Delayed ischaemic contracture onset by empagliflozin associates with NHE1 inhibition and is dependent on insulin in isolated mouse hearts

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Aims
Sodium glucose cotransporter 2 (SGLT2) inhibitors have sodium–hydrogen exchanger (NHE) inhibition properties in isolated cardiomyocytes, but it is unknown whether these properties extend to the intact heart during ischaemia–reperfusion (IR) conditions. NHE inhibitors as Cariporide delay time to onset of contracture (TOC) during ischaemia and reduce IR injury. We hypothesized that, in the ex vivo heart, Empagliflozin (Empa) mimics Cariporide during IR by delaying TOC and reducing IR injury. To facilitate translation to in vivo conditions with insulin present, effects were examined in the absence and presence of insulin.

Methods and results
Isolated C57Bl/6NCrl mouse hearts were subjected to 25 min I and 120 min R without and with 50 mU/L insulin. Without insulin, Empa and Cari delayed TOC by 100 and 129 s, respectively, yet only Cariporide reduced IR injury [infarct size (mean ± SEM in %) from 51 ± 6 to 34 ± 5]. Empa did not delay TOC in the presence of the NHE1 inhibitor Eniporide. Insulin perfusion increased tissue glycogen content at baseline (from 2 ± 2 m mol to 42 ± 1 m mol glycosyl units/g heart dry weight), amplified G6P and lactate accumulation at end-ischaemia, thereby decreased mtHKII and exacerbated IR injury. Under these conditions, Empa (1 m M) and Cariporide (10 m M) were without effect on TOC and IR injury. Empa and Cariporide both inhibited NHE activity, in isolated cardiomyocytes, independent of insulin.

Conclusions
In the absence of insulin, Empa and Cariporide strongly delayed the time to onset of contracture during ischaemia. In the presence of insulin, both Empa and Cari were without effect on IR, possibly because of severe ischaemic acidification. Insulin exacerbates IR injury through increased glycogen depletion during ischaemia and consequently mtHKII dissociation. The data suggest that also in the ex vivo intact heart Empa exerts direct cardiac effects by inhibiting NHE during ischaemia, but not during reperfusion.

Keywords
Empagliflozin • Ischaemia • Reperfusion • Sodium-hydrogen exchanger • Isolated heart

1. Introduction

Previous work in isolated cardiomyocytes has shown that the kidney-targeted sodium glucose cotransporter 2 inhibitors (SGLT2) have sodium–hydrogen exchanger (NHE) inhibition properties.1,2 These NHE inhibition properties may, at least partly, contribute to the cardiovascular benefits associated with SGLT2 inhibitors in large clinical trials, i.e. reductions in heart failure-related hospitalization and cardiovascular mortality.3–6 NHE is a pH regulating membrane protein with increased activity in several pathological, chronic conditions, such as diabetes and heart failure3 and during acute ischaemia–reperfusion (IR) conditions.7 NHE
gets activated to prevent severe acidosis and can contribute to IR injury through increased sodium loading in cardiomyocytes. This increased cellular sodium can then contribute to calcium overload during IR due to reversed or impaired action of the sodium/calcium exchanger. Compounds that specifically target and inhibit NHE have been shown, although not unambiguously, to confer protection against IR injury in both pre-clinical and clinical studies. Molecular docking studies indicated that SGLT2i may inhibit NHE through high-affinity binding towards the extracellular Na+-binding site of NHE1, which is different from the binding site of classically developed NHE inhibitors, including Cariporide. This raises the question to what extend SGLT2i may inhibit NHE during IR conditions, and as such may provide protection against acute IR injury. Indeed, recent studies in high-fat-treated animals reported that SGLT2i protect the heart against IR injury in vivo. However, it is uncertain whether these effects are due to an improved metabolic milieu in the systemic circulation and/or due to direct cardiac effects of SGLT2i. Yu et al. reported that hyperglycaemia exacerbates cardiac IR injury in vivo in dogs, indicating that glycaemic control is an indirect factor that markedly affects infarct size. Therefore, in the present study, we employed the ex vivo isolated heart model to examine possible direct cardiac effects of SGLT2i empagliflozin (Empa), and compared these effects with the NHE1 inhibitor Cariporide. We specifically focus on (i) the development of rigour contracture during ischaemia and (ii) infarct size after 2 h reperfusion. We hypothesized that Empa influences both phenomena through its NHE-inhibition ability in a similar manner as Cariporide. Although both the onset of rigour contracture [time to onset of contracture (TOC)] during ischaemia and infarct size are interrelated and affected by NHE activity, they reflect distinct mechanisms. The TOC coincides with cessation of glycolysis, a critically low level of ATP and the rising of intracellular calcium; whereas infarct size in the acute phase of reperfusion is determined by the degree of mitochondrial damage and calcium overload occurring during reperfusion. Since the field of cardioprotection is challenged by poor translatability of pre-clinical findings, with a 12 h day/night cycle, and food and drinking water ad libitum. One hundred and two animals were used for this study, of which 10 were excluded due to unstable hearts on the Langendorff apparatus (see next section), and two were excluded from 2,3,5-triphenyltetrazolium chloride (TTC) and/or lactate dehydrogenase (LDH) analysis because of poor staining and insufficient sample.

2. Methods

2.1 Animals

All experiments were approved by the animal ethics committee of the Academic Medical Center, Amsterdam, The Netherlands and performed conform the Guide for the Use and Care of Laboratory Animals. C57Bl/6NCrl male mice (10–14 weeks old, 25.4 ± 0.1 g, Charles River, Lyon, France) were housed for at least 1 week under standard housing conditions, with a 12 h day/night cycle, and food and drinking water ad libitum. One hundred and two animals were used for this study, of which 10 were excluded due to unstable hearts on the Langendorff apparatus (see next section), and two were excluded from 2,3,5-triphenyltetrazolium chloride (TTC) and/or lactate dehydrogenase (LDH) analysis because of poor staining and insufficient sample.

