbidities and/or aging may modify endothelial ischaemia/reperfusion and, consequently, alter the infarct-sparing effects conferred by IPC. Although older age is associated with (cardiovascular) co-morbidities, it seems unlikely that the age-related loss in preconditioning can be solely explained by the presence of such co-morbidities (Boengler *et al.*, 2009) since some studies report attenuated efficacy of IPC in older individuals free from co-morbidities and/or cardiovascular (CV) risk factors (van den Munckhof *et al.*, 2013). Furthermore, animal work has linked age-related changes in Connexin 43 to a heart less resilient to the prevailing preconditioning stimulus (Boengler *et al.*, 2007). Finally, it is important to emphasise that the age-related loss of preconditioning efficacy is likely reversible, given the ability of diet and/or exercise training to (partly) restore the reduction in IPC effect (Abete *et al.*, 2010; Devan *et al.*, 2011).

2. What are the cardiovascular adaptations to *repeated* IPC?

2.1 Repeated IPC and myocardium

Only a few studies on repeated IPC have directly focused on the myocardium and the ability of episodic IPCs to impact the magnitude of myocardial damage. For example, 40 patients with coronary artery disease who were scheduled for coronary artery bypass graft (CABG) surgery were randomised to a 20-day period of repeated RIPC (3 IPC sessions daily, n=20) or a control intervention prior to surgery (n=20) (Liang *et al.*, 2015). Patients undergoing

repeated RIPC demonstrated ~50% lower troponin expression levels after CABG compared to the control group. The ability of repeated RIPC to reduce post-surgery cardiac troponin is in line with previous work on single RIPC scheduled prior to CABG (see section 1.2).

Currently there are clinical trials underway in the UK (NCT01664611) and Canada (NCT01817114) examining the impact of 28-days of daily repeated RIPC in patient's post-myocardial infarction. Both trials are applying RIPC on the limb and exploring the impact upon left ventricular ejection fraction (LVEF), measured using cardiac magnetic resonance imaging. These trials represent a logical follow-up on some recent studies performed in animals. One of these studies explored different protocols of RIPC in rats undergoing planned cardiac ischaemia, including a single episode of RIPC and repeated (every 3 days *versus* daily) RIPC across 28 days post-injury (Wei *et al.*, 2011). Although reduction in infarct size at day 4 and 28 was comparable across the protocols, repeated RIPC was associated with a dose-dependent protection against adverse remodelling and improved survival. In parallel with these findings, a recent study from Yamaguchi *et al.* (2015) divided post-myocardial infarction rats into a 4-week repeated RIPC-group and control-group and reported that repeated RIPC prevents adverse cardiac remodelling (and fibrosis in the boundary region). Yamaguchi and co-workers further explored the potential underlying mechanisms by examining expression of miR-29a (anti-fibrotic effects) and insulin-like growth factor 1 receptor (IGF-1R; prevents progression of cardiac remodelling) in the myocardium and serum exosomes (Yamaguchi *et al.*, 2015). Interestingly, repeated RIPC restored the down-regulation of miR-29a in the infarcted myocardium, and increased miR-29a expression in serum exosomes. The lower fibrosis after RIPC may therefore occur through exosome-mediated transmission of miR29-a, with subsequent endocytosis of miR-29a in the heart. Similarly, expression of IGF-1R was markedly increased in the hind limb (exposed to

repeated RIPC), serum exosomes and the myocardium, further supporting the observation that repeated RIPC may initiate exosome-mediated intercellular communication. Therefore, animal data exploring the effects of repeated RIPC suggest a potential impact of this novel intervention to improve clinical outcomes post-myocardial infarction.

One previous study examined the magnitude of infarct size after 60-minutes of coronary artery occlusion in pigs that either underwent a preceding sham intervention, traditional IPC or repeated episodes of coronary ischaemia and reperfusion (6 cycles of 90-min occlusion, followed by 12-h reperfusion) (Shen *et al.*, 2008). Whilst the sham-intervention resulted in an infarct size of 42% from the area at risk, traditional IPC and repeated IPC significantly reduced the infarct size relative to the area at risk to 16% and 6%, respectively. These data demonstrate that repeated IPC protocol conferred cardioprotection against lethal myocardial ischaemia that may exceed the benefits of a single IPC. Interestingly, microarray gene expression level analysis was used to understand the underlying mechanisms, and highlight that the mechanisms for protective effects of single day and repeated IPC may differ given the marked differences in gene expression levels (Shen *et al.*, 2008; Depre *et al.*, 2010). Future studies are required to better understand the potential benefits of repeatedly performing IPC cycles *versus* single IPC to confer the infarct-sparing effects.

2.2 Repeated IPC and vasculature: healthy individuals

In addition to the potential effects of repeated RIPC on reducing the magnitude of cardiac damage following infarction and prolonged ischaemia, repeated RIPC may demonstrate a generalised effect on endothelial function. These potential effects of repeated RIPC on

vascular function are of particular importance, since previous studies have demonstrated that improvement in peripheral and coronary vascular function is related to lower risk for future cardiovascular events (Schachinger *et al.*, 2000; Green *et al.*, 2011).

