Combination therapy with remote ischaemic conditioning and insulin or exenatide enhances infarct size limitation in pigs

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Aims
Remote ischaemic conditioning (RIC) has been shown to reduce myocardial infarct size in patients. Our objective was to investigate whether the combination of RIC with either exenatide or glucose–insulin–potassium (GIK) is more effective than RIC alone.

Methods and results
Pigs were submitted to 40 min of coronary occlusion followed by reperfusion, and received (i) no treatment, (ii) one of the following treatments: RIC (5 min ischemia/5 min reperfusion × 4), GIK, or exenatide (at doses reducing infarct size in clinical trials), or (iii) a combination of two of these treatments (RIC + GIK or RIC + exenatide). After 5 min of reperfusion (n = 4/group), prominent phosphorylation of Akt and endothelial nitric oxide synthase (eNOS) was observed, both in control and reperfused myocardium, in animals receiving GIK, and mitochondria from these hearts showed reduced ADP-stimulated respiration. 1H NMR-based metabonomics disclosed a shift towards increased glycolysis in GIK and exenatide groups. In contrast, oxidative stress (myocardial nitrotyrosine levels) and eNOS uncoupling were significantly reduced only by RIC. In additional experiments (n = 7–10/group), ANOVA demonstrated a significant effect of the number of treatments after 2 h of reperfusion on infarct size (triphenyltetrazolium, % of the area at risk; 59.21 ± 3.34, 36.64 ± 3.03, and 21.04 ± 2.38% for none, one, and two treatments, respectively), and significant differences between one and two treatments (P = 0.004) but not among individual treatments or between RIC + GIK and RIC + exenatide.

Conclusions
GIK and exenatide activate cardioprotective pathways different from those of RIC, and have additive effects with RIC on infarct size reduction in pigs.

Keywords
Insulin • Myocardial infarction • Reperfusion injury • GLP-1 • Remote ischaemic conditioning

1. Introduction
Although emergent reperfusion therapy has reduced mortality and morbidity in patients with ST-segment elevation myocardial infarction (STEMI), extensive areas of myocardial necrosis still occur in a high proportion of these patients and result in substantial mortality and incidence of heart failure and arrhythmias. This is mainly due to the difficulty of shortening total ischaemic time, but also to the fact that part of the beneficial effect of reperfusion is lost due to a certain amount of additional cell death caused by reperfusion itself, a phenomenon called myocardial reperfusion injury.1 During the last years, experimental investigations have identified a number of therapeutic strategies potentially able to attenuate myocardial reperfusion injury and some have been found able to limit infarct size in humans. However, none of them is so far part of standard clinical practice.2 This failure of translation is due, at least in part, to the fact that the effect of these available cardioprotective interventions on infarct size is in general weak and/or inconsistent, and is often influenced by clinical conditions as age, comorbidities, and concomitant medications.3

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Activation of endogenous mechanisms of protection by ischaemic stimuli appears to be a potentially effective strategy to reduce infarction in patients with STEMI. Remote ischaemic conditioning (RIC), induced by brief episodes of ischaemia and reperfusion applied in an arm or leg, appears particularly promising. RIC is safe, inexpensive, and applicable at the point of care, has no drug-related side effects, and has consistently been found beneficial in a series of pilot clinical trials in patients with STEMI and during coronary surgery. However, the protective effect of RIC is relatively weak and may be attenuated in patients with diabetes or metabolic syndrome, and by concomitant treatments.

Another approach to cardioprotection against reperfusion injury in patients with STEMI is pharmacological treatment, including drugs targeting an end-effector of cell death: the mitochondrial permeability transition pore, like cyclosporine, drugs that increase the cGMP/PKG signalling pathway, like the atrial natriuretic peptide, drugs used in the treatment of diabetes, to increase glucose uptake and metabolism, like glucose–insulin–potassium (GIK), 13,14 or exenatide, 15 and treatments expected to interfere with leucocyte-mediated injury, like metoprolol. 16

To the best of our knowledge, the usefulness of combining RIC and pharmacological protection to obtain a stronger cardioprotective effect has not been investigated. A combination therapy has several potential advantages when compared with single interventions. An additive effect can be expected if the mechanisms of action of the combined interventions are different, or in cases when maximal doses of each intervention cannot be applied (like lack of time to administrate the full RIC protocol). In addition, combined therapy may be beneficial when comorbidities or concomitant treatments reduce the efficacy of one of the individual treatments being combined.

