Pre-treatment with the Na\(^+\)/H\(^+\) exchange inhibitor cariporide delays cell-to-cell electrical uncoupling during myocardial ischemia

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Abstract

Objective: Inhibition of Na\(^+\)–H\(^+\) exchange (NHE) delays the onset of myocardial rigor contracture during ischemia. The aim of this study was to analyse the effects of NHE inhibition on cell-to-cell electrical uncoupling during myocardial ischemia/reperfusion.

Methods: Twenty-six isolated rat hearts and 23 in situ porcine hearts were submitted to no-flow ischemia followed by reperfusion, with or without pre-treatment with cariporide (7 \(\mu\)M in rats and 3 mg/kg in pigs). Ischemic rigor and hypercontracture, conduction velocity and myocardial electrical impedance were monitored.

Results: Pre-treatment with cariporide delayed ATP depletion (luminescence assay in rat myocardium) and onset of rigor contracture (tension recordings or ultrasonic crystals) during ischemia both in rat and pig hearts \((P<0.05)\). In addition, cariporide delayed the onset of sharp changes in tissue resistivity and phase angle in impedance recordings (four-electrode probes) from 10±1 to 13±1 min \((P<0.001)\) in rat hearts, and from 22±1 to 38±2 min \((P<0.001)\) in pigs. Blockade of impulse propagation (transmembrane action potentials in rat hearts) was also markedly delayed by cariporide (from 14±1 to 20±1 min, \(P<0.001)\). Reperfusion-induced LDH release in rat hearts and infarct size in pigs were markedly reduced by pre-treatment with cariporide. Conclusions: Inhibition of NHE with cariporide slows the progression of ischemic injury during myocardial ischemia, and delays the onset of cell-to-cell electrical uncoupling.

Keywords: Conduction (block); Gap junctions; Ischemia; Na/H-exchanger; Reperfusion

1. Introduction

There is solid evidence that inhibitors of Na\(^+\)/H\(^+\) exchange (NHE) protect myocardium against cell death secondary to ischemia/reperfusion. NHE inhibitors have been consistently found to limit reperfusion-induced enzyme release and infarct size in a variety of experimental models [1–7]. Although the efficacy of NHE inhibitors in patients with transient myocardial ischemia has not been definitively established, the available information is consistent with a protective effect similar to that observed in other animals [8,9].

Despite the large number of published studies investigating the mechanism of the protective effect of NHE inhibitors, the exact mechanism of action of these drugs remains unclear. NHE inhibitors were originally thought to exert their protective effects during the initial minutes of reperfusion [1,4], by slowing normalization of intracellular pH and reducing Na\(^+\) influx associated with it [4,10,11], thus preventing cardiomyocyte hypercontracture and sarcolemmal rupture [12,13]. However, a large number of studies have shown that the protective effect of NHE inhibitors against cell death secondary to ischemia-reperfusion is largely dependent on the administration of these drugs before the onset of ischemia [6,7], suggesting the drugs act mainly during ischemia [5–7]. In support of this view, it has been shown that pre-treatment with NHE inhibitors preserve ATP concentration [11,14,15] and attenuates rigor contracture in myocytes or myocardial tissue submitted to ischemia or energy depletion [5,7].

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In this context, the effects of NHE inhibition on electrophysiological derangements caused by ischemia are not well known. The only studies performed on electrophysiological variables indicate that NHE inhibition reduces epicardial activation-recovery interval dispersion during ischemia in isolated rabbit hearts [14], and prevents the transient shortening of action potential duration observed during reperfusion [16]. However, the effects on action potential duration during ischemia are contradictory [2,3,16]. The aim of this study was to elucidate the effects of cariporide on cell-to-cell electrical uncoupling, as assessed by analysis of electrical tissue impedance or conduction blockade, during ischemia-reperfusion. The effects of cariporide were assessed in isolated rat hearts in which action potential characteristics, conduction velocity, and tissue electrical impedance were simultaneously measured. To further evaluate the relevance of the findings obtained in this model, the effects of cariporide were assessed in the in situ pig heart submitted to transient coronary occlusion. Cariporide is a highly selective extensively investigated NHE inhibitor which has been shown very effective in inhibiting NHE1, the only Na\(^+/\)H\(^+\) exchanger isoform that has been detected in the sarcolemma of heart cells [17].

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23m revised 1996). The study was approved by the ethics committee of our institution.

