Glucagon-like peptide-1 (GLP-1) mediates cardioprotection by remote ischaemic conditioning

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Aims

Although the nature of the humoral factor which mediates cardioprotection established by remote ischaemic conditioning (RIC) remains unknown, parasympathetic (vagal) mechanisms appear to play a critical role. As the production and release of many gut hormones is modulated by the vagus nerve, here we tested the hypothesis that RIC cardioprotection is mediated by the actions of glucagon-like peptide-1 (GLP-1).

Methods

A rat model of myocardial infarction (coronary artery occlusion followed by reperfusion) was used. Remote ischaemic pre- (RIPre) or perconditioning (RIPer) was induced by 15 min occlusion of femoral arteries applied prior to or during the myocardial ischaemia. The degree of RIPre and RIPer cardioprotection was determined in conditions of cervical or subdiaphragmatic vagotomy, or following blockade of GLP-1 receptors (GLP-1R) using specific antagonist Exendin(9–39). Phosphorylation of PI3K/AKT and STAT3 was assessed. RIPre and RIPer reduced infarct size by ~50%. In conditions of bilateral cervical or subdiaphragmatic vagotomy RIPer failed to establish cardioprotection. GLP-1R blockade abolished cardioprotection induced by either RIPre or RIPer. Exendin(9–39) also prevented RIPre-induced AKT phosphorylation. Cardioprotection induced by GLP-1R agonist Exendin-4 was preserved following cervical vagotomy, but was abolished in conditions of M3 muscarinic receptor blockade.

Conclusions

These data strongly suggest that GLP-1 functions as a humoral factor of remote ischaemic conditioning cardioprotection. This phenomenon requires intact vagal innervation of the visceral organs and recruitment of GLP-1R-mediated signalling. Cardioprotection induced by GLP-1R activation is mediated by a mechanism involving M3 muscarinic receptors.

Keywords

Cardioprotection • Glucagon-like peptide-1 • Myocardial infarction • Myocardial ischaemia • Parasympathetic • Remote ischaemic conditioning • Reperfusion • Vagus nerve

1. Introduction

Powerful innate mechanisms of cardioprotection can be recruited by remote ischaemic conditioning (RIC), which can be established by cycles of ischaemia/reperfusion applied to an organ/tissue distant from the heart. In several animal models significant reduction of myocardial ischaemia/reperfusion injury was demonstrated when RIC stimulus was applied either before (remote ischaemic preconditioning, RIPre) or during myocardial ischaemia (remote ischaemic perconditioning, RIPer), or after the onset of reperfusion (remote ischaemic postconditioning). Clinical trials have demonstrated the efficacy of RIC in reducing infarct size in patients with an acute myocardial infarction (AMI), in reducing...
myocardial damage during cardiac surgery and improving long term prognosis in both patient cohorts. Although the exact mechanisms underlying Rlc cardioprotection are not fully understood, a number of studies suggested the involvement of both humoral and neural signaling pathways. Several candidate humoral factors of Rlc have been proposed, including stromal cell-derived factor-1α, nitrite/nitric oxide, interleukin-10, microRNA-144, apolipoprotein A-1 and alpha-ketoglutarate-dependent dioxygenase Egln1. However, the full extent of Rlc cardioprotection cannot be explained by the actions of any of these factors alone.