Kimura and colleagues were the first to examine the effects of repeated RIPC following 28 days of IPC on forearm resistance artery endothelial function in healthy individuals (Kimura *et al.*, 2007) (Figure 3). They provided evidence that repeated RIPC enhanced resistance artery endothelial function via increases in nitric oxide (NO) production, whilst daily repeated RIPC augmented both circulating endothelial progenitor cells (EPCs) and plasma levels of vascular endothelial growth factors (VEGF). Luca *et al*. (2013) followed this work by exploring whether repeated (daily) IPC alters the efficacy of a single IPC against endothelial ischaemia reperfusion injury. They also found that 7-days of daily IPC provided sustained protection against ischaemia reperfusion injury of the endothelium of peripheral arteries (Luca *et al.*, 2013). More recently, we extended these findings of enhanced vascular endothelial function and found that 7-days of daily IPC improved FMD as well as perfusion to the skin (e.g. enhanced microvascular function) in the ipsi- (i.e. localized to arm receiving the IPC-stimulus) and contralateral arms (i.e. remote from arm receiving the RIPC-stimulus). Effects of repeated IPC persisted for 7-days following the cessation of the intervention (Jones *et al.*, 2014) (Figure 4). In addition to these effects on the vasculature, previous work suggests anti-inflammatory effects of repeated RIPC, since 10-day daily RIPC reduced neutrophil adhesion (Shimizu *et al.*, 2010).

It has been suggested that infarct-sparing effects from IPC are present for 1-2 h following an IPC episode (early phase), whilst these protective effects return after 24 h for a period of 3-4 days (late phase). Although described in relation to protection of the ischaemic myocardium,

this pattern may also affect peripheral vessels. Therefore, repeated IPC bouts may be timed 24-72 h apart to ensure that tissue will be exposed to the 'early' *and* 'late' phase of protection, at the same time. Combining these effects may be of clinical relevance, since the 'early' and 'late' phases may have distinct protective mechanisms (Bolli, 2000). Given the relatively long duration of the 'late' phase of protection, we explored whether less frequent (and arguably more practical) exposure to repeated IPC is sufficient to induce vascular adaptation (Jones *et al.*, 2015). We found that 8-weeks of IPC (3 cycles per week) significantly improved brachial artery endothelial function, and that this effect was evident after 2-weeks (i.e. 6 sessions of IPC) of the 8-week intervention. Presence of (sustained and systemic) improvement in peripheral endothelial function may be clinically relevant, particularly given the agreement with coronary endothelial function (Takase *et al.*, 1998; Takase *et al.*, 2005) and its prognostic value for future CV events (Green *et al.*, 2011; Ras *et al.*, 2013). Given the clinical benefits of adaptations in coronary arteries for cardioprotection, future studies are recommended to further explore these effects of repeated IPC.

2.3 Repeated IPC and vasculature: cardiovascular disease

Studies using repeated IPC interventions have also revealed positive effects on vascular function in those with pre-existing cardiovascular disease. For example, improved brachial artery FMD was observed in patients with coronary artery disease who were scheduled for coronary artery bypass graft (CABG) surgery and underwent a 20-day period of repeated RIPC (3 IPC sessions daily) (Liang *et al.*, 2015). Moreover, vascular tissue harvested during CABG surgery demonstrated that patients who underwent repeated RIPC had increased expression of both eNOS mRNA and STAT-3, and also found increased levels of endothelial progenitor cells (Liang *et al.*, 2015). These findings may provide a mechanistic explanation for improvement in vascular function after repeated RIPC. Whilst the majority of papers

focused on peripheral vascular effects, Kono and co-workers explored the effects of 7-days of bilateral RIPC on coronary flow reserve in patients with congestive heart failure and healthy individuals (Kono *et al.*, 2014). The findings of that study demonstrate significant improvement in coronary flow reserve after repeated RIPC. These observations are of clinical relevance, since improvements in coronary flow reserve are linked to reduction in cardiovascular risk.

2.4 Impact of repeated IPC: clinical outcomes

The ability of repeated RIPC to alter vascular and cardiac function, and also impact cardiac remodelling, raises questions about its impact on clinical endpoints. Meng and colleagues were the first to perform a repeated RIPC intervention in a clinical setting, assessing the effects of 300-days of twice daily RIPC on stroke recurrence and cerebral perfusion in patients with intracranial arterial stenosis (Meng *et al.*, 2012). Lower stroke recurrence was found in the IPC group at 90 and 300 days (5.0 and 7.8%, respectively) compared to the control group (23.3 and 26.7%, respectively). Patients receiving the repeated RIPC-protocol also demonstrated a significantly shorter time to recovery and improved cerebral perfusion. Improvement in event-free survival in the group that received repeated RIPC was recently reinforced by another study from the same group. In this study, significant protection against stroke recurrence was observed after 180-days of twice daily RIPC in symptomatic intracranial arterial stenosis patients (Meng *et al.*, 2015).

Another recent study examined the impact of repeated RIPC on diabetic wound healing in diabetes mellitus type 1 and 2 patients (Shaked *et al.*, 2015). Patients with foot ulcers were

randomised to 6-week bilateral repeated RIPC (upper limbs) or a control intervention. The ratio of patients who reached complete healing of their ulcer was significantly better in those who received repeated RIPC compared to the control group (41% *vs* 0%), whilst the remaining ulcer area was smaller (25% *vs* 61%, respectively). A higher prevalence of complete healing is clinically relevant since 20% of patients with diabetic foot ulcers ultimately require amputation (Eldor *et al.*, 2004). Taken together, these data suggest a potential clinical relevance of repeated RIPC in patient groups. Nevertheless, randomised controlled trials are required to further understand the clinical relevance of repeated RIPC in patient groups.

3. IPC: Potential mechanisms

Studies exploring the mechanisms for cardioprotection from repeated IPC are currently lacking. In the following sections, we have distinguished between the distinct local and remote effects of IPC *versus* RIPC, respectively. Within these sections, we have described the mechanisms which mediate the beneficial effects of a *single* episode of IPC or RIPC to mediate the infarct-sparing effects. Repeated activation of these effects may represent the first explanation for the benefits of repeated IPC. Nevertheless, it is important to acknowledge that mechanisms that contribute to the cardioprotection achieved through single episodes of IPC do not necessarily relate to the observations after repeated exposure to IPC (Shen *et al.*, 2008; Depre *et al.*, 2010).

