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Remote ischemic conditioning protects against endothelial ischemia-reperfusion injury via a glucagon-like peptide-1 receptor-mediated mechanism in humans☆

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ABSTRACT

Background: Remote ischemic conditioning (RIC), i.e. short cycles of ischemia and reperfusion in remote tissue, is a novel approach to protect against myocardial ischemia-reperfusion injury in ST-elevation myocardial infarction. The nature of the factors transmitting the protective effect of RIC remains unknown, and both neuronal and hormonal mechanisms appear to be involved. A recent study indicated involvement of glucagon-like peptide-1 (GLP-1) regulated by the vagal nerve in RIC in rats. In the present study we aimed to investigate whether the protective effect of RIC is mediated by a GLP-1 receptor-dependent mechanism in humans.
Methods: Endothelial function was determined from flow-mediated dilatation (FMD) of the brachial artery before and after 20 min of forearm ischemia and 20 min of reperfusion in twelve healthy subjects on three occasions: (A) ischemia-reperfusion without intervention, (B) ischemia-reperfusion + RIC and (C) iv administration of the GLP-1 receptor antagonist exendin(9-39) + ischemia-reperfusion + RIC.
Results: Ischemia-reperfusion reduced FMD from 4.7 ± 0.8% at baseline to 1.5 ± 0.4% ($p < 0.01$). RIC protected from the impairment in FMD induced by ischemia-reperfusion (4.6 ± 1.1% at baseline vs. 5.0 ± 1.1% following ischemia-reperfusion). Exendin(9-39) abolished the protection induced by RIC (FMD 4.9 ± 0.9% at baseline vs. 1.4 ± 1.3% following ischemia-reperfusion; $p < 0.01$) but did not affect basal FMD. Plasma GLP-1 levels did not change significantly between examinations.
Conclusion: The present study is the first to suggest that RIC protects against endothelial ischemia-reperfusion injury via a GLP-1 receptor-mediated mechanism in humans.

1. Introduction

Successful treatment of patients with ST-segment elevation myocardial infarction (STEMI) is based on rapid reperfusion of the occluded coronary artery by primary percutaneous coronary intervention (PCI) to disrupt the ischemic insult to the heart. However, the return of blood flow paradoxically also has deleterious effects on the myocardium. This is known as reperfusion injury and significantly contributes to the final infarct size [1]. While there is still no established therapy to limit the reperfusion injury in clinical practice, several mechanical and pharmacological interventions are being explored [2]. Remote ischemic conditioning (RIC) is a promising approach by which short cycles of ischemia and reperfusion in remote tissue protects against ischemia-reperfusion injury. Effective cardioprotection was demonstrated in various animal models when the RIC stimulus was applied either before myocardial ischemia (remote ischemic preconditioning), during ischemia (remote ischemic perconditioning) or after the onset of reperfusion (remote ischemic postconditioning) [3–5]. Several clinical studies have demonstrated the efficacy of RIC in reducing infarct size in STEMI patients [6–9] and preventing endothelial ischemia-reperfusion injury [10,11]. Recent studies and meta-analyses have also indicated improved prognosis of STEMI patients following RIC [12,13]. By contrast, conflicting results with no reduction in infarct size in STEMI patients randomized to RIC exist [14]. This highlights the importance of clarifying the mechanisms underlying the protective effects of RIC in order to successfully translate preclinical findings to clinical benefit [15]. The mechanism/s by which the RIC stimulus may induce tissue protection has been proposed to involve both neural