In this study, we investigated the potential usefulness of combining RIC with either GIK or exenatide, a mimetic of the incretin glucagon-like peptide-1 (GLP-1), to attenuate reperfusion injury and reduce infarct size after transient coronary occlusion in a pig model. Exenatide has consistently been found to be protective and safe in clinical trials and has consistently been found to be protective and safe in clinical trials far in patients with suspected acute coronary syndrome demonstrated a marked beneficial effect on infarct size in a pre-specified subgroup of patients with STEMI. 13

2. Methods

The present study conforms to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and the NIH Guide for the Care and Use of Laboratory Animals (NIH publications N°. 85-23, revised 1996, updated in 2011). The study was approved by the Ethics Committee of our institution (reference numbers: 53/11 CEEA and 13/15 CEEA). A complete description of the methods used can be found in Supplementary material online.

2.1 Animals and instrumentation

Seventy hybrid farm pigs (25–30 kg, 12 h fasting) were premedicated with tiletamine–zolazepam (4–6 mg/kg, IM) and xylazine (1–2 mg/kg, IM), anaesthetised with propofol–lipuro 1% (1.5–2.5 mg/kg, IV, followed by continuous infusion at 11 mg/kg/h) and fentanyl (5 μg/kg, IV, followed by continuous infusion at 3–6 μg/kg/h), and mechanically ventilated. A mid-sternotomy was performed, and the left anterior descending (LAD) coronary artery was dissected free at its midpoint to place a coronary ligature. Lead II of ECG, left ventricular (LV) pressure and LV dP/dt, coronary blood flow, and regional myocardial function were measured as previously described. At the end of the experiments, animals were sacrificed by a pentobarbital overdose (100 mg/kg, IV).

2.2 Study protocols

Mechanisms of protection were assessed in 24 pigs submitted to 40 min of LAD coronary artery occlusion followed by 5 min of reperfusion (n = 4/group). Myocardial tissue samples were quickly obtained from both a control region and the area at risk and processed as described below, to analyse mitochondrial respiration and the degree of activation of RISK, SAFE, and cGMP/PKG pathways by western blot analysis. To assess the effects of treatments on infarct size, 46 animals were submitted to 40 min of LAD coronary artery occlusion followed by 2 h of reperfusion (n = 7–10/group).

Blood glucose concentration was measured before ischaemia and at 5 min of reperfusion using a commercially available kit (Glucocard G⁺ meter, Arkray Factory, Shiga, Japan). Insulin was measured in plasma samples taken at 5 min of reperfusion using a colorimetric ELISA kit (ab100578, Abcam, Cambridge, UK) at 450 nm. Animals were allocated to one of six groups, as detailed below.

2.2.1 Control ischaemia–reperfusion

Animals received an intravenous infusion of 5% glucose, at 1.5 mL/kg/h, starting 10 min after the onset of ischaemia and continuing until the end of the experiment. No additional manoeuvre was performed.

2.2.2 Remote ischaemic conditioning

Animals were submitted to four cycles of 5 min of right lower limb ischaemia followed by 5 min of reperfusion, using an elastic snare placed around the right femoral artery and starting 40 min before reperfusion.

2.2.3 Glucose–insulin–potassium

A continuous intravenous infusion of GIK (Actrapid, Novodisk A/S, Bagsvaerd, Denmark), and 80 mEq KCl/L, at 1.5 mL/kg/h, given intravenously, starting 30 min before reperfusion and continuing for the entire duration of reperfusion, as described in the IMMEDIATE trial.