A second explanation for the benefits of repeated IPC is the activation of pathways and/or stimuli that contribute to improvement in vascular structure and function. To understand these adaptations, it is important to acknowledge that repeated IPC induces local *and* remote improvements in (cardio)vascular function (Table 1). Adaptations in local areas may be the

result of changes in haemodynamics induced by the IPC stimulus and/or by activation of pathways involved in the protective effects of IPC. In addition, alternative stimuli, such as blood-borne factors, must be present to mediate (cardio)vascular adaptation in remote areas.

3.1 Local effects IPC: infarct-sparing effects

As a consequence of IPC, signalling molecules are released that activates a mediator to transmit the cardioprotective signal that attenuates ischaemic injury. Consistent evidence supports the involvement of adenosine, bradykinin, and opioids (Liu *et al.*, 1991; Schulz *et al.*, 1998; Hu *et al.*, 2007; Shimizu *et al.*, 2009), which are released by cells directly exposed to IPC. Other signalling molecules have been identified, such as NO, hydrogen sulfide and reactive oxygen species (Heusch, 2015). The importance of these signalling molecules has typically been demonstrated by exploring myocardial infarct size after prolonged ischaemia of an animal heart after IPC during specific pharmacological blockade, or infusion of agonists.

Expression of signalling molecules leads to activation of mediators that transmit the protective signal in the cytosol. An important mediator contributing to cardioprotective effects of IPC is protein kinase C (Liu *et al.*, 1994). Others suggest an important role for NO in protecting against ischaemic injury through cGMP formation and PKG activation (Oldenburg *et al.*, 2004; Yang *et al.*, 2013). Given the potent anti-atherogenic and vasodilator effects of this substance, the involvement of the NO-pathway is clinically relevant. Furthermore, IPC-mediated protection also occurs via the Reperfusion Injury Salvage Kinases (RISK)-pathway (involving activation of PI3K, Akt and ERK) and Survivor Activating Factor Enhancement (SAFE)-pathway (involving TNFα and STAT-3), whilst

evidence also supports a role for protein kinases, hypoxia-inducible factor 1α, and microRNA-144.

Ultimately, most, if not all, of the signalling pathways converge at the mitochondria, the most important effector of protection induced by IPC. Periods of ischaemia have deleterious effects on the mitochondria, ultimately limiting the production of ATP and leading to cell necrosis. A crucial step in this process is the opening of the mitochondrial permeability transition pore (MPTP), a finding supported by preclinical evidence showing infarct sparing effects when infusing MPTP inhibitors (see review (Ong *et al.*, 2015)). Also, ATP-dependent potassium channels (K_{ATP}), especially those on the inner mitochondrial membrane, contribute to the protective effects of IPC against prolonged ischaemia (Ardehali & O'Rourke, 2005). Whilst IPC has well-established local infarct-sparing effects on the myocardium, the mechanisms underlying the translation of IPC to remote tissues is somewhat unclear.

3.2 Local effects IPC: vascular adaptation

IPC has a characteristic temporal nature, involving an acute protection that disappears ~2 hr after the preconditioning stimulus, and a delayed window of protection that appears after ~24 hr that lasts longer, but may be less protective (Marber *et al.*, 1993; Heusch, 2015). Whilst the acute protection depends on immediate recruitment of signaling molecules, increased expression of protective proteins is a hallmark of delayed protection (Bolli *et al.*, 2007). Whilst endogenous NO, generated from endothelial and inducible NOS, seem not a requisite for immediate protection, it is involved in the delayed protective effects of IPC (Bolli *et al.*, 1998; West *et al.*, 2008). In support of this, infarct-sparing effects of the late phase of IPC are abolished by a nonselective NOS inhibitor and selective iNOS inhibitor (Takano *et al.*, 1998).

Expression of other cardioprotective proteins is also upregulated during delayed protection (e.g. COX-2, superoxide dismutase and heme oxygenase) (Zhou *et al.*, 1996; Guo *et al.*, 2000; Brooks *et al.*, 2014). These effects on the upregulation of (cardioprotective) proteins may contribute to the sustainability of the IPC stimulus and vascular adaptation. Therefore, repetitive upregulation of these proteins, typically observed during the delayed protective phase, may be of relevance in understanding the effects of repeated IPC on sustainable improvement in vascular function. Whether these effects indeed translate to the peripheral vascular beds, also taking into consideration the presence of potential deleterious remodeling, is currently unknown.

An alternative explanation for local adaptations in vascular function and structure induced by repeated IPC relates to the cyclical exposure to changes in local haemodynamics induced by the episodes of ischaemia and reperfusion, such as elevations in blood flow (or shear stress). Previous work has demonstrated that repeated increases in shear represent an important stimulus for vascular adaptations in function and structure (Green *et al.*, 2004; Tinken *et al.*, 2010). The perception that vascular adaptations are mediated by shear stress dependent mechanisms is well supported by both in *vivo* animal models and human experiments (Hambrecht *et al.*, 2003; McAllister *et al.*, 2005; Green *et al.*, 2010). In addition, repeated exposure to short periods of hypoxia (or ischaemia) may also contribute to cardiac and vascular adaptations (Laughlin *et al.*, 2008; Newcomer *et al.*, 2011). Given that repeated exposure to elevations in blood flow (or shear stress) and hypoxia are key characteristics of IPC in a limb exposed to the stimulus, any localised vascular adaptations in the limb directly exposed to IPC could be mediated by increased shear stress and also the hypoxic release of local metabolic byproducts.