2.2 Heart isolation and perfusion

Mice were intraperitoneally heparinized (15 IU) and anaesthetized with an initial anaesthetic bolus containing 125 mg/kg body weight S(+)–ketamine and 0.1 mg/kg body weight dexmedetomidine. Anaesthetic depth was tested with multiple toe pinches and response by the animals indicated the necessity for supplementary anaesthesia administration, resulting in an average dose of 151.7 mg/kg body weight S(+)–ketamine and 0.124 mg/kg body weight dexmedetomidine. Mice were intratracheally ventilated with 50% O2 and 50% N2, which was followed by in- chest aortic cannulation for immediate perfusion of hearts. Hearts were perfused under constant flow (perfusion pressure stabilized to 80 mmHg at start of experiment) and submerged with filtered Krebs–Henseleit buffer (KHB; composition in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.5 EDTA, 2.25 CaCl2, 7 D-glucose, 0.5 L-glutamine, 1 lactate, and 0.1 pyruvate, 37°C), oxygenated with 95% O2 and 5% CO2. We previously reported the necessity of adding glutamine, pyruvate, and lactate to mouse heart perfusions for generating stable preparations. Insulin was diluted in KHB containing 0.1% albumin to a final concentration of 50 mU/L. Pilot experiments demonstrated the necessity of adding small amounts of albumin to the perfusion buffer to prevent complete loss of insulin due to it sticking to glassware and tubing of the perfusion system. A water-filled polyethylene balloon (4–5 mm length) was inserted into the left ventricular cavity; end-diastolic pressure [end-diastolic pressure (EDP); corrected for balloon pressure]; and left ventricular developed pressure [left ventricular developed pressure (LVEDP) (=systolic pressure–EDP)] were set at 2–5 mmHg and at least 80 mmHg, respectively. Rate pressure product was calculated from LVEDP × heart rate. Contracture onset was defined as the time(s) from the start of ischaemia when EDP progressed above 7.5 mmHg.

2.3 Langendorff protocols

Hearts were stabilized for 20–30 min prior to start of each experiment, after which one of the following protocols was executed.

(1) Ischaemia reperfusion injury (IRI) protocol with or without insulin (Figures 1A and 2A): following stabilization, hearts were subjected to 20 min baseline, 25 min global ischaemia, and 120 min reperfusion. A 1 μM Empa (n = 6/group with insulin, n = 13/group without insulin), obtained from MedChemExpress, Monmouth Junction, NJ, USA, 10 μM Cariporide (n = 6/group with insulin, n = 15/group without insulin, kindly gifted by Aventis Pharma/Sanofi, Paris, France), or 0.02% Dimethyl sulfoxide (DMSO) (vehicle; n = 5/group with insulin, n = 14/group without insulin) was added from t = 2 min baseline until t = 10 min after start of reperfusion. Coronary effluent was collected during reperfusion to measure LDH release from cells. Hearts were weighed and stored at -20°C for no more than 1 week before determination of infarct size with TTC staining occurred.

(2) IRI protocol equal to protocol 1 investigating Empa effects under NHE1 inhibiting conditions (Figure 3A, n = 6/group): non-insulin-perfused hearts were subjected to 2.5 μM NHE1 inhibitor Eniporide or 2.5 μM Eniporide + 1 μM Empa (dissolved in 0.02% DMSO).

(3) Baseline protocol with or without insulin and with or without 1 μM Empa (Figure 4A): hearts (n = 3–5/group) were perfused for 20 min with or without 1 μM Empa. Hearts were either immediately submerged in liquid nitrogen (whole cell) or were first horizontally sliced in half. The half containing the apex was further homogenized to obtain mitochondrial fractions. The other half (whole cell) was frozen in liquid nitrogen. All fractions were stored at -80°C until further processing.

(4) End-ischaemia protocol with or without insulin (Figure 4All): all hearts (n = 6/group) were subjected to 20 min baseline perfusion...
Figure 1 Empa and Cariporide effects during IR in insulin-perfused hearts. Hearts were subjected to IR and 1 μM Empa, 10 μM Cariporide, or vehicle in the presence of insulin (A). All three conditions display similar time points of ischaemic contracture development (B + C). No changes in any IR injury parameters were detected by Empa or Cariporide, including global infarct size (D), RPP recovery at t = 2 h reperfusion relative to baseline RPP (E), end-diastolic pressure (F), and total LDH release during reperfusion (G). Vehicle n = 5, Empa n = 6, and Cariporide n = 6. Scale bar = 0.5 mm. LDH AUC, Lactate dehydrogenase activity area under the curve during reperfusion, normalized to heart wet weight; Pdia, diastolic pressure; RPP, rate pressure product; TOC, time of onset contracture. Statistical testing by one-way ANOVA.
Figure 2  Empa and Cariporide effects during IR in non-insulin-perfused hearts. Hearts were subjected to IR and 1 μM Empa, 10 μM Cariporide, or vehicle in the absence of insulin (A). Both Empa and Cariporide delayed ischaemic contracture development (B + C), whereas only Cariporide administration proceeded to improvements in IR injury, measured as global infarct size (D), RPP recovery at t = 2 h reperfusion relative to baseline RPP (E), end-diastolic pressure (F), and total LDH release during reperfusion (G). Vehicle n = 14, Empa n = 13, and Cariporide n = 15. Scale bar = 0.5 mm. LDH AUC, Lactate dehydrogenase activity area under the curve during reperfusion, normalized to heart wet weight; Pdia, diastolic pressure; RPP, rate pressure product; TOC, time of onset contracture. *P < 0.05, **P < 0.01 by one-way ANOVA with Dunnett’s post hoc vs. vehicle except for Figure 2F (Pdia), which was tested by the Kruskal–Wallis test and the Mann–Whitney U test with Bonferroni correction.
and 25 min global ischaemia. Each heart was horizontally sliced in half, of which the half containing the apex was further homogenized to obtain mitochondrial fractions. The other half (whole cell) was frozen in liquid nitrogen and all fractions were stored at -80°C until further processing.

