Original article

Cardioprotective Effect of Ranolazine in the Process of Ischemia-reperfusion in Adult Rat Cardiomyocytes

Eva M. Calderón-Sánchez,a Alejandro Domínguez-Rodríguez,a,b José López-Haldón,c Manuel F. Jiménez-Navarro,d Ana M. Gómez,b Tarik Smani,a and Antonio Ordóñez.a,c,*

A B S T R A C T

Introduction and objectives: Ranolazine is used as a complementary treatment for angina in symptomatic patients who are inadequately controlled with first-line antianginal therapies. Ranolazine inhibits sodium voltage-dependent channels, suggesting their possible involvement in the reperfusion process by preventing the sodium and calcium overload that occurs during ischemia. In this study, we characterized the effect of ranolazine on calcium homeostasis in isolated adult cardiomyocytes from rats subjected to a simulated ischemia and reperfusion protocol.

Methods: The effects of ranolazine on changes in intracellular calcium concentration were evaluated at different times using field electrostimulation. The study of intracellular calcium was performed using microfluorimetry with the fluorescent indicator, Fura-2, and by confocal microscopy with the indicator, Fluo-3.

Results: We found that cardiomyocytes subjected to ischemia-reperfusion showed an increase in the diastolic calcium concentration and a decrease in the amplitude of intracellular calcium transients. The application of ranolazine during ischemia significantly improved intracellular calcium handling, preventing intracellular calcium overload, decreasing the diastolic calcium concentration, increasing the sarcoplasmic reticulum calcium load, and preserving the amplitude of the intracellular calcium transient, which was reflected by successful recovery in the process of excitation-contraction coupling during reperfusion. However, these effects of ranolazine did not occur when it was applied during reperfusion or when applied in both ischemia and reperfusion.

Conclusions: Ranolazine shows beneficial effects in cardiomyocytes exposed to ischemia/reperfusion but only when applied during ischemia. This effect is achieved through its improvement of calcium handling during ischemia.

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Palabras clave: Isquemia-reperfusión Cardioprotección Ca2+ Ranolazina
INTRODUCTION

Ischemic heart disease is the leading cause of death in the western world.1 The most devastating expression of this disease is ST-segment elevation myocardial infarction, which is due to acute coronary artery occlusion known to cause ischemic myocardial cell death. After ST-segment elevation myocardial infarction, rapid myocardial reperfusion by thrombolytic therapy or primary percutaneous coronary intervention is the most effective strategy to reduce myocardial infarct size and thus improve clinical outcome.2 Reperfusion therapy has a substantial impact on early mortality after ST-segment elevation myocardial infarction. However, a high percentage (20% to 30%) of patients develops adverse remodeling in our hospital.3 Early and effective reperfusion limits the extent of myocardial necrosis by reducing the incidence of left ventricular remodeling and dysfunction, but even with the best reperfusion therapy, paradoxically, a proportion of cardiomyocytes dies due to restoration of blood flow.4 This phenomenon is called ischemia/reperfusion (I/R) injury.5

Oxygen deprivation and calcium overload during cardiac ischemia and reactive oxygen species production during reperfusion cause cardiomyocyte death by necrosis and apoptosis.6 The major adverse changes that occur in the ischemic myocardi um consist of an increase in intracellular Na+ concentration due to failure of the sarcolemmal Na+/K+ pump in the absence of energy and acidification of cytosol by anaerobic glycolysis. The cell attempts to solve this intracellular Na+ increase through the Na+/Ca2+ exchanger (NCX) acting in the reverse mode, extruding Na+ and introducing Ca2+ inside the cells. This leads to an intracellular Ca2+ overload and a mishandling of Ca2+ by the cells.7

Ranolazine is a piperazine derivative with a novel mechanism of action that was first approved by the Food and Drug Administration in 2006 for the symptomatic treatment of patients with chronic angina. Due to its pharmacological properties, it is able to block the late Na+ current (I_{nat}) in cardiomyocytes and steer the oxidation of fatty acids toward glucose oxidation, making oxygen use more efficient in the heart.8,9 However, the mechanism of action of ranolazine is not yet precisely known. The beneficial effects of ranolazine reside in its action of reducing the Na+ influx into myocardial cells through Na+ channels, which in pathologic situations fail during their inactivation or else they open again.10 Ranolazine has also been suggested to decrease calcium overload in myocardial cells during ischemia by blocking the I_{nat} current.9 At therapeutic plasma concentrations (≤ 10–21 mmol/L), ranolazine selectively inhibits I_{nat}, reduces intracellular accumulation of Na+ and subsequent Na+-induced Ca2+ overload, as well as mechanical, electrical, and metabolic abnormalities in ischemic or insufficient myocardium.11 However, at this concentration, ranolazine does not alter the peak of Na+ current responsible for step 0 of the action potential, the input current of Ca2+, or the activity of NCX and Na+/H+ exchanger.12

Nowadays, the use of ranolazine has been approved as an adjunctive therapy for symptomatic angina in patients who are inadequately controlled with first-line antianginal therapies.12 The development of a substance capable of inhibiting or reducing the deleterious effects of a pathological increase in intracellular Ca2+ concentration in cardiomyocytes during ischemia processes would be a hugely important clinical and therapeutic contribution. In this study, we hypothesized that ranolazine might have a novel action in reperfusion procedures, preventing the Na+ and Ca2+ overload that occurs in ischemic hearts and helping cells to improve Ca2+ handling at reperfusion.

METHODS

Animals were handled in accordance with the recommendations of the Royal Decree 53/2013 in agreement with Directive 2010/63/EU of the European Parliament. The study was approved by the local Ethics Committee on Human Research of the Virgen del Rocío University Hospital of Seville and the Animal Research Committee of the University of Seville.