2.1. Isolated rat hearts

2.1.1. Experimental preparation

Thirty-six adult male Sprague–Dawley rats (280–350 g) were killed by an intraperitoneal overdose of sodium thiopental. Whole hearts were rapidly removed and retrogradely perfused through the aorta with an oxygenated (95% O\(_2\)–5% CO\(_2\)) Krebs solution (in mM: NaCl 118, KCl 4.7, MgSO\(_4\) 1.2, CaCl\(_2\) 2.5, NaHCO\(_3\) 25, KH\(_2\)PO\(_4\) 1.2, and glucose 11, pH 7.4) at 37°C. After removing both atria, the right ventricular free wall and the septum were incised from the cardiac base to the apex. Opened hearts were pinned to a silicon membrane placed at the bottom of an organ bath, thus exposing the endocardial surface of the left ventricle. A 2.0 silk snare was placed in the septum of the hearts and was connected to horizontal isometric force transducers (FSG-01, and SG-M DC bridge amplifier module, Experimetria, London, UK). Resting tension was 0.5 g. Preparations were paced from the base of the heart using rectangular pulses of 2.5 ms duration and 4 V of amplitude, at 400 ms basic cycle length. Rigor contracture was detected as an increase in resting tension during ischemia. Its onset was defined as the time when this increase reached a 10% of resting tension measured after 10 min of ischemia.

2.1.2. Transmembrane action potential recordings

Transmembrane action potentials were recorded as previously described [18], from the apical region of the heart. Conduction time was measured as the time elapsed between the stimulus artefact and the onset of the rapid depolarization of the action potential. Using this method, the estimated conduction velocity represents an average velocity between the stimulus site and the recording electrode, as the pathway of activation is not known. However, it permits one to compare different groups of treatment in order to assess changes in the time of conduction blockade. Conduction blockade was understood as complete loss of excitability. Distance between the stimulating electrodes and the recording microelectrode was between 0.5 and 0.7 cm.

2.1.3. Measurement of myocardial electrical impedance

Measurement of myocardial electrical impedance and its two components, tissue resistivity (R) and phase angle (\(\theta\)), was performed using a four-electrode probe placed in the septum, as previously described, at 7 KHz [19,20]. The impedance probe consisted of a linear array of four platinum electrodes (length: 5 mm, diameter=0.4 mm), placed at an interelectrode distance of 2.5 mm. Previous studies have demonstrated that after an initial period with only slight changes, ischemia induced a second phase characterized by a sharp increase in tissue resistivity and a marked decrease in phase angle [19]. The onset of this second phase has been related to onset of cell-to-cell electrical uncoupling [21–23]. The time of the onset of these abrupt changes in both tissue resistivity and phase angle was determined as the time of intersection of the two tangent lines superimposed on the original recording.

2.1.4. Lactate dehydrogenase (LDH) release and ATP content

LDH release was measured during reperfusion in five hearts of each group [24]. Myocardial ATP content was determined in four additional rat hearts from each group at 10 min of ischemia by a quantitative bioluminescent method (Sigma–Aldrich, USA).

2.1.5. Experimental protocol

After 40 min of equilibration, fourteen hearts (controls) were submitted to 1 h acute no-flow ischemia. During the ischemic insult the preparation was superfused with hypoxic Krebs (bubbled with 95% N\(_2\)–5% CO\(_2\), pH 7.4), lacking glucose, in order to maintain temperature (35.5–36°C). In other 12 hearts (cariporide treated hearts), the ischemic insult was preceded by perfusion for 10 min with a Krebs solution containing cariporide, 7 \(\mu\)M. After the 60 min of ischemia, five hearts of each group were reperfused
for 90 min. This concentration was chosen as we have previously demonstrated that it reduces the proportion of cardiomyocytes developing ischemic rigor contracture [5], prevents reoxygenation edema in the isolated rat hearts [24], and reduces infarct size in pigs [7]. To exclude the possibility that lower or higher concentrations of cariporide might cause different responses, eight additional rat hearts were pre-treated with 0.1 μM (n = 3), 1 μM (n = 3) or 100 μM (n = 2), and concentration–response curves were calculated. Stability of the preparation during 1 h normoxic perfusion was assessed in the remaining two hearts.

2.2. Studies in the in situ pig heart

2.2.1. Animals and instrumentation.

Twenty-three Large-White pigs (39.3±1.3 kg) were anaesthetised with sodium thionental (30 mg/kg, i.v., plus continuous infusion) and mechanically ventilated. A mid-sternotomy was performed and the left anterior descending (LAD) coronary artery was dissected free at its midpoint and surrounded by an elastic snare. End-diastolic segment length (EDSL), end-systolic segment length (ESSL), and systolic shortening (SS) in the area at risk and in control myocardium were monitored by means of ultrasonic crystals inserted into the inner third of the left ventricle free wall as previously described [7]. Rigor contracture development was estimated from the reduction in the amplitude of the segment length change (maximal–minimal value) [7]. Left ventricular (LV) pressure and coronary blood flow at the occlusion site were monitored as previously described [7]. Tissue electrical impedance was measured at the centre of the area at risk using the same four-electrode probe employed in the isolated rat heart model.