**Figure 3** Empa effects under NHE1 inhibiting conditions during IR in non-insulin-perfused hearts. Hearts were subjected to IR and 2.5 μM Eniporide, with or without 1 μM Empa in the absence of insulin (A). Empa did not further delay ischaemic contracture onset (B + C). No differences between groups were observed in global infarct size (D), RPP recovery at t = 2 h reperfusion relative to baseline RPP (E), end-diastolic pressure (f), and total LDH release during reperfusion (G). N = 6 condition. Scale bar = 0.5 mm. LDH-AUC, Lactate dehydrogenase activity area under the curve during reperfusion, normalized to heart wet weight; Pdia, diastolic pressure; RPP, rate pressure product; TOC, time of onset contracture.

(5) IRI protocol equal to protocol 1 without insulin, and with 3 μM (n = 5) or 10 μM (n = 6) Empa and vehicle (n = 5; Figure 6A); due to the low solubility of higher concentrations of Empa in DMSO, Empa was dissolved in cyclodextrin (Sigma Aldrich, Saint Louis, MO, USA; final concentration 0.044%) to obtain 3 and 10 μM.
Figure 4 Presence of insulin results in increased baseline glycogen, ischaemic glycogen depletion, G6P, lactate accumulation, and mtHKII dissociation at end-ischaemia. Hearts were subjected to 20 min baseline (A) and 25 min ischaemia (AII). Insulin extensively delayed contracture development during ischaemia (B). Cardiac glycogen content (C), G6P (D), and lactate (E) were determined at baseline (n = 3/4 per group) and end-ischaemia (n = 6 per group) for insulin- and non-insulin-perfused hearts. Typical examples of HKII and VDAC western bands for mitochondrial compartment (F). MtHKII protein content normalized to VDAC (G + H) and HK activity normalized to CS activity (I + J) in baseline and end-ischaemic hearts, respectively. G6P, glucose-6-phosphate; HKII, hexokinase 2; VDAC, voltage-dependent anion channel. *P < 0.05, **P < 0.01, and ***P < 0.001 by one-way ANOVA with Bonferroni post hoc.
2.4 LDH activity measurement
LDH activity in coronary effluent at \( t = 5, 10, 15, 30, 45, 60, 90, \) and 120 min of reperfusion was determined by spectrophotometry in a blinded fashion. Briefly, conversion of NADPH to NADP\(^+\) at 340 nm and 30\(^\circ\)C in the presence of pyruvate was measured for 3 min to calculate LDH activity.\(^{25,26}\) LDH activity was corrected for heart wet weight and baseline coronary flow.

2.5 Infarct size determination
Hearts were cut in 1-mm thick slices across the short-, transverse axis, and immediately incubated with 22.5 mM TTC in phosphate-buffered saline 37\(^\circ\)C in the dark for 20 min. Afterwards, the slices were fixed in 4% formalin overnight at room temperature in the dark and scanned the next morning. Blinded analyses occurred using SigmaScan Pro5 to measure global infarct size.

2.6 Tissue preparation
Whole cell homogenates were collected after hearts are freeze dried, weighted, treated with 4% perchloric acid and neurtrimix (Tri-ethanol amine 300 mM, KOH 900 mM, and KCl 30 mM) and pH was adjusted to 7. Mitochondrial fractions were obtained after homogenization of the tissue in HEPES buffer, containing 250 mM sucrose and 100 mM dithiothreitol. Samples were centrifuged at 10,000 g for 10 min at 4\(^\circ\)C. The pellet was lysed with 0.5% Triton. The 100 mM glucose-6-phosphate (G6P) was added for hexokinase (HK) detachment from mitochondrial membranes. The samples were sonicated for 4 s and centrifuged for 1 min at 4\(^\circ\)C and 10,000 g. The supernatant contained components of the mitochondrial fraction.\(^{26,27}\)

2.7 Spectrophotometric assays
G6P in whole cell homogenates was measured in assay mix that contained NADP\(^+\) and G6P dehydrogenase and the formation of NADPH indicated G6P content of each sample. Lactate was measured in the presence of NAD\(^+\), glycine, and hydrazine hydrate and the amount of NADH formed pointed to the lactate content. Both assays were performed at 30\(^\circ\)C and 340 nm. HK activity was measured through the conversion of NAD to NADH in the presence of G6P dehydrogenase, glucose, ATP, and NAD\(^+\) at 340 nm and 25\(^\circ\)C, and was corrected for citrate synthase activity, which was measured by the formation of thionitrobenzoic acid in the presence of acetyl-CoA oxaloacetate and dithionitrobenzoic acid at 410 nm and 25\(^\circ\)C.\(^{26,27}\)

2.8 Glycogen content
Glycogen content was determined using the glycogen Colorimetric/Fluorometric Assay Kit (Biosvision Inc., Milpitas, CA, USA). Samples were corrected for background glucose concentration and spiked with an additional 0.8 \( \mu \)g glycogen per sample to correct for interference from endogenous compounds.