There is strong evidence that parasympathetic (vagal) mechanisms are critically important for RIPre cardioprotection. RIPre was reported to be abolished by selective genetic inhibition of brainstem vagal preganglionic neurons, muscarinic receptor blockade, bilateral cervical vagotomy or sectioning of the posterior gastric branch of the vagus nerve. Electrical stimulation of the whole vagus nerve at the cervical level or isolated posterior gastric branch of the vagus establishes cardioprotection. These data suggest that visceral organs, innervated by the posterior gastric branch of the vagus nerve, are the likely source of a humoral factor (or factors) of Rlc cardioprotection. Glucagon-like peptide-1 (GLP-1) is the most notable of all humoral factors, which originate from the visceral organs and known to have cardioprotective properties. GLP-1 is an incretin hormone released by the L-cells of the intestine in response to the ingestion of food. Release of GLP-1 is modulated by vagal efferent (motor) activity and there is also evidence that GLP-1 may interact with vagal sensory fibres innervating the visera. GLP-1 actions appear to be mediated via glucagon-like peptide-1 receptor (GLP-1R)-dependent and independent mechanisms, although the existence of the latter is debated. Studies conducted in animal models demonstrated potent cardioprotection by GLP-1R activation. The efficacy of GLP-1R agonists in reducing infarct size was also shown in human studies. Recent study conducted in patients with type 2 diabetes demonstrated significant reduction in frequency of adverse cardiovascular events and death from cardiovascular causes by treatment with GLP-1 analogue liraglutide. The molecular weight of GLP-1 is ~3.3 kDa and it appears to satisfy the key criteria of the humoral preconditioning factor (including molecular weight of less than 8 kDa), suggested by Lang and colleagues on the basis of the proteomic analysis of blood samples obtained from experimental animals receiving the RIPre stimulus.

This study was designed to test the hypothesis that cardioprotection established by Rlc is mediated by the actions of GLP-1. Taken into the account that the parasympathetic nervous system appears to be critically important for RIPre cardioprotection, we also investigated the significance of vagal mechanisms in establishing cardioprotection induced by the GLP-1R agonist Exendin-4 (Ex4).

2. Methods

All the experiments were performed in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the respective Institutional Animal Care and Use Committees.

2.1 Animal preparation

Adult male Sprague Dawley rats (280–320 g) were anaesthetized with pentobarbital sodium (induction 60 mg kg⁻¹ i.p.; maintenance 15 mg kg⁻¹ h⁻¹ i.v.). Adequate anaesthesia was ensured by maintaining stable levels of the arterial blood pressure and heart rate and confirmed by the absence of a withdrawal response to a paw pinch. The right carotid artery and left jugular vein were cannulated for the measurement of the arterial blood pressure and administration of anaesthetic or test compounds, respectively. The trachea was cannulated to allow mechanical ventilation with room air using a positive pressure rodent ventilator (tidal volume ~8–10 ml kg⁻¹, frequency ~60 strokes min⁻¹). Partial pressures of O₂ and CO₂ as well as pH of the arterial blood were measured regularly. A standard lead II ECG was recorded. The body temperature was maintained at 37.0 ± 0.5 °C with a servo-controlled heating blanket.

2.2 Model of myocardial infarction

An established rat model of myocardial ischaemia/reperfusion injury was used. The heart was exposed via a left thoracotomy and a 5–0 monofilament polypropylene suture was passed around the left anterior descending coronary (LAD) artery to induce a temporary occlusion. LAD artery was occluded for 30 min followed by reperfusion lasting 120 min.

2.3 Infarct size measurement

At the end of the reperfusion period, the LAD artery was ligated, and 5% Evans Blue dye solution (0.2 ml) was infused via the jugular vein to determine the area at risk. The animal was then given an anaesthetic overdose (pentobarbital, 200 mg kg⁻¹ i.v.), the heart was excised, the left ventricle (LV) was isolated, frozen, and sectioned into 5–6 transverse slices from the apex to the base. The area at risk was demarcated by the absence of Evans Blue staining. LV slices were then incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in Tris buffer (pH 7.4) for 15 min at 37 °C and fixed in 4% formalin for 24 h. Viable myocardium is stained red by TTC, whereas necrotic myocardium appears white. The area at risk and the necrotic area were determined by computerized planimetry, normalized to the weight of each slice, with the degree of necrosis (i.e. infarct size) expressed as the percentage of area at risk, as described.