2.2.4 Exenatide

A continuous intravenous infusion of exenatide (Byetta 10, Bristol-Myers Squibb/AstraZeneca EEEG, Middlesex, UK; 10 μg in 100 mL of saline) was administered during the last 15 min of ischaemia at 72 mL/h (0.12 μg/min) and then reduced to 26 mL/h (0.043 μg/min) during the entire reperfusion period, as previously used in humans.

2.2.5 RIC + GIK

A GIK solution was administered while RIC was performed at the right lower limb, using the previously described protocols.

2.2.6 RIC + exenatide

Exenatide was combined with RIC using the protocols described before.

2.3 Analysis of myocardial metabolism

Cardiac metabolites were extracted using the methanol : chloroform mixture followed by 5 min of reperfusion, using an elastic snare placed around the right femoral artery and starting 40 min before reperfusion.

2.4 Activation of cytosolic signalling pathways

The degree of activation at both myocardial areas, and under the different treatments. The RISK (Akt, ERK1/2, GSK3β, p38 MAPK, and AMPK) and SAFE (STAT3) pathways was investigated by western blot analysis according to standard procedures, by determining the phosphorylation state (ratio phosphorylated/total form) of the proteins. The role played by the cGMP/PKG pathway was assessed by western blot analysis of the
phosphorylation state of eNOS, dimer:monomer eNOS ratio, and nitrotyrosine formation, as previously described.\textsuperscript{21}

\subsection*{2.5 Analysis of mitochondrial respiration}
Mitochondria were isolated from myocardial tissue samples 5 min after reperfusion and mitochondrial oxygen consumption was measured with an oxymeter (Clark-type oxygen electrode, Hansatech, Norfolk, UK) as previously described.\textsuperscript{22}

\subsection*{2.6 Area at risk and infarct size}
At the end of the 2 h reperfusion period, the LAD was reocluded and the size of the area at risk and of infarction were determined by 10% fluorescein and 1% 2,3,5-triphenyltetrazolium chloride staining, respectively, as previously described.\textsuperscript{18}

\subsection*{2.7 Statistical analysis}
Data are expressed as mean ± SEM. Differences in the mechanistic variables analysed were assessed by one-way ANOVA, and predictors for mitochondrial activity and western blot data were determined by stepwise regression analysis. Analysis of temperature, infarct size, and area at risk was performed by hierarchical ANOVA. The superior level was the number of cardioprotective treatments applied (0, 1, or 2). A second level was constituted by the different groups with individual treatments or treatment combinations. Changes in the time course of myocardial systolic shortening, heart rate, or haemodynamic variables were studied by repeated-measures ANOVA (MANOVA) and Dunnett’s post hoc test. Differences were considered significant when $P < 0.05$. For pattern recognition purposes, NMR spectra were imported into the Simca V13 software (Umetrics) where both unsupervised (principal component analysis) and supervised orthogonal projections to latent structures (OPLS) methods were applied.

\section*{3. Results}

\subsection*{3.1 Mechanisms of protection}

\subsubsection*{3.1.1 Blood glucose concentration}
Glycaemia was significantly decreased during the coronary occlusion period, with no differences between groups (Figure 1). Plasma insulin concentration was increased only in animals treated with GIK (Figure 1).

\subsubsection*{3.1.2 Myocardial metabolism}
Myocardium from area at risk showed a marked decrease in ADP, ATP, and glutamate concentrations, and a significant increase in lactate, succinate, and alanine, with no differences between groups (see Supplementary material online, Figure S1).

Supervised classification discriminated myocardial samples from control and RIC groups from those receiving GIK or exenatide ($P < 0.05$, $Q^2 = 0.129$ and 0.002, respectively). It was not possible to further discriminate between samples receiving GIK and exenatide treatment. When samples treated with GIK or exenatide were grouped, the OPLS model obtained had a high predictive value ($Q^2 = 0.421$; Figure 2A), suggesting that both GIK and exenatide had similar effects on the cardiac metabolic pattern. Using this model, samples corresponding to metabolic interventions alone (Figure 2B), used as a positive control, were classified as those corresponding to metabolic interventions alone (Figure 2B). However, we could not detect a single metabolite that was responsible for the differences between treatments.