2.9 Protein determination and western blot
Protein contents were determined using the Lowry method.\(^{28}\) Mitochondrial fractions were immunoblotted with the Citerion Western Blotting system (Biorad, Hercules, CA, USA). In brief, 4 \( \mu \)g sample in sample buffer was run through sodium dodecyl sulfate-polyacrylamide gel with gel electrophoresis. The proteins were transferred to polyvinylidine fluoride membrane with tank blotting (100 V for 30 min). Non-specific binding was blocked for 1 h with Odyssey blocking buffer (Li-cor, Lincoln, NE, USA). Membranes were incubated overnight at 4\(^\circ\)C with primary antibodies (HKII from Cell Signalling, #2867, 1:1000, voltage-dependent anion channel (VDAC) from Calbiochem, PC548, 1:5000), washed three times with cold PBS-0.1% Tween. The next morning, membranes were incubated for 1 h with a secondary antibody (IRDye\(^\text{TM}\) 680RD Goat anti-Rabbit IgG from Li-cor, 926-68071), washed three times with PBS-0.1% Tween and scanned with the Odyssey scanner (Li-cor).

2.10 NHE activity
NHE activity in healthy cardiomyocytes (\( n = 8 \) cardiomyocytes/condition from five mice) was measured in the presence and absence of 50 mIU/L insulin and 0.1% albumin. Cardiomyocytes were isolated as described previously\(^{25}\) and loaded with 10 \( \mu \)M pH-sensitive SNARF-fluorescence (Molecular probes, Eugene, OR, USA; S80/640 nm emission; 515 nm excitation) and subsequently seeded on 0.1 g/L poly-S-lysine treated coverslips. Cells were pre-incubated for 15 min with HEPES buffer containing 7 mM glucose, 1.8 mM CaCl\(_2\) with or without insulin and albumin in a temperature controlled (37\(^\circ\)C) perfusion chamber, which was staged on an inverted fluorescence microscope (Nikon Diaphot, Tokyo, Japan). As index of NHE activity, the recovery of pH at 5 min following a 20 mM NH\(_4\)\(^+\)-pulse of 5 min was determined. The 1 \( \mu \)M Empa or 10 \( \mu \)M Cariporide were present during the NH\(_4\)\(^+\)-pulse and recovery phase. Throughout the experiment, myocytes were field stimulated at 2 Hz frequency, and dual wave length emission wave length was measured during 100 ms light flashes at 1 kHz, and corrected for fluorescence of unloaded myocytes.

2.11 Statistical analysis
Data are expressed in mean \( \pm \) SEM. Throughout this manuscript, \( n \) applies to the number of animals used unless stated otherwise. For the IR experiments, an initial \( n = 6 \) was chosen to be able to detect a clinically relevant decrease of 30% in infarct size with an \( \alpha = 0.05, \) SD = 15, and a power of 0.9. For the baseline and ischaemia experiments, unpublished data from a different series of experiments indicated that glycogen levels between baseline insulin- vs. non-insulin-perfused mouse hearts equalled 0.082 \( \mu \)mol glycosyl units/mg dry weight. We wanted to detect this difference, for which we needed \( n = 4 \) experiments/group with \( \alpha = 0.05, \) SD = 0.032, and power = 0.9. Hearts subjected to vehicle, Empa or Cariporide were randomly assigned to different parts of the day (morning/afternoon). Normality distribution of data was tested with the Shapiro–Wilk test. The Student’s t-test was used for comparisons between insulin and non-insulin groups for HKII analyses. Comparisons between more than two groups were performed by one-way ANOVA followed by a Bonferroni or Dunnnett’s post hoc test if data was normally distributed. Not normal data were analysed with the Kruskal–Wallis test and the Mann–Whitney U test with Bonferroni correction. Analyses were performed using IBM SPSS statistics version 24 (International Business Machines Corp., Armond, NY, USA) and figures were created in GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA).
USA). Significant values are indicated with asterisk symbols; *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1 Empa and Cariporide similarly altered rigour contracture development during ischaemia, yet only Cariporide reduced IR injury in the absence of insulin

Empa and Cariporide effects were determined and compared for TOC during ischaemia, and for IR injury during 2h reperfusion (Figure 1A). Baseline mechanical and physiological values of the isolated hearts for vehicle and intervention groups with and without insulin are summarized in Table 1. In insulin-perfused hearts subjected to vehicle, 1 μM Empa or 10 μM Cariporide, the time of contracture development (TOC) during ischaemia occurred late and at similar time points (Figure 1B and C in seconds, vehicle 1056 ± 66, Empa 900 ± 44, and Cariporide 880 ± 84). The 25-min ischaemia and 2-h reperfusion resulted in extensive injury; 59.3 ± 5.6% infarct size, 14.8 ± 3.3% rate-pressure product (RPP) recovery, 66.4 ± 9.2 mmHg end-diastolic pressure, and 73.9 ± 2.4 μmol/g heart LDH accumulated release in the vehicle condition. Both Empa and Cariporide were ineffective in reducing any of the IR outcome parameters for insulin-perfused hearts (Figure 1D–G).

Cariporide was supposed to serve as a positive control for the effects of a classical NHE inhibitor, but was unexpectedly unable to delay TOC and reduce IR injury in insulin-perfused mouse hearts. Because of this, we hypothesized that prolonged glycogen breakdown due to glycogen loading with insulin-perfusion was causing late TOC and extended IR injury, possibly overruling any cardioprotective effects of Cariporide (see Discussion section). We, therefore, decided to repeat the experiments in the perfused hearts in the absence of insulin (Figure 2A). Indeed, TOC started much earlier in non-insulin (459 ± 25s) vs. the insulin-perfused hearts (1056 ± 66s), and both Empa and Cariporide now delayed TOC (Figure 2B and C in seconds, Empa 559 ± 22 and Cariporide 588 ± 31). However, only Cariporide, but not Empa, significantly improved infarct size (% vehicle 51.2 ± 5.5, Empa 50.7 ± 3.9, and Cariporide 34.1 ± 4.9), RPP recovery (% from baseline, vehicle 52.9 ± 4.9, Empa 58.0 ± 3.9, and Cariporide 73.6 ± 4.7), and showed non-significant improvements for EDP (mmHg, vehicle 30.0 ± 8.7, Empa 20.7 ± 3.6, and Cariporide 13.5 ± 3.5), and LDH release (μmol/g heart, vehicle 33.9 ± 7.3, Empa 33.5 ± 7.0, and Cariporide 20.4 ± 5.2 (Figure 2D–G).