3.3 Systemic effects of remote IPC: infarct-sparing effects

In the classic view on the cardioprotective effects of RIPC, a trigger is released (i.e. in the occluded limb) and acts as the stimulus to activate a mediator, which transmits a protective signal onto an effector that attenuates injury in remote (cardiac and vascular) areas in response to ischaemia-reperfusion. Given the complexity of the activation of these pathways, involvement of many signals, and also how they confer subsequent protection, we refer to recent reviews that provide a comprehensive overview of these mechanisms (Heusch, 2015; Heusch *et al.*, 2015). Below, we have provided a short, condensed overview of the most important pathways involved in the immediate infarct-sparing effects of RIPC. Although speculative, these pathways may contribute to the infarct-sparing effects of repeated IPC.

The stimulus for RIPC relevant in the model of repeated IPC originates from the episodes of ischaemia and reperfusion applied to a limb. Subsequent transduction of the local signal to remote tissues is dependent on (intact) neural pathways and humoral pathways that are able to reach the remote target tissue or organ (Gho *et al.*, 1996; Lim *et al.*, 2010). For example, several studies have demonstrated the role of neural pathways in signal transduction in RIPC (including somatosensory system, the spinal cord and the autonomic system), by reporting abrogation of protection after pharmacologically or surgically affecting neural pathways (Gho *et al.*, 1996; Loukogeorgakis *et al.*, 2005; Jones *et al.*, 2009). Similarly, cardioprotection of RIPC is also mediated through circulating, blood-borne hormones that are able to protect remote (cardio)vascular regions against prolonged ischaemia (Dickson *et al.*, 1999), with recent evidence supporting a potential role for NO, microRNA-144 and stromal derived factor 1α. In the target organ, signal transduction pathways are activated that ultimately

contribute to the protection against ischaemic injury. In the heart, signal transduction of RIPC 'shares' that of local IPC, with at least significant involvement of NO (and eNOS), PKC and the RISK-pathway that ultimately work on the mitochondria (Heusch *et al.*, 2015).

3.4 Systemic effects of remote IPC: vascular adaptation

The production of circulating hormones represents one logical mechanism that might contribute to the effects of repeated IPC on both the ability to ameliorate damage after prolonged ischaemia *and* sustained adaptation in remote vascular function. The role of circulating hormones in the protection against myocardial injury was first highlighted by Dickson *et al.*, who found that coronary effluent from a pre-conditioned heart induced cardioprotection in a naïve acceptor heart (Dickson *et al.*, 1999) (Figure 1). Several subsequent studies, typically adopting the Langendorff bioassay, suggested the presence of a blood-borne substance that confers protection when infused in an organ exposed to prolonged ischaemia. Despite the scientific and clinical importance, identifying the substance, or substances, that explain the effects of RIPC has proven challenging. At least some part of the remote protection has been attributed to microRNA-144, stromal-derived factor-1α (SDF-1α) and NO (Pickard *et al.*, 2015). Increased bioavailability of NO, via conversion from circulating nitrite, may reduce myocardial damage during prolonged ischaemia, whilst the effects of RIPC were diminished in animals deficient in endothelial NO synthase (Rassaf *et al.*, 2014). Similarly, cardioprotection provided by RIPC was partially abolished using an inhibitor of SDF-1α (Davidson *et al.*, 2013). Finally, recent work suggests that RIPC-induced release of microRNA-144 contributes to the cardioprotection, since the efficacy of RIPC is reduced when combined with an antagomir to microRNA-144 (Li *et al.*, 2014). Whilst NO, SDF-1α and microRNA144 are capable, at least partly, of explaining some of the infarct-

sparing effects of RIPC, these circulating hormones may also contribute to adaptations in peripheral and/or coronary arteries.

As indicated earlier, due to the temporal differences in the effects of IPC (i.e. early *versus* late preconditioning), candidates contributing to the acute benefits of IPC may not necessarily contribute to more sustainable adaptations. For example, similar to IPC, RIPC is associated with sustainable decreases in iNOS (Wei *et al.*, 2012). Furthermore, RIPC did not mediate acute mobilisation of endothelial progenitor cells, making it unlikely they contribute to the immediate protective effects, whilst increases in these cells were observed 12- and 24-hr after IPC (Kamota *et al.*, 2009). This makes endothelial progenitor cells a potential candidate to contribute to improvement in vascular function in response to repeated IPC, especially given their ability to improve vascular function. Some support for this is provided by the observation of elevated levels of endothelial progenitor cells after 28-days of repeated IPC, which coincided with improvement in (peripheral) vascular function (Kimura *et al.*, 2007).

RIPC also represents a potent stimulus for inflammatory pathways. Despite its role in inflammation, upregulation of cytokine interleukin (IL)-6 is mandatory to mediate (remote) IPC (Zuurbier *et al.*, 2012). The role for low levels of cytokines in the protection mediated by (remote) IPC is interesting, especially since excessive levels of cytokines are detrimental for ischaemic injury and vascular function. Such paradoxical observations can also be observed for TNFα and reactive oxygen species. Furthermore, expression of IL-10, a potent anti-inflammatory cytokine, is upregulated during delayed protection of RIPC and late protection against ischaemia may be mediated by IL-10, since blockade of IL-10 receptors abolished cardioprotective effects whilst IL-10 infusion induced benefit (Cai *et al.*, 2012). These data indicate that RIPC leads to sustainable changes in inflammatory pathways, possibly

contributing to prolonged protection against ischaemic injury and/or adaptation in vascular function/structure.

Taken together, early and/or late effects of RIPC may contribute to infarct-sparing effects, but also to the generalized improvement in vascular function associated with repeated IPC, and therefore reduction in risk for CV events. The remote effects of IPC are an attractive explanation of the improvement in vascular function and/or structure, especially given sustained increases in circulating hormones during the late phase of RIPC that have direct relevance for vascular adaptation and health (e.g. NO, endothelial progenitor cells, IL-10). However, understanding the potential sustainable effects of repeated IPC is more complicated than simply exploring the early *versus* late effects of RIPC (Whittaker & Przyklenk, 2014). For example, previous work from Depre *et al.* found single and repeated RIPC to lead to comparable preconditioning effects, but with marked differences in gene expression (Depre *et al.*, 2010). Furthermore, exposure to a large number of ischaemic episodes may cause local collagen damage, potentially leading to inflammation and fibrosis (Whittaker & Przyklenk, 2014). Future work is required to explore the underlying mechanisms to understand the impact of repeated IPC.