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and humoral pathways [16,17]. A recent study demonstrated that selective sectioning of the posterior gastric branch of the vagal nerve abolished the protective effect of RIC, while stimulation of this branch evoked cardioprotection [18]. These data suggest that visceral organs innervated by the posterior gastric branch of the vagal nerve may serve as a source of a tissue protective factor. These organs release several peptide hormones, some of which protect from ischemia-reperfusion injury and improve endothelial function. Glucagon-like peptide-1 (GLP-1) is an incretin hormone released by the L-cells of the small intestine under the regulation of vagal efferent activity [19,20]. Several experimental studies have demonstrated cardioprotective effects of GLP-1 and GLP-1 receptor agonists in the setting of ischemia-reperfusion [21]. It was also shown that the GLP-1 agonist exenatide reduces infarct size in patients with STEMI when administered during and following PCI [22]. Furthermore, we were recently able to demonstrate that GLP-1 functions as a mediator of cardioprotection induced by RIC in a rat model of myocardial ischemia-reperfusion injury [23]. Based on these observations, we hypothesized that protective effects of RIC involves GLP-1 receptor-dependent mechanisms in humans. To test this we used a validated model of endothelial ischemia-reperfusion injury in humans to evaluate whether the GLP-1 receptor antagonist exendin(9-39) attenuates the protective effect of RIC.

2. Methods

2.1. Study subjects

Healthy male volunteers were recruited. For the main study fourteen subjects participated in three experimental protocols to evaluate whether the GLP-1 receptor antagonist exendin(9-39) attenuates the protective effect of RIC. Two subjects were excluded from analyses due to insufficient image quality of the ultrasound registration. In an additional protocol to evaluate the effect of exendin(9-39) on endothelial function per se, eleven healthy male volunteers (nine of which also participated in the main study) were examined. All subjects arrived in the laboratory after an overnight fasting period. They were informed to refrain from tobacco products on the study day. They were given oral and written information and gave their written consent. The study was approved by the regional ethics committee in Stockholm and was conducted according to the Declaration of Helsinki [24]. The clinical trial registration no. was NCT02746757 (clinicaltrials.gov).

2.2. Flow-mediated dilatation

All examinations were performed in the morning in a quiet dimly lit temperature-controlled (21–24 °C) room. A venous catheter was introduced in a superficial vein in the right antecubital fossa for drug administration and a deep vein in the left antecubital fossa for collection of blood samples during the examinations. Endothelial function was assessed by determination of flow-mediated dilatation (FMD) of the brachial artery of the non-dominant arm (left for all subjects) with an 11 MHz (output 12 MHz) linear-array transducer connected to a Vivid E9® (GE, Waukesha, Wisconsin, USA). The transducer was connected to a flexible tripod to prevent movement of the probe. ECG-gated images of baseline artery diameter at end-diastole were recorded every 3 s for 1 min and a mean value was calculated. A blood pressure cuff was placed around the upper part of the forearm and inflated to 200 mm Hg for 5 min. The diameter of the brachial artery was continuously recorded for 3 min during the hyperemia occurring following deflation of the cuff. The three frames displaying maximum dilatation at end-diastole were used to calculate a mean diameter [25]. Images were analyzed using the Brachial Analyzer software (Medical Imaging Applications, Iowa City, IA, USA). The distance between the intima of the near and far vessel walls defined the lumen diameter and the maximum diameter was found through beat-to-beat analysis. FMD was calculated as percentage increase from baseline diameter according to the following formula: diameter following cuff deflation − baseline diameter / baseline diameter × 100. Pulsed Doppler was used to determine peak blood flow velocities in hyperemia calculated as the mean of three different cardiac cycles. All evaluations were performed by a technician blinded to the randomization. Intra-observer variability for FMD was 0.1 ± 3.9 (bias and SD).