3.2 Empa does not increase ischaemic rigour contracture development during co-administration with Eniporide

To determine if Empa’s effect on TOC was mediated through NHE1 inhibition, non-insulin-perfused hearts were perfused with the NHE1 inhibitor Eniporide (Figure 3A). Table 2 indicates the baseline mechanical and physiological values for both groups. Eniporide administration resulted in a TOC of 570 ± 50.8s (Figure 3B and C), which was not further delayed by administration of Empa (560 ± 50.8s). Additionally, reperfusion injury parameters were not affected by Empa in the Eniporide-treated hearts (Figure 3D–G), including infarct size (% Eniporide 45.1 ± 4.0, Eniporide + Empa 46.9 ± 3.9), RPP recovery (% from baseline, Eniporide 83.6 ± 4.1, Eniporide + Empa 73.6 ± 10.4), EMPH (mmHg, Eniporide 12.6 ± 2.9, Eniporide + Empa 16.8 ± 3.7) and LDH release (μmol/g heart, Eniporide 15.9 ± 4.7, Eniporide + Empa 22.2 ± 5.4). Haemodynamic parameters, including EDP, perfusion pressure, RPP and DLVP, were monitored throughout these experiments and are summarized in Supplementary Figures S1–S3.

Table 1 Baseline characteristics for isolated insulin- and non-insulin-perfused hearts that received 1 μM Empa, 10 μM Cari, or 0.02% DMSO (vehicle)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Cariporide</th>
<th>1 μM Empa</th>
<th></th>
<th>Vehicle</th>
<th>Cariporide</th>
<th>1 μM Empa</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDP (mmHg)</td>
<td>4.0 ± 0.3</td>
<td>4.1 ± 0.7</td>
<td>4.5 ± 0.6</td>
<td>2.9 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>2.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>DLVP (mmHg)</td>
<td>126.2 ± 8.1</td>
<td>124.7 ± 6.2</td>
<td>123.9 ± 7.7</td>
<td>114.3 ± 3.6</td>
<td>112.5 ± 5.0</td>
<td>111.4 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>HR (b.p.m.)</td>
<td>342 ± 21</td>
<td>380 ± 29</td>
<td>366 ± 14</td>
<td>383 ± 11</td>
<td>365 ± 11</td>
<td>373 ± 12</td>
<td></td>
</tr>
<tr>
<td>RPP (HR × DLVP)</td>
<td>43369 ± 4389</td>
<td>47155 ± 3628</td>
<td>45539 ± 3811</td>
<td>43778 ± 1962</td>
<td>40829 ± 1908</td>
<td>41716 ± 2031</td>
<td></td>
</tr>
<tr>
<td>Flow (mL/min/g HW)</td>
<td>11.4 ± 1.0</td>
<td>11.6 ± 1.2</td>
<td>11.9 ± 0.9</td>
<td>14.8 ± 1.2</td>
<td>12.6 ± 0.5</td>
<td>11.9 ± 0.7</td>
<td></td>
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<tr>
<td>Temperature (°C)</td>
<td>36.6 ± 0.1</td>
<td>36.4 ± 0.0</td>
<td>36.7 ± 0.1</td>
<td>36.6 ± 0.0</td>
<td>36.6 ± 0.0</td>
<td>36.6 ± 0.0</td>
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</table>

No differences were observed in baseline parameters within each intervention in the presence and absence of insulin.

Table 2 Baseline characteristics for isolated non-insulin-perfused hearts that received 2.5 μM Eniporide or 2.5 μM Eniporide with 1 μM Empa

<table>
<thead>
<tr>
<th></th>
<th>2.5 μM Eni</th>
<th>2.5 μM Eni + 1 μM Empa</th>
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<tbody>
<tr>
<td>EDP (mmHg)</td>
<td>2.4 ± 0.4</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>DLVP (mmHg)</td>
<td>116 ± 2</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>HR (b.p.m.)</td>
<td>387 ± 30</td>
<td>416 ± 21</td>
</tr>
<tr>
<td>RPP (HR × DLVP)</td>
<td>44881 ± 3090</td>
<td>48464 ± 3632</td>
</tr>
<tr>
<td>Flow (mL/min/g HW)</td>
<td>11.3 ± 0.3</td>
<td>12.2 ± 1.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.7 ± 0.0</td>
<td>36.6 ± 0.1</td>
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</table>

No differences were observed in baseline parameters within each intervention in the presence and absence of insulin.

DLVP, developed left ventricular pressure; EDP, end-diastolic pressure; g HW, heart wet weight in grams; HR, heart rate; RPP, rate-pressure product.
3.3 Late TOC and extensive IR injury in insulin-perfused hearts are associated with increased baseline glycogen loading and decreased end-ischaemic mtHKII

We observed late TOC (Figures 1, 2, and 4B) and extensive IR injury in insulin vs. non-insulin-perfused hearts. Knowing that increased glycogen at the start of ischaemia can delay TOC, and that increased glycogen breakdown during ischaemia can contribute to IR injury due to dissociation of HKII from mitochondria, we hypothesized that insulin perfusion increased glycogen loading in the isolated mouse hearts. Indeed, baseline glycogen levels were 23-fold higher in insulin-perfused hearts compared to non-insulin-perfused hearts. At end ischaemia, glycogen was fully depleted for both (Figure 4D and E), indicating greater glycogen depletion during ischaemia for the insulin-perfused hearts. As a consequence of the increased glycogen breakdown, both G6P and lactate levels were elevated at end-ischaemia in insulin-perfused hearts, while no differences in G6P and lactate were present at baseline (Figure 4D and E). Because it is known that increases in G6P and lactate (acidification) dissociate HKII from mitochondria, and that reduced mtHKII is a main determinant of infant size, we determined the association of HK with mitochondria at end-ischaemia. Indeed, both mtHKII protein levels and total mitochondrial HK activity were decreased in the insulin-perfused hearts (Figure 4F–J). To investigate whether Empa would change baseline glycogen loading, non-insulin and insulin-perfused hearts were treated with 1 μM Empa for 20 min. Empa was without effect on tissue glycogen content under these conditions (Supplementary Figure S4).