\subsubsection*{3.1.3 Activation of cytosolic signalling pathways}
Treatment with GIK induced an increased Akt and STAT3 phosphorylation (Figure 3). Analysis of the phosphorylation state of the different proteins (i.e. their activation state) by stepwise regression showed a significant increase of ERK1/2, GSK3β, and p38 MAPK phosphorylation in reperfused myocardium (see Supplementary material online, Figure S2), and a reduction for Akt and STAT3 (Figure 3).

\subsubsection*{3.1.4 eNOS activity}
The phosphorylation state of eNOS was reduced in the area at risk (Figure 4A), whereas no change was observed for the dimer:monomer eNOS ratio (Figure 4B). GIK increased eNOS phosphorylation in both reperfused and control myocardium, whereas exenatide increased eNOS phosphorylation in reperfused myocardium only (Figure 4A).

\subsubsection*{3.1.5 Mitochondrial respiration}
Mitochondria from control pigs showed reduced ADP-stimulated oxygen consumption in the reperfused when compared with control myocardium (29.3 ± 9.2% reduction; Figure 5), without differences between groups. However, in mitochondria from control myocardium, a reduction in ADP-stimulated oxygen consumption was observed with GIK treatment (Figure 5).

\subsubsection*{3.1.6 Myocardial nitrotyrosine levels}
Nitrotyrosine levels were elevated in reperfused myocardium (Figure 5C) and significantly reduced by RIC (Figure 4C). RIC was also the only treatment associated with a higher dimer:monomer eNOS ratio (Figure 4B).

\subsection*{3.2 Infarct size studies}

\subsubsection*{3.2.1 Haemodynamic variables and LAD coronary blood flow}
No significant differences were observed between the different groups in the time course of these variables during ischaemia—reperfusion (see Supplementary material online, Table S1, and Figures S3 and S4).

\subsubsection*{3.2.2 Regional myocardial function}
There were no differences between groups in regional myocardial shortening (see Supplementary material online, Table S1 and Figure S5). Reperfusion-induced hypercontracture was maximal in the control group (Figure 6), and significantly reduced by RIC (Figure 6, lower panel).
3.2.3 Infarct size
No differences were observed between groups in the size of the area at risk or body temperature (Figure 7A and B). Infarct size in control pigs submitted to 40 min of LAD coronary artery occlusion followed by reperfusion averaged 59.21 ± 3.17% of area at risk (Figure 7C). Factorial analysis demonstrated a significant effect of the number of treatments (none, one, or two) on infarct size (P = 0.001), with no significant differences between individual treatments or treatment combinations. Combination therapy (two treatments) was more effective in reducing infarct size than individual treatments (P = 0.004; Figure 7D).

4. Discussion
This study shows that RIC, GIK, and exenatide have different impacts on key cardioprotective pathways, RIC reducing more markedly oxidative stress, as assessed by nitrotyrosination, while GIK sharing with exenatide an effect on myocardial metabolism of glucose, and having, in addition, a prominent effect on the Akt–eNOS axis (RISK pathway). Combination therapy (RIC with either GIK or exenatide) was more effective than individual treatments in limiting infarct size. These results indicate that combination therapy may be advantageous respect to RIC to prevent reperfusion injury in patients with STEMI.

4.1 GIK and exenatide have similar effects on myocardial metabolism
Both insulin and GLP-1 play key roles in various aspects of cardiovascular metabolism, promoting glucose uptake and glycolysis,23,24 and have cardioprotective effects.25–27 In the case of insulin, these effects have been linked to increased myocardial ATP, creatine phosphate, and glycogen content,23 whereas protection induced by GLP-1 has been
found to be sensitive to iodo-acetate, implicating stimulation of glycolysis, and associated with activation of the glycolytic enzyme phosphofructokinase-2.28 Our present NMR data confirm that both GIK and exenatide act on myocardial metabolism and share a common metabolic fingerprint.