2.3. Study protocol

After measurement of basal FMD the left arm was subjected to 20 min of ischemia by inflating a blood pressure cuff placed around the upper arm to 200 mm Hg for 20 min. After 20 min of reperfusion a second FMD was performed. Each subject was examined on three occasions (protocols A, B and C as illustrated in Fig. 1) with at least six days between visits. The order of protocols was randomized for each subject. In protocol A (control ischemia-reperfusion) no intervention took place. In protocol B, (ischemia-reperfusion + RIC) RIC was performed during ischemia and reperfusion with a blood pressure cuff around the left thigh. The cuff was connected to an automated device (PeriVasc Cuff Unit, EBIDA, Göteborg, Sweden) which controlled inflation to 200 mm Hg for 5 min and deflation for 5 min for four cycles. In protocol C, (exendin(9-39) + ischemia-reperfusion + RIC), the GLP-1 receptor antagonist exendin(9-39) (Bachem, Bubendorf, Switzerland) was administered in the contralateral arm to the one used for FMD measurement as a bolus of 7500 pmol/kg iv given 10 min before the first FMD measurement and immediately followed by a continuous iv infusion of 500 pmol/kg/min throughout the remaining protocol. This dose of exendin(9-39) has previously been shown to antagonize the effect of GLP-1 in humans [26,27]. RIC was performed as described above. RIC protocols employing cycles of 5 min ischemia and 5 min reperfusion of the leg have previously been shown to protect from forearm endothelial dysfunction induced by ischemia-reperfusion [28]. In an additional protocol D the same dose of exendin(9-39) was administered after an initial FMD measurement to evaluate the effect of exendin(9-39) on FMD per se (Fig. 1). The FMD measurement was repeated 40 min after the start of the infusion of exendin(9-39). No forearm ischemia or RIC was performed in this protocol. Endothelium-independent vasodilatation was determined in 11 of the subjects by administration of 0.4 mg of nitroglycerine at the end of the examinations. Nitroglycerine was not administered before ischemia due to that its long-acting vasodilatation may influence baseline radial artery diameter following ischemia. The effect of exogenously administered NO donors is not affected by ischemia-reperfusion [29]. Therefore, only one time-point at the end of the protocol was used to evaluate the effect of RIC on endothelium-independent vasodilatation.

2.4. Blood sample analysis

Blood samples were collected before forearm ischemia, 1 min and 16 min after the onset of reperfusion. The blood samples for analyses of plasma GLP-1 were immediately placed in tubes prepared with the dipeptidyl peptidase 4 inhibitor diprotin (final concentration 0.1 mmol/L) to avoid cleavage. Plasma GLP-1 was analyzed using a commercially available ELISA according to the manufacturer's instructions (ELISA No EZGLP1T, Merck). This ELISA measures total GLP-1 including both the active form (7-36) and fragments of GLP-1. It gives high recoveries of all amidated isoforms and non-amidated forms of GLP-1 [30]. The intra-assay variation is <5%. All samples were analyzed using the same lot. Blood samples for analysis of insulin were collected in EDTA tubes before FMD at baseline and at the end of reperfusion and plasma insulin was analyzed by electrochemiluminescence immunoassay.

2.5. Statistical analysis

The primary endpoint of the study was change in FMD after ischemia-reperfusion from baseline. Based on our own pilot studies, assuming a relative improvement in FMD of 30%, twelve subjects were needed to achieve 80% power at a significance level of 5%. To compensate for possible dropouts and examinations with poor image quality fourteen volunteers were recruited. Normality of data was tested using D'Agostino and Pearson normality test. Data were analyzed with one-way ANOVA for comparison of treatment effect and two-way ANOVA for comparison of FMD and plasma levels of GLP-1 between baseline and following ischemia-reperfusion and between groups (treatment and time) using GraphPad Prism version 6.05 for Windows (GraphPad software, La Jolla California USA, www.graphpad.com). Data are presented as means ± SEM. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Study subjects

Subjects were 30.5 ± 2.1 years old with a body mass index of 24 ± 0.7, fasting glucose of 5.1 ± 0.1 and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) of 1.5 ± 0.3. Self-assessed exercise capacity was normal or good for all participants. One subject reported regular intake of dietary supplements (vitamin C and omega 3) and two were smokers. All subjects tolerated the examination protocols well. Plasma glucose was unaffected by the infusion of exendin(9-39).