3.4 Insulin and NHE activity in cardiomyocytes

We investigated whether the presence of insulin affects NHE activity and/or the effectiveness of Empa or Cariporide to inhibit NHE. Cariporide strongly impaired pH recovery following NH₄⁺ pulse, indicating NHE inhibitor capacity. Empa mimicked Cariporide effects in pH recovery. However, insulin was without effect on NHE activity and did not affect the NHE inhibition abilities of Empa and Cariporide (Figure 5, in pH, without insulin: vehicle 7.09 ± 0.02, Empa 6.78 ± 0.04, and Cariporide 6.82 ± 0.04; and with insulin: vehicle 7.13 ± 0.03, Empa 6.78 ± 0.03, and Cariporide 6.74 ± 0.05).

3.5 Higher Empa concentrations does not reduce injury at end reperfusion

To examine whether Empa’s neutral effects on IR injury were due to low dosing, we studied non-insulin-perfused hearts administered with 3 μM and 10 μM Empa during IR (Figure 6A). Table 3 summarizes the baseline values for each condition. We found that higher concentrations of Empa also delayed contracture onset during ischaemia compared to vehicle (Figure 6B and C). However, the higher dosages showed no sign of improvement in damage parameters at the end of reperfusion (Figure 6D–G; infarct size (%), vehicle 55.0 ± 3.9, 3 μM Empa 64.1 ± 3.6, and 10 μM Empa 55.6 ± 2.1, RPP recovery (% from baseline), vehicle 482 ± 4.6, 3 μM Empa 40.3 ± 6.9, and 10 μM Empa 52.7 ± 6.6, EDP (mmHg), vehicle 37.7 ± 6.6, 3 μM Empa 26.8 ± 6.1, and 10 μM Empa 32.5 ± 4.3 and LDH release (μmol/g heart), vehicle 39.8 ± 3.8, 3 μM Empa 65.3 ± 12.5, and 10 μM Empa 45.7 ± 9.2). Haemodynamic parameters, including EDP, perfusion pressure, RPP, and LVDP, were monitored throughout these experiments and are summarized in supplementary Figure S5.

4. Discussion

We investigated whether the SGLT2 inhibitor Empa mimicked the effects of the classical NHE inhibitor Cariporide during cardiac IR conditions in the isolated intact mouse heart, by focusing on TOC and IR injury. We observed that (i) Empa and Cariporide had similar effects on contracture development during ischaemia, suggesting a direct cardiac effect of Empa similar to what we expect from NHE1 inhibition during ischaemia, (ii) Cariporide, but not Empa, protected against acute IR injury, (iii) Empa administration on top of NHE1 inhibition by Eniporide did not further delay contracture development, suggesting that Empa and the classical NHE1 inhibitor Eniporide delay contracture development through similar mechanisms, (iv) classical NHE inhibitor effects on cardiac IR injury are strongly dependent on the degree of ischaemic glycogen breakdown, (v) the extended cardiac IR injury in the insulin- vs. non-insulin-perfused hearts was associated with increased glycogen at baseline, enhanced glycogen breakdown during ischaemia, and consequently increased G6P, increased lactate and decreased mtHKII end-ischaemia, and (vi) incubating isolated cardiomyocytes with Empa or Cariporide impaired NHE activity, independent from the presence of insulin. The similar (NHE-associated) actions of Empa and Cariporide during stagnant conditions (ischaemia, cardiomyocyte incubation) vs. the divergent actions of Empa and Cariporide during turbulent (reperfusion) conditions suggest a differential NHE interaction between a specific NHE inhibitor and Empa.
Figure 6 Increasing Empa dosages further delayed TOC, but did not result in reduced IR injury (as compared to Figure 3). Hearts were subjected to IR and 3 μM Empa, 10 μM Empa or vehicle in the absence of insulin (A). Contracture development during ischaemia (B), TOC (C), global infarct size (D), RPP recovery at \( t = 2 \) h reperfusion relative to baseline RPP (E), end-diastolic pressure (F), and total LDH release during reperfusion (G) after 2 h reperfusion. \( N = 5 \) for vehicle, \( n = 5 \) for 3 μM Empa, and \( n = 6 \) for 10 μM Empa. Scale bar = 0.5 mm. LDH AUC, Lactate dehydrogenase activity area under the curve during reperfusion, normalized to heart wet weight; Pdia, diastolic pressure; RPP, rate pressure product; TOC, time of onset contracture.
obtained in cardiomyocytes, we did not find reduced cardiac IR injury in
with a recent study in isolated non-diabetic rat hearts showing no im-
Further studies are needed to examine to what extent these effects can
data indicate that these effects are mediated through NHE inhibition.
reduction in IR injury. Our study confirmed these observations in
contracture develops. The delayed contracture observed with Empa
ischemia-reperfusion isolated heart

| Table 3 Baseline characteristics for isolated non-insulin-perfused hearts that received 3 μM Empa, 10 μM Empa, or 0.044% cyclodextrin (vehicle) |
|-----------------|-----------------|-----------------|
|                 | Vehicle         | 3 μM Empa       | 10 μM Empa      |
| EDP (mmHg)      | 2.9 ± 0.2       | 2.6 ± 0.3       | 2.0 ± 0.2+      |
| DLVP (mmHg)     | 124.7 ± 5.9     | 101.8 ± 9.3     | 129.8 ± 5.8     |
| HR (b.p.m.)     | 403 ± 11        | 376 ± 9         | 378 ± 11        |
| RPP (HR × DLVP) | 50.074 ± 2128   | 37.653 ± 2991*  | 48.622 ± 2101   |
| Flow (mL/min/g HW) | 11.7 ± 0.4  | 8.9 ± 1.1       | 11.9 ± 0.8      |
| Temperature (°C) | 36.7 ± 0.0      | 36.6 ± 0.0      | 36.7 ± 0.1      |

Differences were present for EDP between vehicle and 10 μM Empa and for RPP between vehicle and 3 μM Empa. DLVP, developed left ventricular pressure; EDP, end-diastolic pressure; g HW, heart wet weight in grams; HR, heart rate; RPP, rate-pressure product.