4.2 GIK signals through Akt and STAT3

Binding of insulin to its receptor induces its autotransphosphorylation and the phosphorylation of downstream targets, including PI3K–Akt and MAPK pathways,23 whereas stimulation of GLP-1 receptors has been suggested to activate several prosurvival pathways, including kinases as PI3K and p22/44 MAPK27 or PI3K and AMPK.28 Our study demonstrates an increased activation of both Akt and eNOS in groups treated with GIK, both in the area at risk and in control myocardium. In addition, a role for the SAFE pathway in GIK protection cannot be discarded, as STAT3 activation was also increased in the area at risk after this treatment. In contrast to GIK, western blot analysis indicates that the signalling pathways activated by both treatments are, at least in part, different, as exenatide did not increase Akt phosphorylation. These differences in cell signalling are not contradictory with similar metabolic effects, as it has been proposed that exenatide may stimulate glucose uptake and metabolism by Akt-independent mechanisms.29 On the other hand, the increased phosphorylation of eNOS in the absence of Akt phosphorylation in the area at risk of exenatide-treated groups is not easy to explain. One possibility is...
that eNOS may also become activated independently of Akt, as it has been suggested in hypertrophied cardiomyocytes, where activation involved AMPK and SIRT1. Another possibility is that there is a temporal dissociation between Akt activation and eNOS phosphorylation in the case of exenatide. Our samples were taken at 5 min of reperfusion, but transient activation of Akt before this time cannot be discarded.

Since inhibition of mitochondrial respiration can limit myocardial reperfusion injury, we examined respiration in mitochondrial preparations from the different treatment groups. Infusion of GIK, but not exenatide, had an inhibitory effect on mitochondrial respiration, despite similar metabolic fingerprints of both treatments. The effect of GIK was specifically manifested at complex 1-mediated respiratory activity. A potential mechanism for the effect of GIK on mitochondrial oxygen consumption is the posttranslational nitrosation of respiratory proteins. In agreement with this hypothesis, we observed a clear increase in phosphorylation state of eNOS in GIK-treated hearts.

Complex 1 is a major source of reactive oxygen species at reperfusion and its transient inhibition reduces reperfusion injury. In particular, S-nitrosation at mitochondrial complex 1 has been demonstrated to decrease oxidative damage and necrosis in vivo by slowing the reactivation of mitochondrial respiration during the first minute of reperfusion. However, the possibility exists that changes in respiration were, in fact, not directly related to protection. In this regard, we have to consider that changes in respiration in the control area were quantified after a long period of exposure to GIK (35 min), whereas changes in the area at risk were measured after 5 min of exposure to this treatment. This alone could explain that effects in respiration were more subtle in the area at risk than in control myocardium. Furthermore, although the decrease in state 3 respiration in the control area in the group treated with GIK + RIC did not reach significance, there is a trend towards a change in the same direction than in control tissue, i.e. a reduction in respiration.

4.3 RIC, but not GIK or exenatide, markedly reduces nitrotyrosination in myocardial tissue

Previous studies have demonstrated that superoxide generated during the first minute of reperfusion combines with NO at a very fast rate to generate peroxynitrite (ONOO$^-$). Peroxynitrite can react with different biological targets of the cell and, in addition, reduces the availability of free NO and the activity of NOS by acting on its haem group and by oxidizing its cofactor tetrahydrobiopterin. Inhibition of ONOO$^-$ synthesis and the use of ONOO$^-$ scavengers or
decomposition catalysts have been associated with reduced reperfusion injury in different experimental models. Recently, our group has demonstrated that attenuation of oxidative stress during reperfusion and subsequent preservation of the cGMP/PKG pathway represents a primary cardioprotective mechanism of ischaemic postconditioning. The present study demonstrates that RIC, but not GIK or exenatide, markedly reduces nitro-oxidative stress. These results are consistent with the increased eNOS coupling observed in the group receiving RIC, indicative of attenuated oxidation of eNOS and tetrahydrobiopterin.