3.2. Flow-mediated dilatation

Baseline brachial artery diameter or maximal flow rate during hyperemia did not differ between the study protocols (Table 1). There was no difference in baseline FMD between the three protocols (Fig. 2). In protocol A, FMD was reduced from 4.7 ± 0.8% at baseline to 1.5 ± 0.4% after forearm ischemia-reperfusion ($p < 0.01$). RIC applied during ischemia-reperfusion in protocol B protected from the impairment of FMD induced by ischemia-reperfusion (Fig. 2). Administration of exendin(9-39) in protocol C abolished the protective effect of RIC against ischemia-reperfusion induced endothelial dysfunction (FMD 4.9 ± 0.9% at baseline vs. 1.5 ± 1.3% after ischemia-reperfusion; $p < 0.01$). Thus, FMD following ischemia-reperfusion was significantly greater in protocol B (ischemia-reperfusion + RIC) than in protocol A (control ischemia-reperfusion) ($p < 0.05$) and protocol C (exendin(9-39) + ischemia-reperfusion + RIC) ($p < 0.05$). Furthermore, the change in FMD from baseline to

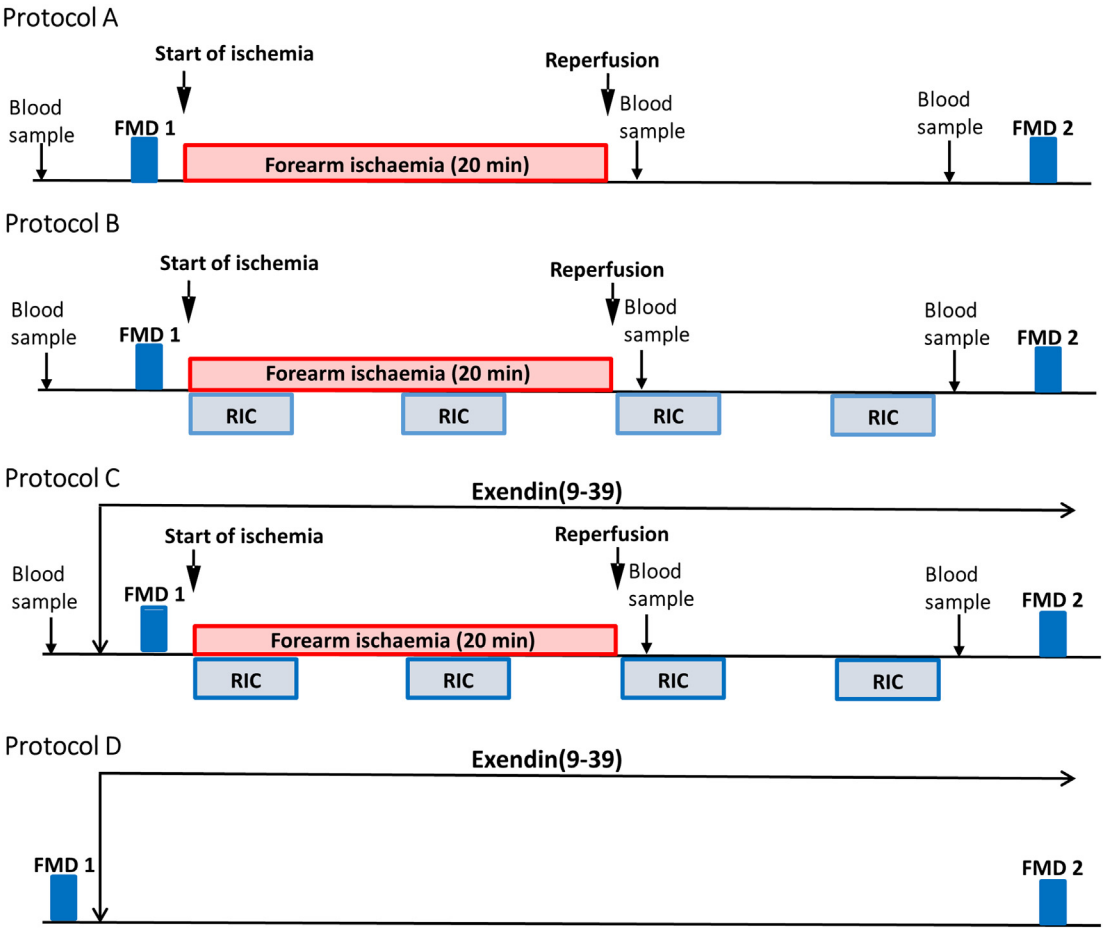

Fig. 1. Study protocol. Protocol A = Ischemia-reperfusion. Protocol B = Ischemia-reperfusion + RIC. Protocol C = Exendin(9-39) + ischemia-reperfusion + RIC. Protocol D = Exendin(9-39). FMD = flow mediated dilatation; GLP-1 = glucagon like peptide 1; RIC = remote ischemic conditioning.

post ischemia-reperfusion was significantly different between the protocols (protocol A vs. B $p < 0.05$, protocol B vs. C $p < 0.01$). In protocol D, bolus dose and 40 min infusion of exendin(9-39) did not affect FMD per se (4.1 ± 0.3% at baseline vs. 4.2 ± 0.5% after 40 min infusion). Neither brachial artery diameter (3.3 ± 0.1 vs. 3.4 ± 0.1 mm) or peak flow (57 ± 4.9 vs. 62 ± 4.8 cm/s) was affected by the exendin(9-39) infusion.

3.3. Nitroglycerine-induced dilatation

Endothelium-independent dilatation of the brachial artery induced by sublingual nitroglycerine did not differ significantly between the study protocols (protocol A: 11.8 ± 1.7%; protocol B: 11.4 ± 1.8%, protocol C: 15.2 ± 3.3%) and protocol D (16.2 ± 1.5%).