4. Summary, future directions and the potential clinical relevance of repeated IPC

Previous work regarding IPC has largely focused on understanding the clinical benefits and mechanisms contributing to the effects of a single episode of IPC. Given the potency of these effects, more recent studies have explored the potential benefits associated with *repeated* IPC. Whilst this field is in its infancy, current evidence suggests that repeated IPC is associated with both local and remote adaptations in vascular function which likely relate to

lower risk for future CV events and protection against ischaemic injury. The ability to benefit from the protective effects of IPC against sustained myocardial ischaemic injury *and/or* to mediate improvements in vascular function makes repeated IPC a novel strategy that is feasible, low cost and easy applied. Some recent and provocative studies have linked repeated exposure to IPC to clinical cardio- and cerebro-vascular outcome benefits.

Studies examining the mechanisms underlying the clinical effects of repeated IPC are scant. Current evidence suggest that both early (0-1 h) and late (24-72 h) effects are apparent in terms of attenuating and/or preventing ischaemic injury. Repeated IPC is also associated with both local (i.e. repeated increases in shear) and systemic mechanisms (i.e. circulating hormones, cytokines and growth factors) that contribute to improvement in vascular function and/or structure. By virtue of improved vascular function, lower risk for future CV events is possible. Future work is required to better understand the differences between the mechanisms underlying *single* and *repeated* IPC. The potential dual effects of repeated IPC on the prevention of events *and* attenuation of ischaemic injury makes this strategy potentially suitable in both primary and secondary preventive settings.

The impact of repeated preconditioning has also assessed in relation to adaptations in exercise physiology (Thijssen *et al.*, 2010). Interestingly, recent work in animals (Michelsen *et al.*, 2012) and humans (Seeger *et al.*, 2014b) has demonstrated the ability of exercise to protect against cardiac and endothelial ischaemia-reperfusion injury. Moreover, we demonstrated the ability of IPC to enhance exercise performance (de Groot *et al.*, 2010), whilst others found effects of IPC in various types of exercise. IPC may be especially advantageous in exercise where hypoxia is a limiting factor (e.g. swimming) (Crisafulli *et al.*, 2011). Accordingly, it is tempting to postulate that exercise, either alone or in combination

with IPC, may serve as a preconditioning stimulus in clinical research and/or as a strategy to restore preconditioning in clinical populations.

Taken together, increasing the 'dose' of a single IPC by repeated performance of this procedure represents an attractive, easy applicable and novel strategy. The ability to induce both local and systemic adaptations that may be associated with lower risk for future CV events, in addition to attenuation of the impact of ischaemic events, is highly attractive from a clinical viewpoint. Moreover, an increased 'dose' of IPC may overcome some of the issues associated with exposure to a single bout of IPC, such as attenuated efficacy in clinical groups. At the very least, this work deserves further exploration regarding the time course, sustainability and validity of potential benefits of repeated IPC in both healthy and clinical populations. Practical considerations such as the delivery of the stimulus, optimal dose and long-term safety remain to be addressed.

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Table 1. Overview of published studies investigating impact of repeated IPC

Author + Year	Group	Dose of IPC	IPC/RIPC	Uni-/Bilateral	Control	Duration	Findings
Kimura 2007	Healthy young	6x5 min daily	RIPC	Uni	Control	4-weeks	Vascular function (VOP-infusion) ↑ Endothelial progenitor cells ↑
Shimizu 2010	Healthy middle-aged	3x5 min daily	RIPC	Uni	None	10-days	Neutrophil adhesion ↓ Phagocytosis ↓
Luca 2013	Healthy young	3x5 min daily	RIPC	Uni	None	7-days	Vascular function (FMD) ↑
Kono 2014	Healthy middle-aged	4x5 min 2/day	RIPC	Uni	None	7-days	Coronary flow reserve ↑ LV end-diastolic volume =
Jones 2014a	Healthy young	4x5 min daily	IPC/RIPC	Uni	None	7-days	Vascular function (FMD) ↑ (bilateral) Skin perfusion ↑ (bilateral)
Jones 2015	Healthy young	4x5 min 3/week	IPC/RIPC	Uni	Control	8-weeks	Vascular function (FMD) ↑ (bilateral) Skin perfusion =
Wei 2011	Rats – myocardial infarction	4x5 min daily	RIPC	Uni	Sham	28-days	Infarct size ↓ LV remodelling ↓
Meng 2012	Intracranial arterial stenosis	5x5 min daily	RIPC	Bi	Control	300-days	Stroke recurrence ↓ Brain perfusion ↑
Kono 2014	Heart failure	4x5 min 2/day	RIPC	Uni	None	7-days	Coronary flow reserve (CFR) ↑ LV end-diastolic volume =
Liang 2015	Coronary heart disease	4x5 min 3/day	RIPC	Uni	Control	20-days	Vascular function (FMD) ↑ eNOS mRNA levels ↑ STAT-3 levels ↑ Endothelial progenitor cells ↑
Yamaguchi 2015	Rats – myocardial infarction	5x5 min daily	RIPC	Bi	Control	4-weeks	LVEF ↑ LV diastolic function ↑
Meng 2015	Intracranial arterial stenosis	5x5 min 2/day	RIPC	Bi	Sham	180-days	Stroke recurrence ↓
Shaked 2015	Type 1 & 2 diabetics	3x5 min 1/14 days	RIPC	Bi	Sham	6-weeks	Diabetic ulcer wound size ↓