Previous studies have associated protection by RIC with activation of the RISK pathway. Hausenloy et al. demonstrated an increased Akt phosphorylation 5 min after reperfusion in pigs submitted to 60 min of ischaemia followed by reperfusion, and protection by RIC was abolished by wortmannin, a PI3K inhibitor. Our study did not detect this effect of RIC. However, it was not aimed at investigating the mechanisms of RIC, but to detect potential differences in the effect of tested treatments on key cardioprotective pathways. Altogether, the previous data study suggests that the predominant mechanisms of cardioprotection induced by the treatments investigated in this study may be indeed different (Figure 8).

4.4 Combination therapy is superior to individual therapies to limit infarct size
RIC has been found effective in a large series of studies, including clinical trials, and lacks pharmacological side effects, which makes it an ideal candidate to be combined with other treatments. However, few studies have analysed the effect of combined therapies to enhance the beneficial effect of RIC on infarct size after transient coronary occlusion. Xin et al. observed additive protection between RIC and local ischaemic postconditioning in vivo in rats. Also, Schmidt et al. identified an additive protective effect between glucose–insulin and remote ischaemic preconditioning in neonatal porcine hearts, where both treatments had no or deleterious effects when given alone. Our data in the mature porcine heart clearly indicate that combining RIC with either GIK or exenatide is more effective in reducing infarct size than any of these interventions alone.

Figure 7 Size of the area at risk (A) and body temperature during ischaemia (B) in pigs submitted to 40 min of LAD coronary occlusion followed by 120 min of reperfusion. (C) Infarct size in the same groups of animals. (D) Analysis by hierarchical ANOVA of infarct size. *P < 0.001 indicates significant differences vs. control pigs. $P < 0.01 indicates significant differences between groups treated with one and two cardioprotective manoeuvres.
4.5 Study limitations

Application of treatments at the onset of reperfusion is more feasible than application before reperfusion in many clinical settings and, in particular, in STEMI. However, several previous studies have demonstrated that application of cardioprotective strategies in the ambulance, or during procedures prior to coronary artery recanalization, is possible. In fact, in this study, we used protocols identical to those found protective in pilot clinical trials. In the study by Botker et al., RIC was applied soon after symptom onset, during transportation to the hospital in the ambulance and before reperfusion. In a similar way, and to ensure the earliest possible drug administration, in the IMMEDIATE study, GIK was given during transportation to the hospital, well before reperfusion, whereas exenatide was given 15 min before reperfusion. Thus, the interventions and combinations we tested are clearly feasible in patients with STEMI. One additional consideration is that we cannot rule out that part of the observed effects was due to actions during the ischaemic phase rather than during the reperfusion period, although it appears unlikely that a significant amount of the administered drugs can reach the ischaemic tissue in our experimental setting.

We cannot rule out that the additive effect of interventions detected in this study could be attenuated by increasing the doses of the individual interventions. However, increasing the number of RIC cycles beyond 4 was not associated with a significant increase in myocardial salvage in rats submitted to transient coronary occlusion. Similarly, the doses and protocols of GIK and exenatide administration were those found safe and effective in human studies. The present study cannot determine which combination, RIC + GIK or RIC + exenatide, is more advantageous since a much larger number of animals would have been necessary to answer this question.

Our animal model included the use of propofol, a drug that has been described to interfere with RIC cardioprotection in patients undergoing coronary artery bypass surgery. A larger cardioprotective effect of RIC could be thus expected in the absence of propofol. However, the effect of RIC in this study is in line with that reported in clinical trials.

5. Conclusions and implications

This study shows that a combined therapy with RIC and GIK or exenatide, administered at the doses that have been found to reduce infarct size in human studies, is superior to these interventions administered individually in limiting infarct size in the pig model. Investigation of these combined therapies in patients with STEMI appears warranted.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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