*P < 0.05.

4.1 Empa mimics classic NHE1 inhibitors during ischaemia of the isolated heart
Several studies have shown that inhibition of NHE can delay ischaemic rigour contracture. Knowing that Empa inhibited NHE in isolated cardiomyocytes, we hypothesized that Empa could delay rigour contracture during ischaemia in the isolated heart. Indeed, in non-insulin-perfused hearts, Cariporide delayed contracture development and this effect was mirrored by Empa. Empa was unable to delay contracture development when NHE1 inhibitor Eniporide was present, suggesting that Empa delays the contracture development through inhibition of NHE1. During ischaemia, cardiomyocytes operate entirely through glycolysis. Once glycolysis is impaired, ATP content becomes depleted and rigour contracture develops. The delayed contracture observed with Empa suggests that Empa and Cariporide have a direct cardiac, ATP preserving effect during ischaemia. The major message of our study is the demonstration of a direct and acute effect of Empagliflozin on the ischaemic intact heart, as reflected by the delay in contracture development. Our data indicate that these effects are mediated through NHE inhibition. Further studies are needed to examine to what extent these effects can contribute to the beneficial cardiovascular effects observed in the large clinical trials.

4.2 Empa effects on cardiac IR injury of the isolated heart
Increased activity of NHE is a well-recognized contributor to cardiac IR injury, due to intracellular sodium-loading that leads to calcium overload. We recently reported in isolated cardiomyocytes direct cardiac effects of Empa through intracellular sodium and calcium lowering and NHE inhibition. NHE inhibition is shown, although not unambiguously, to protect against cardiac IR injury. Despite the direct actions previously obtained in cardiomyocytes, we did not find reduced cardiac IR injury in isolated hearts given 1 μM, 3 μM, or 10 μM Empa. Our findings are in line with a recent study in isolated non-diabetic rat hearts showing no improvement in infarct size after IR using 5 μM Empa. Interestingly, this study demonstrated direct cardiac effects of Empa, because post-ischaemic mitochondrial function showed increased permeability of the inner mitochondrial membrane and increased respiratory activity through complex 1 and 2. It is unknown whether these mitochondrial effects were mediated through NHE1 inhibition. In contrast, Andreadou et al. showed that Empa protected the heart from IR injury in vivo. Their experiments, performed in high-fat-treated animals, showed lowered plasma glucose concentration with Empa treatment. The glucose-lowering effect of Empa, amongst other systemic changes, could possibly explain its protective action on the heart. Interestingly, in that study Empa did improve cell viability and ATP content after hypoxia/reoxygenation in cultured H9C2 and endothelial cells, suggesting that Empa can employ direct cardiac cell effects, independent of SGLT2, glucose levels, or glucotoxicity. Recent reports also showed that empagliflozin treatment after transverse aortic constriction prevented worsening of cardiac functioning in non-diabetic mice, suggesting that Empa does not alter heart failure pathways associated only with diabetes per se. Yet, these studies did not examine direct cardiac effects of Empa in the intact organ. Understanding the mechanisms that lead to cardioprotection, whether these are related to systemic changes and/or direct intracellular changes, may ultimately aid in the developments of novel and effective therapies against heart failure and myocardial infarction. Previous investigators have also reported suppression of apoptosis and ROS, and improved cell function and viability by SGLT2 inhibitors in kidney tissue. These effects most likely act through inhibition of SGLT2. Since SGLT2 is absent in the heart, the drug mechanisms of Empa in the kidney and the heart could be dissimilar. When considering the cardio-renal syndrome, these effects on the kidney may also evolve into cardiac improvements.

The discrepancies between Empa and Cariporide effects on IR injury in the present study might be explained by different binding sites of Empa and Cariporide at the NHE1. While Empa binds to the extracellular Na\(^+\)-binding site of NHE1, Cariporide binds to an extracellular region that regulates the interactions between transmembrane (TM) IV and TM X1. Furthermore, our previous docking studies estimated an extracellular binding site for Cariporide distinct from the extracellular Na\(^+\)-binding site of Empa (unpublished data). At the moment of reperfusion, when extracellular pH is restored and glycolysis is activated again, NHE1 becomes activated with an accelerated influx of Na\(^+\) through the extracellular Na\(^+\)-binding site. Possibly, competitive binding between Na\(^+\) and Empa at this site may eliminate NHE1 inhibition by Empa, whereas this mechanism does not apply to the classic NHE1 inhibitors acting at a different site. Seemingly, Empa can function as a NHE1 inhibitor for the intact heart in conditions of homeostasis and relative stable flow, but less so in conditions of rapidly changing flows and ion fluxes.