FMD, flow mediated dilation; LVEF, Left Ventricular Ejection Fraction

AUTHOR CONTRIBUTIONS

All authors contributed to the conception or design of this review, the consideration of studies included, drafting of the manuscript and its critical revision. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Finally, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

FIGURE LEGENDS

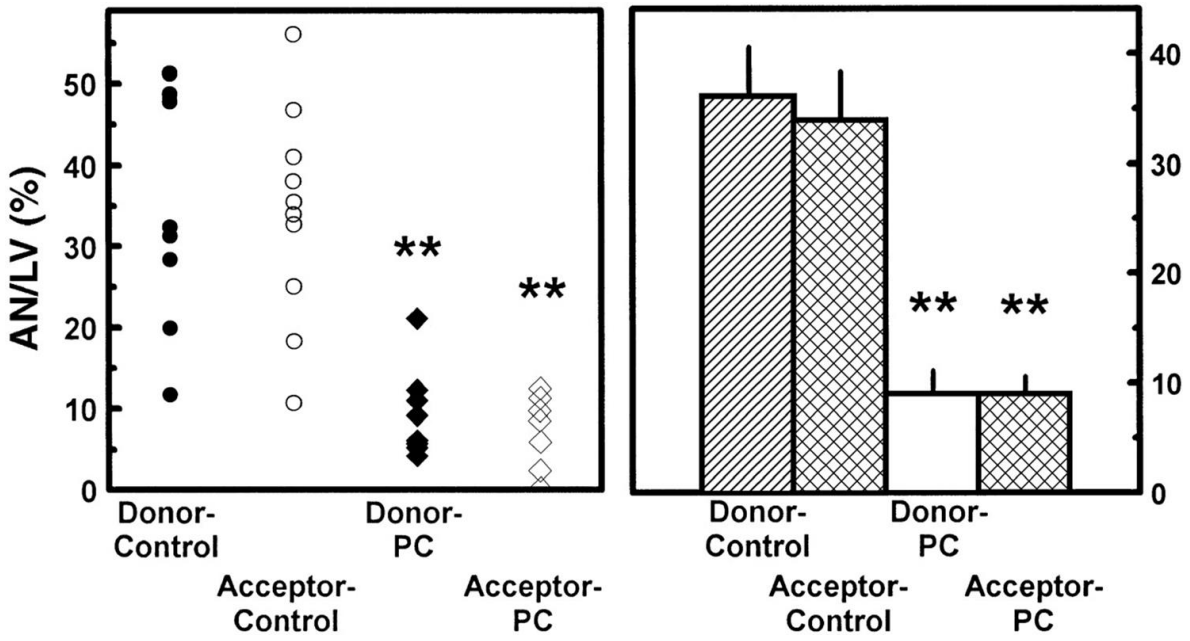
FIGURE 1. Area of necrosis (AN) expressed as a percentage of total left ventricular (LV) weight (AN/LV) in rabbit hearts after a control (Control) or ischaemic preconditioning (PC) in the donor heart or in an acceptor heart (i.e. hearts that received effluent from donor). Derived from Dickson *et al.* (1999).

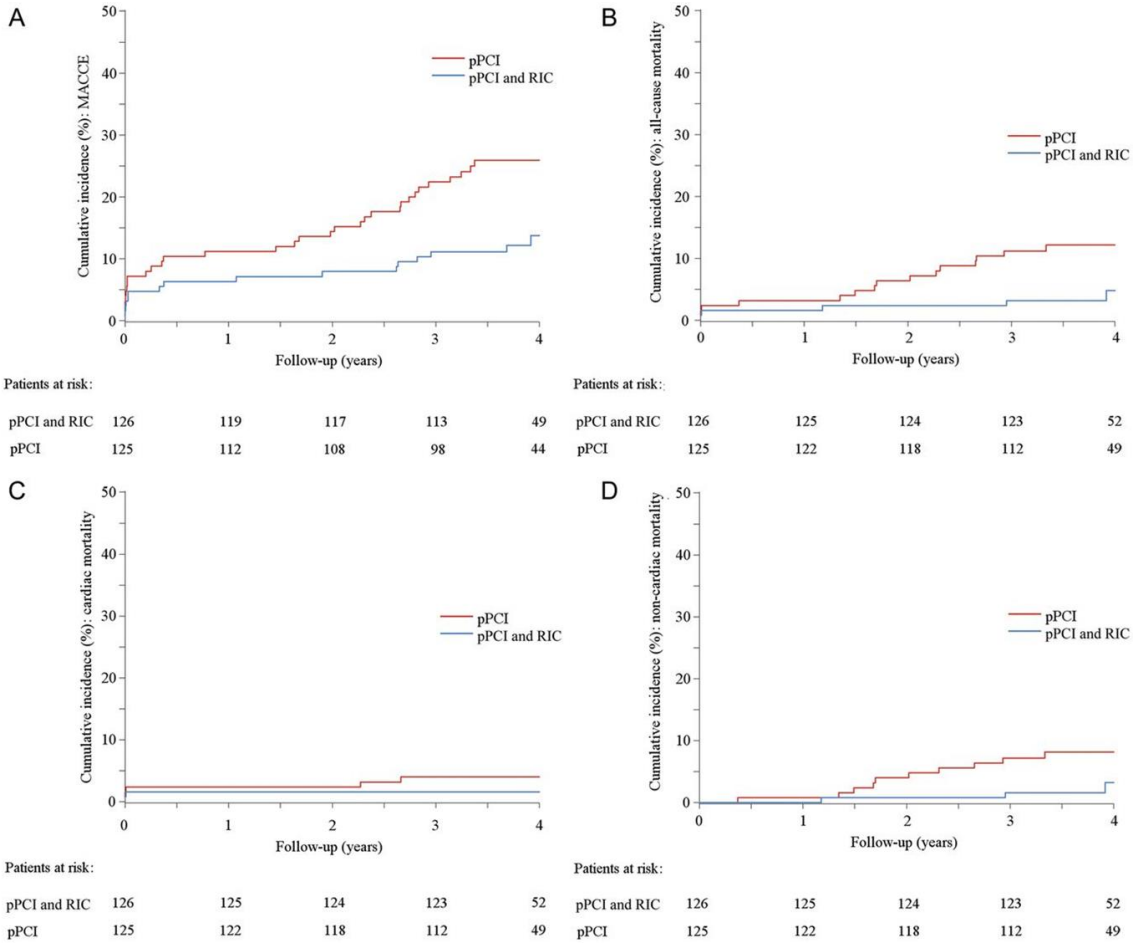
FIGURE 2. Cumulative incidence (%, by year since randomisation, per-protocol analysis): (*A*) Major adverse cardiac and cerebrovascular events (MACCE) at P=0.010, (*B*) All-cause mortality at P=0.019, (*C*) Cardiac mortality at P=0.248, and (*D*) Non-cardiac mortality at P=0.045. Derived from Sloth *et al.* (2014).