2.2.2. Study protocol

Animals were submitted to a coronary occlusion of the LAD of 48 min followed by 2 h of reperfusion. Ten minutes before coronary occlusion, animals received an intravenous bolus of saline (n = 12) or saline containing 3 mg/kg of cariporide (n = 11). Previous studies have shown that this dose of cariporide resulted in reproducible plasma drug levels, peaking at 7 μM 5 min after injection, which were still elevated 90 min later (2.7 μM) [7].

2.2.3. Area at risk and infarct size

Area at risk and infarct size were measured using 10% fluorescein and 1% triphenyltetrazolium chloride, respectively, as previously described [7], 2 h after reperfusion.

2.3. Statistical analysis

Statistical analysis was performed using commercial available software (SPSS for Windows 8.0). Data are expressed as mean±S.E.M. Repeated measures analysis of variance (ANOVA) and Student’s t-test were used to assess differences between groups, that were considered significant when P<0.05.

3. Results

3.1. Isolated rat hearts

3.1.1. Baseline effects of cariporide

Stability of the preparation was confirmed in two hearts in which no changes in any variable were observed during 1 h normoxic perfusion.

Pre-treatment during 10 min with 7 μM of cariporide in isolated rat hearts did not induce any significant change in action potential characteristics (Fig. 1), developed tension (from 589±48 to 527±49 mg), tissue resistivity (from 96.21±5.11 to 93.44±5.06 Ω·cm), or in phase angle values (from −0.56±0.20 to −0.50±0.20 °).

3.1.2. Effects of cariporide during no-flow myocardial ischemia

3.1.2.1. Ischemic rigor contracture

Developed tension decreased during ischemia, to reach a minimal value several min later. Rigor contracture, detected as an abrupt increase in diastolic tension, occurred at 15.54±1.21 and 21.17±1.64 min of ischemia in control and cariporide pre-treated hearts, respectively (P=0.014) (Fig. 2).

3.1.2.2. Transmembrane action potential and conduction velocity

Ischemia in the 14 control rat hearts caused a significant reduction in resting membrane potential, action potential amplitude and duration, and maximal rate of depolarization (dV/drmax), and a progressive decrease in conduction velocity (Fig. 1). Pre-treatment with cariporide did not modify the time course of action potential characteristics, although there was a non-significant trend towards an attenuated shortening of repolarization in treated hearts (Fig. 1). A significant delay in conduction blockade, referred as a complete loss of excitability, was observed in cariporide pre-treated hearts as compared with control hearts (Fig. 2).

3.1.2.3. Tissue impedance

Ischemia induced a marked increase in myocardial resistivity (from 86.46±5.04 to 183.40±12.87 Ω·cm at 45 min of ischemia, and from 93.44±5.06 to 195.56±16.34 Ω·cm, in control and cariporide pre-treated rats, respectively, P<0.001) and a marked negative shift in phase angle (from −0.36±0.26 to −4.62±0.43 ° and from −0.50±0.20 to −3.78±0.26 °, in control and treated animals, respectively, P<0.001). As previously described [19], following an initial period with only slight changes, there was a second phase characterized by a sharp increase in tissue resistivity and a marked decrease in phase angle. The onset of this second phase of sharp changes occurred earlier than conduction blockade.
and rigor onset ($P<0.01$) (Fig. 2). Hearts pre-treated with cariporide showed similar changes in tissue resistivity and phase angle, but the onset of the sharp changes in both variables was significantly delayed as respect to control hearts (Fig. 2).

3.1.2.4. Concentration–response curves to cariporide

The delay in the times of onset of rigor contracture and cell-to-cell electrical uncoupling (determined both in resistivity and phase angle) were calculated at 0.1, 1 and 100 μM in eight additional rat hearts. The three responses fitted very well to sigmoid curves ($r^2 = 0.997, 0.998, \text{and } 0.991, \text{respectively}$). Half-maximal effect concentrations were 2.08 μM for the delay in rigor onset, and 2.38 and 3.14 μM, respectively, for the delays in the onset of tissue resistivity increase and phase angle shift.