3.4. GLP-1 and insulin levels

Plasma GLP-1 and insulin levels did not change significantly in any of the protocols (Table 2).

4. Discussion

The mechanism underlying the protective effect induced by RIC has been a matter of extensive investigation. In the present study we demonstrate that the impairment in FMD caused by ischemia-reperfusion was prevented by RIC. Systemic administration of the GLP-1 receptor antagonist exendin(9-39) abolished this protection. Exendin(9-39) did not affect FMD per se, but rather interfered with RIC-induced protection of endothelial function. These results suggest an important role of the GLP-1 receptor in the protective effect of RIC in humans.

Protection from ischemia-reperfusion injury by RIC has attracted much attention due to its efficacy and clinical feasibility. The protective effect of RIC has been established in many experimental studies [31]. Although most clinical studies have demonstrated protective effects, of RIC neutral results have recently been presented [6–8,12–14]. These apparently inconsistent data highlight the translational challenge associated with this research field [32,33] and underline the importance of identification of underlying mechanisms behind RIC in humans to take full advantage of its therapeutic potential. The forearm model of

Table 1
Brachial artery diameter and peak flow.

Variables	Protocol A		Protocol B		Protocol C	
	Before IR	After IR	Before IR	After IR	Before IR	After IR
Baseline diameter (mm)	3.6 ± 0.1	3.8 ± 0.1	3.5 ± 0.1	3.7 ± 0.1	3.5 ± 0.1	3.7 ± 0.1
Vpeak (cm/s)	58 ± 6	67 ± 5	66 ± 5	69 ± 5	61 ± 6	62 ± 4

Brachial artery diameter and peak flow from protocols A (ischemia-reperfusion), B (ischemia-reperfusion + RIC) and C (exendin(9-39) + ischemia-reperfusion + RIC). $n = 12$. Means and SEM. IR = ischemia-reperfusion; RIC = remote ischemic conditioning.