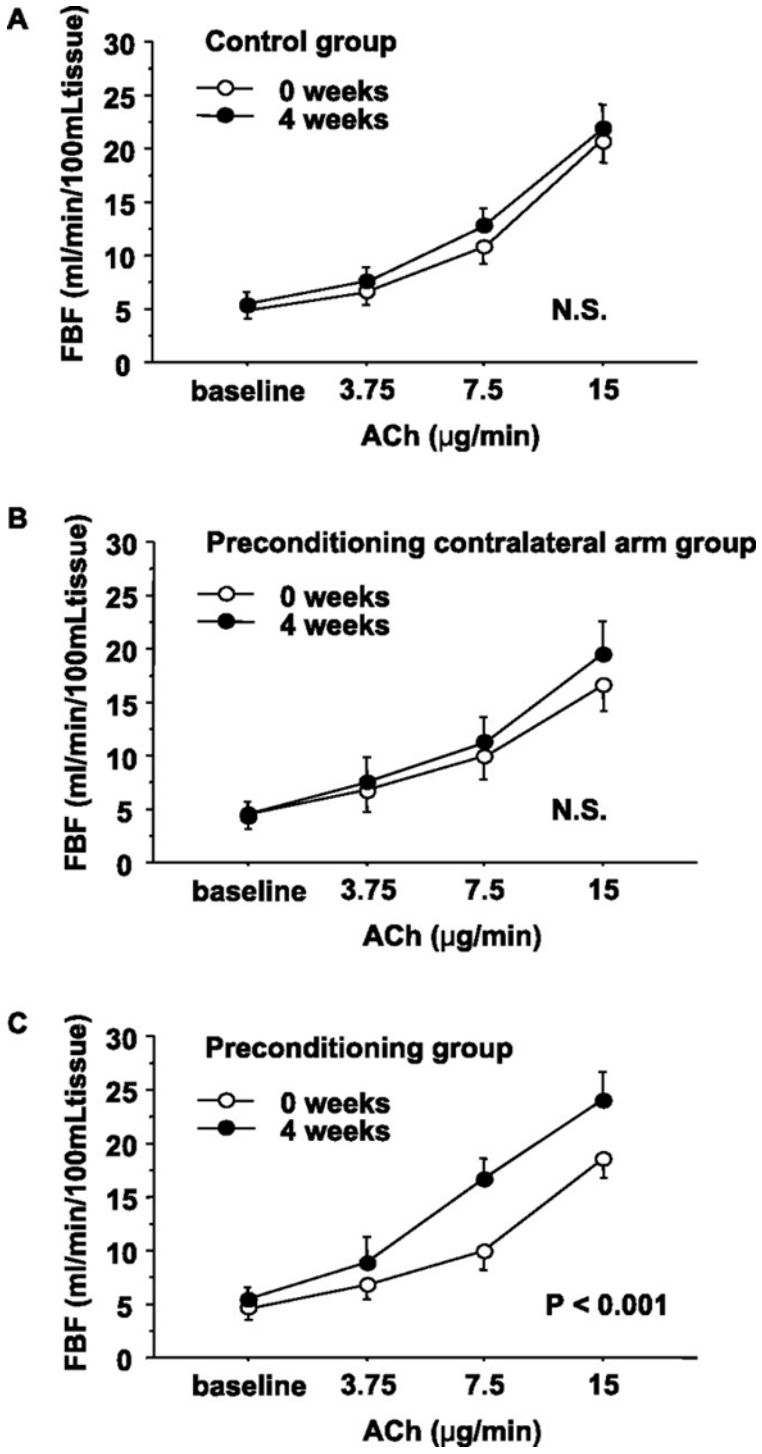
FIGURE 3. Impact of repeated IPC on the vasculature. (a) Brachial artery FMD (%) and (b) resting (baseline) forearm cutaneous vascular conductance (CVC) before (Pre), after (Post), and 8 days after (Post+8) the 7-day daily IPC intervention in the IPC (open circles) and contralateral arm (solid squares) of healthy volunteers (n=13). Error bars represent SE. *Post hoc significantly different from day 0. Derived from Jones *et al.* (Jones *et al.*, 2014).