2.4 Immunoblotting

For the analysis of protein phosphorylation, Western blot was performed on the myocardium from the area at risk. The ventricular tissue was excised, frozen in liquid nitrogen and stored at ~80 °C before the assays. Total phosphatidylinositol 3-kinase AKT (PI3K/AKT), phospho–AKT (Ser473), janus-activated kinase (JAK) signal transducer and activator of transcription (STAT3) and phospho-STAT3 (Tyr705) were immunodetected in cell lysates (whole cell fractions) using specific primary antibodies (all from Cell Signalling Technology, UK). Proteins were electrophoretically separated in SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, USA) according to the manufacturer’s instructions. After antibody labelling, detection was performed (ECL detection system, Amersham Biosciences, USA). Densitometry was used to calculate the ratio of phosphorylated and total protein normalized to the expression of ß-actin (Santa Cruz UK) to control protein loading.
2.5 Experimental protocols

2.5.1 Experiment 1. The effect of vagotomy on RIPer cardioprotection

There is evidence that RIPre cardioprotection is abolished in conditions of either cervical or subdiaphragmatic vagotomy. However, the role of parasympathetic mechanisms in mediating RIPer cardioprotection remains unknown. RIPer was induced by occlusion of both femoral arteries for 15 min starting 10 min after the onset of myocardial ischaemia (see timeline Figure 1A). For bilateral cervical vagotomy, both nerves were exposed at the neck level and sectioned 15 min prior to myocardial ischaemia. To perform total subdiaphragmatic vagotomy, minimal incision was made to gain access to the abdominal cavity, the left lobes of the liver were gently pulled aside, the stomach was retracted caudally to expose the oesophagus, the vagal trunks were carefully dissected under the diaphragm and sectioned.

2.5.2 Experiment 2. The effect of GLP-1 receptor blockade on RIc-induced cardioprotection and phosphorylation of AKT and STAT3

RIc was induced by 15 min occlusion of both femoral arteries, followed by reperfusion, starting either 25 min prior or 10 min after the onset of myocardial ischaemia (see timeline Figure 2A). GLP-1R antagonist Exendin(9–39) (Ex(9–39), 50 µg kg⁻¹, i.v.) was administered 40 min or 15 min before the onset of ischaemia in animals receiving the RIPre or RIPer stimulus, respectively. The effect of Ex(9–39) on cardioprotection established by classical myocardial ischaemic preconditioning (IPre) was also determined. IPre was induced by three episodes of myocardial ischaemia (LAD occlusion; 3 + 5 + 5 min) separated by 5-min periods of reperfusion. Ex(9–39) was given 15 min before the first ischaemic episode. The dose of Ex(9–39) was selected on the basis of previously published reports. In a separate experiment the effect of Ex(9–39) on RIPre-induced phosphorylation of AKT and STAT3 in the myocardium was assessed. The hearts were collected 15 min after the onset of myocardial reperfusion.

2.5.3 Experiment 3. The effect of vagotomy and systemic muscarinic receptor blockade on cardioprotection established by GLP-1 receptor activation

Intravenous administration of GLP-1R agonist Exendin-4 (Ex4) in doses ranging between 1–10 µg kg⁻¹ has been shown to protect the rat heart against myocardial ischaemia/reperfusion injury. In this study we used Ex4 in 5 µg kg⁻¹ dose to determine the efficacy of GLP-1R activation in establishing cardioprotection in conditions of bilateral cervical vagotomy, systemic muscarinic receptor blockade (atropine methyl nitrate; initial bolus dose 2 mg kg⁻¹, i.v., followed by infusion at a rate of 1 mg kg⁻¹ h⁻¹) or M3 muscarinic receptor blockade (4-DAMP; 2 mg kg⁻¹, i.v.). Experimental protocols are illustrated on Figure 3A.