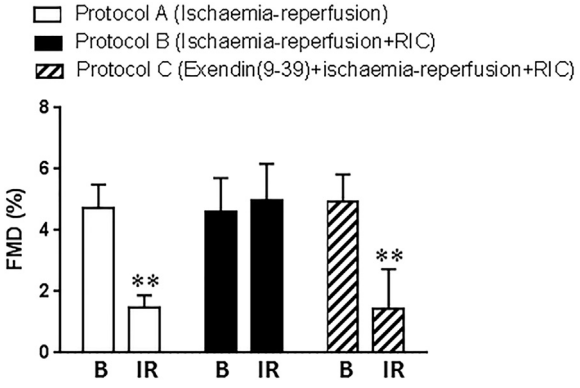

Fig. 2. Impairment of endothelial function following ischemia-reperfusion. Flow-mediated dilatation (FMD) at baseline (filled symbols) and following ischemia-reperfusion (open symbols) in protocols A, B and C depicted as scatter plot, mean values with 95% CI. Significant differences from baseline FMD are depicted. Significant difference between baseline and ischemia-reperfusion in each protocol is depicted; $^{**}p < 0.01$; one-way ANOVA. B = baseline; I/R = ischemia-reperfusion; RIC = remote ischemic conditioning.

ischemia-reperfusion with determination of endothelial function is a validated model for such investigations [10,28]. In line with our present protocol, RIC has been shown to protect from endothelial dysfunction induced by forearm ischemia-reperfusion [28].

The transduction of the conditioning stimulus from remote to end organ is still far from fully understood and several possible pathways have been identified [34]. The observations that dialysate from a remotely conditioned animal exerts cardioprotection in a recipient Langendorff heart [35] even across species [36–38] in different settings provide evidence that one or several humoral factors carry the conditioning effect. In addition, there is strong evidence for the involvement of a neural pathway in RIC. The cardioprotective effect of remote ischemic preconditioning is abolished by sectioning of the sciatic and the femoral nerve [5,39,40], the spinal cord or the vagus nerve [5,41]. It is in addition prevented by the muscarinic cholinoreceptor blocker atropine [41,42] and by the autonomic ganglion blockers hexamethonium [4] and trimetaphan [10]. Intriguingly, the ability to provide cardioprotection through plasma dialysate administered to a recipient heart is lost if plasma dialysate is obtained from diabetic patients with peripheral neuropathy [43]. Furthermore the cardioprotective effect of dialysate from RIC treated rats is lost if the donor is subjected to bilateral vagotomy before the RIC stimulus or if the recipient heart receives hexamethonium or atropine [44]. These data demonstrate an important interplay between the humoral and neural pathways in tissue protection induced by RIC.