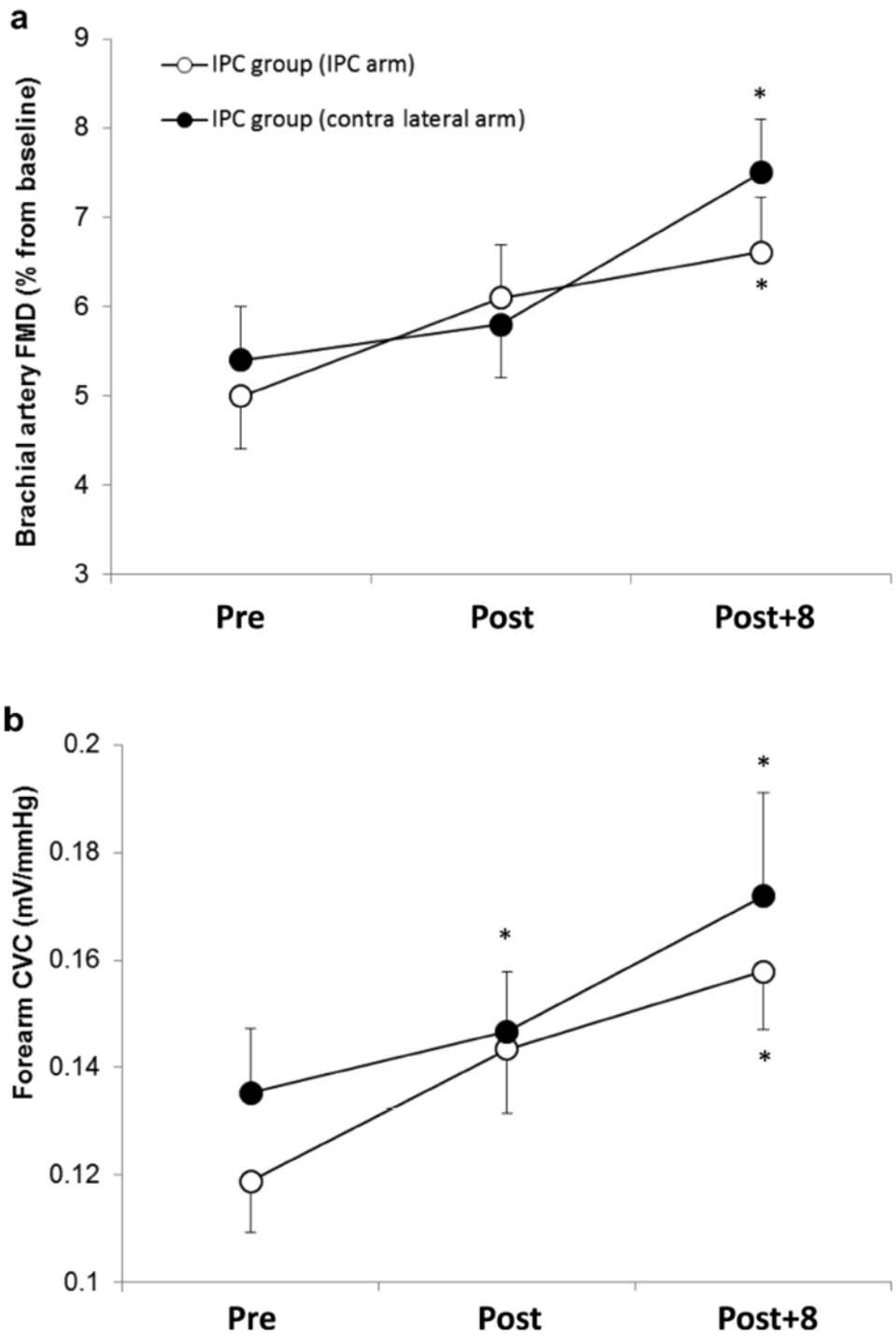
FIGURE 4. Comparison of forearm blood flow (FBF) responses to incremental doses of acetylcholine (ACh) at 0 weeks and 4 weeks of follow-up in the control groups (A) and in the group that received daily unilateral ischaemic preconditioning with data presented for the untreated, contralateral arm (B) and treated,

preconditioned arm (C). Error bars represent SE. Derived from Kimura *et al.* (Kimura *et al.*, 2007).


AN/LV (%)
0
10
20
30
40
50
**
**
Donor-Control
Acceptor-Control
Donor-PC
Acceptor-PC
0
10
20
30
40
**
**
Donor-Control
Acceptor-Control
Donor-PC
Acceptor-PC


A
Cumulative incidence (%): MACCE
pPCI
pPCI and RIC
Follow-up (years)
Patients at risk:
pPCI and RIC 126 119 117 113 49
pPCI 125 112 108 98 44
B
Cumulative incidence (%): all-cause mortality
pPCI
pPCI and RIC
Follow-up (years)
Patients at risk:
pPCI and RIC 126 125 124 123 52
pPCI 125 122 118 112 49
C
Cumulative incidence (%): cardiac mortality
pPCI
pPCI and RIC
Follow-up (years)
Patients at risk:
pPCI and RIC 126 125 124 123 52
pPCI 125 122 118 112 49
D
Cumulative incidence (%): non-cardiac mortality
pPCI
pPCI and RIC
Follow-up (years)
Patients at risk:
pPCI and RIC 126 125 124 123 52
pPCI 125 122 118 112 49


A
Control group
0 weeks
4 weeks
FBF (ml/min/100mLtissue)
30
25
20
15
10
5
0
N.S.
baseline
3.75
7.5
15
ACh (µg/min)
B
Preconditioning contralateral arm group
0 weeks
4 weeks
FBF (ml/min/100mLtissue)
N.S.
baseline
3.75
7.5
15
ACh (µg/min)
C
Preconditioning group
0 weeks
4 weeks
FBF (ml/min/100mLtissue)
P < 0.001
baseline
3.75
7.5
15
ACh (µg/min)


a
Brachial artery FMD (% from baseline)
9
8
7
6
5
4
3
IPC group (IPC arm)
IPC group (contra lateral arm)
*
*
Pre
Post
Post+8
b
Forearm CVC (mV/mmHg)
0.2
0.18
0.16
0.14
0.12
0.1
*
*
*
Pre
Post
Post+8