2.5.4 Experiment 4. The effect of RIc on plasma level of GLP-1

After 12-h overnight fast, the animals were anaesthetized and instrumented as described above. RIc was induced by 15 min occlusion of both femoral arteries, followed by reperfusion. Samples of the arterial blood (300 µl) were collected into pre-chilled EDTA-Eppendorf tubes at 4 time points: 10 min prior to and 5, 20, and 30 min after the onset of limb ischaemia or sham-RIc. Dipeptidyl peptidase-4 (DPP-4) inhibitor (50 µM; EMD Millipore) was added to the collected samples and plasma was separated by centrifugation (10 min; 1000 g; 4°C). Total GLP-1 in plasma was measured using MesoScale Discovery Total GLP-1 (v2) kit (Rockville, USA).
2.6 Statistical analysis
One-way ANOVA (Tukey’s and Bonferroni’s Multiple Comparison Tests) was used for statistical analysis of the data. Values of $P < 0.05$ were considered to be significant.

3. Results
In the Experiment 1, an increase in heart rate was observed in animals subjected to RIPer in conditions of subdiaphragmatic vagotomy during myocardial ischaemia and reperfusion period (Supplementary material online, Table). In the Experiment 3, mean arterial blood pressure increased in Ex4-treated animals subjected to cervical vagotomy before myocardial ischaemia. Heart rate increased before myocardial ischaemia and remained elevated during myocardial ischaemia and during reperfusion (Supplementary material online, Table). No other differences in mean arterial blood pressure and heart rate during ischaemia and reperfusion were observed between the experimental groups of animals (Supplementary material online, Table). Areas at risk were similar between all the experimental groups (data not shown). Figures 1B, 2B,
and 3B illustrate infarct sizes expressed as percentages of the areas at risk.

3.1 Experiment 1. The effect of vagotomy on RIPer cardioprotection

Average infarct size in animals subjected to 30 min of LAD occlusion followed by 120 min of reperfusion was $56 \pm 6\%$ (Figure 1B). RIPer significantly reduced myocardial ischaemia/reperfusion injury ($infarct size 27 \pm 6\%$, $P < 0.001$), but failed to establish cardioprotection in conditions of either bilateral cervical or subdiaphragmatic vagotomy (Figure 1B). Vagotomy per se had no effect on myocardial ischaemia/reperfusion injury (Figure 1B).

3.2 Experiment 2. The effect of GLP-1 receptor blockade on Rlc-induced cardioprotection and phosphorylation of AKT and STAT3

To determine whether GLP-1 may act as a humoral factor of Rlc we next determined the efficacy of IPre, RIPre, and RIPer in establishing cardioprotection in conditions of systemic GLP-1R blockade (Figure 2B). Systemic administration of a specific GLP-1R antagonist Ex(9–39) blocked cardioprotection induced by RIPre and RIPer (infarct sizes $48 \pm 10\%$ and $52 \pm 9\%$, respectively), but had no effect on cardioprotection conferred by classical direct myocardial IPre (infarct size $24 \pm 4\%$) (Figure 2B). RIPre-induced AKT phosphorylation was blocked ($P < 0.05$) by systemic treatment with Ex(9–39) (Figure 2C). RIPre had no significant effect on STAT3 phosphorylation (Figure 2D).

3.3 Experiment 3. The effect of vagotomy and systemic muscarinic receptor blockade on cardioprotection established by GLP-1 receptor activation

We next determined whether GLP-1R activation induces cardioprotection via recruitment of vagal mechanisms. Intravenous infusion of GLP-1R agonist Ex4 significantly reduced (by 40%, $P < 0.01$) the extent of myocardial ischaemia/reperfusion injury (Figure 3B). Ex4-induced cardioprotection was not affected by bilateral cervical vagotomy (infarct size $27 \pm 11\%$, NS vs. Ex4 treatment) (Figure 3B). However, systemic muscarinic receptor blockade (atropine) abolished Ex4-induced cardioprotection (infarct size $52 \pm 9\%$, $P < 0.01$ vs. Ex4). Ex4 also failed to establish cardioprotection in conditions of systemic M3 receptor blockade with 4-DAMP (infarct size $57 \pm 6\%$, $P < 0.001$ vs. Ex4) (Figure 3B).