Among several compounds being explored for pharmacological cardioprotective effects, GLP-1 is one of the most promising [45]. Lønborg et al. [22] showed that the GLP-1 agonist exenatide exerts cardioprotection when added to primary PCI for patients with STEMI. These findings were supported in a subsequent study [46]. Additionally, Alburquerque-Béjar et al. showed that the GLP-1 receptor analogue has an additive cardioprotective effect to RIC in pigs [47]. Interestingly, in a recent study we demonstrated that GLP-1 is causally involved in cardioprotection induced by RIC in a rat model of myocardial ischemia-reperfusion injury [23]. Systemic GLP-1 receptor blockade with exendin(9-39) abolished RIC-induced cardioprotection and phosphorylation of the pro-survival kinase Akt. Based on these data we hypothesized that the protective effect of RIC against endothelial ischemia-reperfusion injury in humans involves activation of the GLP-1 receptor. Accordingly, the present study showed that the specific GLP-1 receptor antagonist exendin(9-39) abolished the protective effect of RIC on endothelial function following ischemia-reperfusion in humans. While our findings suggest that RIC acts through activation of GLP-1 receptors, the localization of these receptors and the interaction between the RIC-induced stimulus and the GLP-1 receptors is still unclear. Previous experimental studies have provided evidence for involvement of the vagal nerve for the protective effect of RIC [5,18,44], and the release of GLP-1 is modulated by vagal nerve activity [19,20,48]. Vagal nerve activation by RIC may stimulate release of GLP-1 from intestinal L-cells [20]. Hence, one possibility is that RIC stimulates vagal activity which results in release of GLP-1. The GLP-1 may act locally to relay the conditioning signal to the end-organ. The observation that circulating GLP-1 levels did not change significantly may support such a local mechanism. Although GLP-1 is known to improve endothelial function via a GLP-1 receptor-dependent mechanism [49], it seems unlikely that the subtle change in plasma GLP-1 levels in our study reflects a direct endothelium-protective effect mediated through circulating GLP-1. RIC may induce activation of non-endothelial GLP-1 receptors through local GLP-1 release at an unidentified site that is not reflected in clear increases in plasma GLP-1 levels and these GLP-1 receptors may further relay the protective signal by neural and/or humoral pathway(s) to eventually evoke end-organ protection. While not reaching statistical significance, the small change (20% increase) in plasma GLP-1 levels was of the same magnitude as those observed in rats subjected to RIC [23]. Another recent study demonstrated that the GLP-1 receptor-dependent endothelium-protective effects of Roux-en-Y gastric bypass surgery in obese rats and patients were associated with only a modest increase in plasma GLP-1 [50]. Remarkably, it has been shown that intra-arterial infusion of GLP-1 does not increase forearm blood flow in healthy subjects even when its concentration is increased by dipeptidyl-peptidase 4 inhibition [51]. Thus, the underlying biology of GLP-1 receptor-mediated mechanisms is complex and the precise way by which GLP-1 exerts its actions on the endothelium is not fully understood [21].

The present trial adds valuable new insight into the complex underlying mechanisms of RIC-induced protection against ischemia-reperfusion injury and may provide an important tool in interpreting the findings of the important RIC trials with clinical end-points soon to be completed i.e. the CONDI-2 (ClinicalTrials.gov NCT01857414) and the ERIC-PPCI (ClinicalTrials.gov NCT02342522) as well as the COMBAT-MI (ClinicalTrials.gov NCT02404376).

Table 2
Plasma GLP-1 and insulin levels.

	Protocol A	Protocol B	Protocol C
Plasma GLP-1			
– Baseline	13.5 ± 3.1	11.8 ± 2.9	11.1 ± 2.9
– At reperfusion	14.2 ± 3.4	14.1 ± 3.0	13.6 ± 3.1
– 16 min after reperfusion	13.8 ± 3.5	12.3 ± 3.0	10.0 ± 2.2
Plasma insulin			
– Baseline	6.4 ± 1.2	5.9 ± 0.7	6.4 ± 0.9
– 16 min after reperfusion	5.3 ± 0.9	7.0 ± 1.5	5.5 ± 0.6

Plasma GLP-1 and insulin levels before ischemia and during reperfusion. Values for GLP-1 are pmol/L and for insulin mIE/L. $n = 12$. Means and SEM. Two-way ANOVA. GLP-1 = glucagon like peptide-1.

4.1. Study limitations

Limitations of our study should be acknowledged. The study was not blinded due to the practical nature of the RIC procedure. However, the analyses of FMD were performed by an examiner blinded to randomization. The endpoint was change in endothelial function and it remains to be established whether similar mechanisms are involved in cardioprotection. However, endothelial dysfunction is a key event in the development of myocardial ischemia-reperfusion injury and improvement in FMD is therefore an appropriate surrogate end point as demonstrated in previous studies [10,52,53]. While this was a small sample study investigating underlying mechanisms of RIC in the setting of endothelial ischemia-reperfusion injury, large sample studies are required to confirm the clinical significance of our findings.

4.2. Conclusions

The present trial is the first to suggest that RIC protects against endothelial ischemia-reperfusion injury in humans through a mechanism involving the GLP-1 receptor. Further studies are warranted for better understanding of the interplay between RIC, GLP-1 and protection against ischemia-reperfusion injury in order to establish effective therapies in the clinical setting.

Conflict of interest

The authors have no conflicts of interest to declare.

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