3.1.2.5. Myocardial ATP concentration

Myocardial content of ATP was greater in cariporide-treated hearts than in control hearts (8.7±0.4 vs. 6.8±0.6 μmol/g dry tissue, or 40.1±1.9 vs. 31.5±3.0% of pre-ischemic values; $P<0.05$).

3.1.3. Reperfusion-induced hypercontracture, LDH release, and functional recovery

In control rat hearts, reperfusion induced a further increase in resting or diastolic tension (hypercontracture) peaking during the first 2–3 min of re-flow, with minimal recovery of developed tension. In hearts pre-treated with cariporide prior to ischemia, peak hypercontracture was markedly reduced, and functional recovery after 1 h of reperfusion was better (Fig. 3).

LDH release showed an early peak in the first 2–3 min of reperfusion, followed by a rapid decay (Fig. 3). In contrast, cariporide pre-treated hearts had a depressed LDH activity during the entire reperfusion.

3.2. Studies in the in situ pig heart

3.2.1. Haemodynamics and myocardial segment length measurements

There were no significant differences in heart rate, mean aortic pressure or coronary blood flow, while LV diastolic...
Fig. 3. Maximal hypercontracture during the first 2–3 min of reperfusion (a), developed tension at 1 h of reperfusion (b) and lactate dehydrogenase (LDH) release (c) during reperfusion in control and cariporide pre-treated rat hearts. (d) Accumulated LDH release during the first 30 min of reperfusion. * (P<0.05) and ** (P<0.01) indicate significant differences between both groups.

pressure after 15 min of reperfusion was significantly lower in the cariporide group (11.1±3.0 vs. 23.7±3.7 mmHg, P<0.05). End-diastolic segment length in the area at risk showed a marked increase during ischemia and a rapid decrease under basal values in early reperfusion, reflecting hypercontracture, that was not observed in the cariporide group (Fig. 4a). Systolic shortening was abolished during ischemia and showed a significantly greater recovery in the cariporide group during reperfusion, compared to the control group (Fig. 4b).

3.2.2. Rigor contracture and myocardial electrical impedance

During coronary occlusion, the amplitude of segment length, a marker of ischemic rigor contracture, was significantly reduced in the control group when compared with cariporide pre-treated hearts (Fig. 5).

Occlusion of the LAD in the control group of pigs induced a significant increase in resistivity values and a decrease in phase angle (Fig. 6). Cariporide pre-treated animals showed a significantly different time course of the changes induced by ischemia in both variables, with a much less pronounced second phase (Fig. 6). The onset of the sharp changes in tissue resistivity and phase angle was significantly delayed (resistivity: from 22.35±1.07 min in control pigs to 38.41±1.58 min in cariporide-treated animals, P<0.001; phase angle: from 22.96±1.10 to 38.27±1.57 min, P<0.001).

3.2.3. Arrhythmias

Ventricular fibrillation (VF) appeared at 34 and 37 min in two out of 12 control animals, and at 33.0 min in one out of 11 cariporide-treated pigs, without significant between-group differences. At immediate reperfusion, VF appeared in 72.7 and 87.5% of the animals in control and cariporide groups, respectively, without significant between-groups differences.

3.2.4. Area at risk and infarct size

The area at risk was nearly identical in both groups of treatment (control pigs: 11.52±0.76% of ventricular mass; cariporide pre-treated pigs: 11.0±0.8%). Infarcts were virtually absent in pigs receiving cariporide, and large in controls (0.54±0.33 and 53.22±10.00% of area at risk, respectively, P<0.001).

4. Discussion

This study demonstrates that inhibition of NHE with cariporide has profound effects on the time course of the alterations in the propagation of electrical impulse and in
Measurement of tissue electrical impedance in two models, involving two different species with distinct ionic regulation, disclosed similar delays in cell-to-cell electrical uncoupling, as determined by the onset of sharp changes in tissue resistivity and phase angle caused by ischemia, in the presence of cariporide. These results are in line with previous observations showing that inhibitors of NHE reduce ischemic rigor contracture, and further demonstrate that these drugs slow the progression of ischemic injury during myocardial ischemia.

4.1. Electrophysiological effects of cariporide during ischemia-reperfusion

Despite the large number of studies analysing the effects of NHE1 inhibition during myocardial ischemia-reperfusion, only few studies have analysed its actions on electrophysiological variables. Inhibition of NHE reduced the dispersion of epicardial activation-recovery intervals occurring during ischemia in isolated rabbit hearts [14], and abolished the monophasic action potential shortening induced by reperfusion in a porcine model [16]. The effect of NHE inhibition on changes induced by myocardial ischemia on action potential has been also investigated, with contradictory results [2,3,16]. However, the effects of cariporide on electrical tissue impedance, onset of electrical uncoupling, and conduction blockade during ischemia-reperfusion had not been previously investigated.