3.4 Experiment 4. The effect of Rlc on plasma level of GLP-1

Moderate increases in the level of circulating (arterial) GLP-1 compared to the baseline values were observed 30 min after the onset of limbs ischaemia (15 min into the limb reperfusion period) ($4.7 \pm 0.9$ vs. $3.0 \pm 0.7$ pg/ml at baseline; $P < 0.05$; Table 1). Arterial GLP-1 levels were not affected by sham-Rlc procedure (Table 1).

4. Discussion

To the best of our knowledge, this is the first experimental study which demonstrated that a particular humoral factor is causally involved in cardioprotection induced by remote ischaemic conditioning. Recent study identified the likely origin of the cardioprotective humoral factor which appears to be produced by the visceral organs innervated by the posterior gastric branch of the vagus nerve.13 Organs of the gastrointestinal tract indeed represent a major source of many factors with known cardioprotective properties, including GLP-1. Here we show that the remote conditioning-induced cardioprotection and phosphorylation of pro-survival kinase AKT are abolished by systemic GLP-1R blockade with Ex(9–39). Ex(9–39) has been used in many published studies, including several seminal reports, which described physiological role and significance of GLP-1R-mediated signalling.40 and off-target (i.e. not on GLP-
structured in a number of studies (for recent experimental reports see and functional cholinergic innervation of the cardiac ventricles was dem-
vagotomy or systemic muscarinic receptor blockade.3,13,14 Anatomical
targeting and silencing of vagal preganglionic neurones, bilateral cervical
RIPre cardioprotection was found to be abolished by selective genetic
conditioning cardioprotection.

4.2 Vagus nerve and remote ischaemic conditioning cardioprotection
Since the importance of vagal mechanisms in mediating cardioprotection
induced by Rlc was first proposed,42 results of several experimental
studies provided strong evidence in support of the idea that the intact
parasympathetic mechanisms are essential for Rlc cardioprotection.
RIPre cardioprotection was found to be abolished by selective genetic
targeting and silencing of vagal preganglionic neurones, bilateral cervical
vagotomy or systemic muscarinic receptor blockade.3,13,14 Anatomical
and functional cholinergic innervation of the cardiac ventricles was dem-
onstrated in a number of studies (for recent experimental reports see Refs.43,44) and it was suggested that RIPre is mediated by the actions of
acetylcholine released from vagal efferent fibres which innervate the LV
myocardium.13 However, the mechanism involving vagally-mediated
reflexes cannot fully explain how RIPre is able to protect the trans-
planted or denervated hearts, or cross species transfer of Rlc cardiopro-
tection by plasma dialysate.8–11 Taken together, the available data
suggest that production and release of a humoral factor of Rlc cardiopro-
tection is under parasympathetic control. In support of this idea, it was
recently demonstrated that RIPre fails to establish cardioprotection in
conditions of selective sectioning of the posterior gastric branch of the
vagus nerve,21 pointing to the likely source of the cardioprotective
humoral factor. Results of the present study show that RIPre cardiopro-
tection also requires intact parasympathetic innervation of visceral
organs. Therefore, the common vagal mechanisms appear to mediate
cardioprotection established by Rlc applied either before or during myo-
cardial ischaemia and require GLP-1R-mediated signalling.