4.3 Loss of cardioprotection by Cariporide in insulin-perfused hearts
Most studies in isolated hearts have shown that Cariporide treatment during ischaemia delayed ischaemic contracture onset and decreased IR injury. The degree of contracture delay was positively regulated with the reduction in IR injury. Our study confirmed these observations in hearts not perfused with insulin. However, in our insulin-perfused hearts, Cariporide was unable to delay contracture onset and reduce IR injury. It should thereby be noted that most isolated heart studies examining Cariporide effects on IR have not incorporated insulin in the perfusate. We noted that with insulin present, ischaemic contracture onset is largely delayed (from 7.5 min to 17.5 min), which can probably be explained by the larger cardiac glycogen content at baseline in the insulin-perfused hearts. The amount of glycogen in the insulin-perfused mouse hearts (0.04 μmol/mg) is similar to the amount present in untreated mouse hearts (0.05–0.08 μmol/mg), indicating that the absence of insulin in isolated mouse hearts perfused with glucose, lactate and pyruvate results in glycogen-depleted hearts. We previously showed that glycogen depletion is even more severe when mouse hearts are perfused with glucose, lactate and pyruvate.
perfused with glucose as sole substrate.24 These data indicate that insulin should be present in IR studies of isolated mouse hearts to take into account the role of glycogen in IR injury. The prolonged delay in contraction due to insulin being present may have prevented any further delay by Cariporide treatment. Cross et al.30 showed that high intracellular glycogen levels prior to ischaemia lead to increased glycogen depletion during ischaemia that consequently increased reperfusion injury in rodent hearts. Ischaemic glycolysis is maintained until intracellular glycogen levels are fully depleted, which in the meantime leads to accumulation of metabolites, including H\(^+\) and lactate, causing exceedingly low pH levels.30 NHE activity is dependent on the membrane H\(^+\)-gradient and intracellular pH levels. In our insulin-perfused hearts, the extended period of ischaemic glycolysis may have led to continuous H\(^+\) production, efflux, and build up. It is possible that due to this, the H\(^+\)-gradient across myocardial cell membranes collapsed, resulting in loss of NHE activity. Indeed, with prolonged ischaemia, NHE activity is inhibited by extracellular acidosis.43 In that case, NHE inhibition by Empa or Cariporide could not salvage the myocardium anymore, as occurring in insulin-perfused hearts. In this regard, a recent study by Kingma30 reported no protection of NHE1 inhibitor EMD-87580 against necrosis in an in vivo canine IR model of prolonged ischaemia. In the context of insulin-perfused isolated hearts, high glycogen breakdown that fuels prolonged glycolysis during ischaemia hampers Cariporide-induced cardioprotection. Further research examining extracellular pH during ischaemia in isolated mouse hearts perfused with or without insulin is warranted to answer this matter adequately.

4.4 Increased glycogen and decreased MtHKII associate with IR injury in insulin-perfused hearts

Cardiac glycogen content has been shown to be an important determinant of post-ischaemic damage: beneficial when not completely depleted during ischaemia, but detrimental when entirely broken down before reperfusion. When glycogen gets completely depleted during ischaemia, a lower glycogen content prior to ischaemia results in decreased IR injury.30 This likely explains the worsened recovery after IR of insulin-perfused vs. non-insulin-perfused hearts. Higher glycogen levels, through insulin administration, maintain a longer glycolytic period during ischaemia before cellular energy reserves are fully depleted, thereby extending the period prior to rigour contracture and resulting in high amount of glycogen-derived metabolites at end-ischaemia.31 It was suggested that the breakdown products of glycogen, i.e. G6P and lactate, mediate the increased IR injury, through HKII dissociation from mitochondria.31 Indeed, we observed accumulation of G6P and lactate in the insulin-perfused heart that was associated with decreased mtHKII at end-ischaemia. Numerous studies have shown the importance of mtHKII in cardioprotection and IR injury.30,49 This seems to be the likely mechanism why our insulin-perfused hearts displayed worse recovery after IR. Therefore, targeting mtHKII dissociation may be a valuable strategy against IR injury.

4.5 Study limitations

In our study, we only investigated the effects of Empa in non-diabetic hearts. Whether Empa may employ different IR effects in the diabetic heart than the non-diabetic heart remains unknown. The perfusate used did not contain fatty acid, which is an important cardiac metabolic substrate. This may alter the translation of our data to the in vivo conditions, although it is yet unknown how fatty acid and insulin together could affect IR injury and cardioprotective strategies in the isolated heart. Further research should examine the interaction of fatty acid and insulin in the isolated IR heart. Infarct size was determined by TTC staining, and fixation occurred for an overnight period. This might have been too long, since the infarct sizes measured have been somewhat high compared with functional recovery. However, our data may support the contention that a dichotomy exists between cell death (infarct size) and mechanical function, since they do not necessarily constitute similar cell processes.50,51 Although the decrease in LDH by Cariporide was rather large (~40%) in the non-insulin-perfused hearts, it did not reach significance. This is likely explained by the large variation in LDH release values between experiments, precluding statistical significance to be reached due to insufficient statistical power. Although statistical significance was not reached for this particular parameter, the combined IR injury parameters (infarct size, rate pressure product, end-diastolic pressure, and LDH release) still consistently showed reduced IR injury with Cariporide. We cannot rule out that the absence of Empa or Cariporide effects in the insulin-perfused hearts might be caused by drug binding to albumin, leading to a lower concentration of the drug to reach the heart. However, in isolated mouse cardiomyocytes, Empa and Cariporide demonstrated strong and similar inhibition of NHE in conditions with and without albumin (Figure 5A vs. B). To further understand the mechanism of Empa during IR, studies with Empa in the presence of a NHE or SGLT2 inhibitor are urgently needed.

5. Conclusion

Empa delayed ischaemic contracture development in the absence of insulin, suggesting that Empa has direct cardiac effects and can inhibit the NHE in the *ex vivo* intact hearts. However, Empa did not protect against IR injury in isolated hearts, whereas Cariporide does. The presence of insulin interferes with the effects of NHE inhibitors and Empa on IR injury parameters in the isolated hearts. These data indicate that Empa and Cariporide may inhibit NHE in different manners.

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