The present observations on impulse propagation and tissue electrical properties of ischemic rat and pig myocardium are consistent in indicating that cariporide delays the onset of electrical uncoupling during ischemia. Previous studies have suggested that the onset of the sharp changes in tissue resistivity and phase angle occurring in ischemic myocardium reflect the onset of cell-to-cell electrical uncoupling [21–23]. Cariporide delayed these changes by approximately 3–4 and 16 min in rat and pig myocardium, respectively. In agreement with these data, conduction blockade was also delayed in the isolated rat heart.

Only one study has analysed the recovery of passive cable-like properties of myocardium during reperfusion. Authors showed, using the voltage-ratio method in rabbit papillary muscles [25], that total resistance recovered rapidly on reperfusion, with a faster recovery in extracellular than in intracellular resistance. In this study we have used the four-electrode technique to record tissue electrical impedance during reperfusion. The results confirm the previous observation of a rapid normalization of tissue resistivity and extend it to phase angle. This very rapid normalization was not clearly influenced by the extent of hypercontracture or cell death, as assessed by LDH release, and was not affected by cariporide.

4.2. Mechanism of the electrophysiological effects

The observed delay in conduction blockade and in
changes in passive electrical properties in ischemic myocardium treated with cariporide, in the absence of effects on transmembrane potentials, suggests that this drug preserves gap-junction mediated intercellular communication during ischemia. The mechanism of this effect cannot be elucidated from this study. Delayed ATP depletion in cariporide treated hearts could play an important role. It has been well established that ATP depletion and increased cytosolic Ca$^{2+}$ concentration contribute to electrical uncoupling during ischemia. A close temporal relationship has been observed between electrical uncoupling and the rise in cytosolic Ca$^{2+}$ [26], while studies in isolated cardiomyocytes have demonstrated that the onset of the rise in cytosolic Ca$^{2+}$ is temporally associated to severe ATP depletion and onset of rigor contracture [27,28]. In the present study, the fall in ATP content during the initial 10 min of ischemia was significantly attenuated by approximately 13% in cariporide treated hearts, and the onset of rigor contracture was clearly delayed, in agreement with previous studies in isolated cells [5], myocardial preparations [4,5,15], and in situ pig hearts [7]. The data are thus consistent with the hypothesis that the effects of cariporide on electrical cell coupling are mediated by preserved ATP depletion resulting in delayed rigor contracture and delayed Ca$^{2+}$ rise. The mechanism by which cariporide delays ATP depletion in ischemic myocardium in this and in previous studies [11,14,15] has not been established. In this, as in previous studies, a close
temporal association was observed between the onset of rigor contracture, alterations of electrical impedance indicating cell-to-cell uncoupling, and conduction blockade [26,29].

4.3. Lack of protection against arrhythmias

Most of the previously published studies have demonstrated a protection of NHE inhibition against ischemic [1,2] and/or reperfusion [1,3,4,7,14] arrhythmias. The reduced dispersion of epicardial activation-recovery intervals during ischemia [14], and attenuated Ca^{2+} overload and triggered activities at the time of reperfusion have been proposed to explain this protective effect [4,10]. However, in this study, in spite of the prominent effect of cariporide on the time course of electrical uncoupling during ischemia, no significant difference in the incidence of ventricular fibrillation was observed in the pigs submitted to transient coronary occlusion. The dissociation between the effects of cariporide on passive electrical properties and ventricular fibrillation cannot be easily explained, and does not fit with the widely accepted view that electrical uncoupling has a prominent role in the genesis of ischemic ventricular fibrillation. The possibility that the failure to detect a protective effect of cariporide against arrhythmias is due to insufficient drug concentration seems unlikely, since the dose used results in plasma concentrations with near maximal effects on rigor contracture, cell-to-cell electrical uncoupling, and infarct size limitation.

4.4. Protection against reperfusion-induced hypercontracture and necrosis

The marked protection afforded by pre-treatment with cariporide against reperfusion-induced increase in diastolic tension and LDH release in the isolated rat hearts, and against myocardial segment shrinkage and myocardial necrosis in the in situ pig hearts is fully consistent with previous studies [1–7,15,24]. The fact that these effects were associated to a prominent delay in the progression of markers of ischemic injury, as ATP depletion, development of rigor contracture and change in passive electrical properties, support previous studies suggesting that most, if not all the protective effect of NHE inhibition against cell death secondary to ischemia-reperfusion, is exerted during the ischemic period [5–7].

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