4.3 Humoral factor(s) of Rlc cardioprotection. The role of
GLP-1R-mediated signalling
A significant number of studies demonstrated successful transfer of
humoral factor responsible for Rlc cardioprotection with plasma or dial-
ysaté, obtained after Rlc, to another animal or isolated hearts, even
across species (see for example Refs.8,10,11,41). The molecular weight of
this humoral factor of cardioprotection appears to be less than 8 kDa,34
and several candidate molecules have been proposed.15–19 Cell-derived
factor-1α,15 nitrite/nitric oxide,16 interleukin-10,17 microRNA-14418
were shown to be involved in cardioprotection; however, Rlc cardiopro-
tection cannot be fully explained by any of the identified factors acting
alone. In addition, SDF-1α has been proposed to act as a circulating
mediator of Rlc.15 However, treatment with a selective inhibitor of SDF-
1α only partially attenuated but did not block Rlc cardioprotection,15
suggesting the existence of other (parallel) mechanisms. Proteome/
sequencing analysis also failed to reveal the nature of this factor.34,45,46
One recent study reported Rlc-induced changes in plasma levels of
seven proteins.46 These factors are involved in the control of haemostas-
isis, lipid transport, iron regulation and inflammation. Some of the identi-
ified proteins (when applied exogenously) could mimic Rlc in
experimental models; however, it remains unknown whether blockade
of their actions has an effect on Rlc cardioprotection.

GLP-1 can activate GLP-1R expressed by visceral vagal afferents acting
in a paracrine manner, but also has endocrine functions.24,25 Originally
identified to be expressed by pancreatic β-cells, GLP-1R is now known
to be widely distributed in many tissues, including the atrial myocardium,
coronary vessels, and possibly, ventricles.47 Several studies demon-
strated high efficacy of GLP-1R agonists in reducing the infarct size.30
However, the mechanisms by which GLP-1R activation protects the
ischaemic myocardium remain largely unknown. Both GLP-1R-depen-
dent and GLP-1R-independent mechanisms have been pro-
posed.28,29 As the majority of the intestinally derived GLP-1 is degraded
by the DPP-4, low levels of circulating GLP-1 are usually reported.24
Due to high rate of enzymatic degradation and renal clearance, GLP-1
has a short half-life (1–2 min) in plasma which represents a major limita-
tion for its detection.24,48 In this study, we observed moderate increases
in plasma GLP-1 level 30 min after the onset of the Rlc stimulus, probably
due to high variability of GLP-1 plasma concentration and short half-life
of GLP-1. Interestingly, the long-lasting beneficial effects of short-term
GLP-1 infusion have been shown to persist for weeks even when the cir-
culating levels of GLP-1 return back to normal levels.49

The central finding of the present study is that highly selective GLP-1R
antagonist Ex(9–39) administered before RIPre or RlPr blocks cardio-
protection induced by both Rlc stimuli. GLP-1R blockade also prevented
the stimulatory effect of RIPre on AKT phosphorylation. It appears that
protection induced by both RIPre and RIPer blocks cardio-
protection also requires intact parasympathetic mechanisms.

Table 1: The effect of remote ischaemic conditioning (RIc) or sham-RIc on plasma level of glucagon-like peptide-1 (in pg ml⁻¹)

<table>
<thead>
<tr>
<th>Time from the onset of limb ischaemia or sham (min)</th>
<th>n</th>
<th>Sham</th>
<th>RIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8</td>
<td>4.1 ± 0.7</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>5.2 ± 1.0</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
<td>4.4 ± 0.9</td>
<td>3.4 ± 0.6</td>
</tr>
</tbody>
</table>

*Significant difference from the baseline value (P < 0.05).
GLP-1 and remote conditioning cardioprotection

1R57 are abolished in conditions of AKT blockade. In rodents, inhibition of either RISK or SAFE pathway blocks cardioprotection1,52,54,56 suggesting that the elements of these two protective pathways may interact. In pigs and humans, cardioprotection appears to be predominantly associated with activation of SAFE pathway.58,59 The data obtained in this study suggest that (at least in rats) GLP-1R-mediated signalling is essential for RIC cardioprotection by being responsible for triggering activation of the pro-survival RISK pathway and, therefore, GLP-1 is likely to act as the key humoral factor of this phenomenon.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

### Conflict of interest

None declared.

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### References