Isolation of Ventricular Myocytes

We used adult male Wistar rats weighing approximately 250 g to 350 g, which were previously heparinized (4 IU/g intraperitoneally) and anesthetized by intraperitoneal administration of sodium thiopental (1 mL/250 g). The heart was quickly removed and mounted on a Langendorff perfusion system with a constant flow. Ventricular myocytes were isolated by perfusion using type I collagenase (251 IU/mL, Worthington Biochemical; Lakewood, New Jersey, United States).13 Cardiomyocytes were maintained in the Tyrode solution (mM): 140 NaCl, 4 KCl, 1.1 MgCl2, 10 HEPES, 10 glucose, 1.8 CaCl2 (pH 7.4), supplemented with 1.8 mM CaCl2. All experiments were conducted on rod-shaped cells at room temperature (24 °C to 26 °C).

Intracellular Ca2+ Measurement With Microfluorimetry

Intracellular Ca2+ transients were recorded using the imaging system Incyt high speed Im2 (Intracellular Imaging Inc.; Imsol, United Kingdom) in freshly isolated cardiomyocytes loaded with the fluorescence Ca2+ dye, Fura-2AM. During experiments, cells
were continuously superfused with normal Tyrode (mM) or with simulated ischemia (mM): 140 NaCl, 3.6 KCl, 1.2 MgCl₂, 1.8 CaCl₂, 20 HEPES, 20 Lactate-Na and 2 NaCN (pH 6.22). To evoke intracellular Ca²⁺ transients, cardiomyocytes were field stimulated throughout the experiment at 0.5 Hz using 2 parallel platinum electrodes, as previously described.¹⁴

**Intracellular Ca²⁺ Measurement With Confocal Microscopy**

Confocal microscopy experiments were performed in freshly isolated cardiomyocytes loaded with Fluo-3AM. Images were obtained with confocal microscopy (Leica TCS SP2 AOBS, objective W.I. 63 x and N.A. 1.2) by scanning the cell with an Argon laser every 1.54 ms. To evoke intracellular Ca²⁺ transients, the cardiomyocytes were field stimulated at 0.5 Hz, as previously described.¹⁴ The sarcoplasmatic reticulum Ca²⁺ load was estimated by rapid caffeine application in Fluo-3-loaded cardiomyocytes using the Leica SP5, objective 40 x W.I., N.A. 1.2, scanning in the line scan mode at 700 Hz, with a white light laser at 500 nm. In both cases, emission was collected at > 510 nm, as previously described.¹⁴ ¹⁵

**Treatment Protocols**

Group 1: ischemia/reperfusion (I/R). After stabilization with control solution, cells were exposed to simulated ischemia by the perfusion of ischemic solution for 6 minutes, followed by 10 minutes of reperfusion with control solution.

Group 2: ranolazine applied at the onset of reperfusion (I/R+Ran). Cells were exposed to 6 minutes of simulated ischemia and 10 min of reperfusion with the control solution containing 10 µM of ranolazine.

Group 3: ranolazine applied at ischemia and during reperfusion (I+Ran/R+Ran). Cells were exposed to 6 minutes of simulated ischemia in the presence of 10 µM of ranolazine followed by 10 minutes of reperfusion with control solution containing 10 µM of ranolazine.

Group 4: ranolazine applied only during ischemia (I+Ran/R). Cells were exposed to 6 minutes of simulated ischemia with 10 µM of ranolazine followed by 10 minutes of reperfusion with control solution.

Using these protocols, 50% to 60% of cardiomyocytes subjected to I/R protocol showed a significant hypercontraction compared with control cells.

**Data Analysis**

Group data are presented as mean ± (standard error of the mean). The single or paired Student’s t test was used to determine the statistical significance of the data. The significance between multiple groups was evaluated using analysis of variance followed by the Tukey test. Results with a P-value < .05 were considered significant. Drugs were purchased from Sigma–Aldrich.

**RESULTS**

**Intracellular Ca²⁺ Changes During Ischemia-reperfusion**

Changes in intracellular Ca²⁺ were examined in cardiomyocytes subjected to I/R that were field stimulated at 0.5 Hz. Ischemia induced a significant decrease in the amplitude of intracellular Ca²⁺ transients that was partially recovered after reperfusion (Figure 1A). This effect was accompanied by an increase in diastolic intracellular Ca²⁺ level during ischemia and during early reperfusion and was not restored at the end of reperfusion (Figure 1B). As depicted, intracellular Ca²⁺ baseline levels were stable in the control situation, but continued to increase during simulated ischemia, impeding the recovery of Ca²⁺ transient at reperfusion.

**Ranolazine Modulates Intracellular Ca²⁺ During Ischemia-reperfusion**

To check the effect of ranolazine on intracellular Ca²⁺ handling, we first sought to determine the appropriate timing of its application. To do this, isolated adult cardiomyocytes were subjected to a protocol of I/R in which the drug was applied in 3 stages: a) at the time of reperfusion; b) during I/R and c) during ischemia only. First, we applied ranolazine (10 µM) at the onset of reperfusion as a treatment suitable for clinic applications during primary angioplasty and therefore cardiac cells were subjected to simulated ischemia for 6 minutes and were then exposed to 10 minutes of reperfusion with a control solution containing 1.8 mM CaCl₂ and 10 µM ranolazine (Figure 2A). Using this protocol, we observed no improvement in the change of intracellular Ca²⁺. Moreover, the increase of diastolic intracellular Ca²⁺ caused by ischemia further increased with the application of the drug at the time of reperfusion.

Next, to determine whether ranolazine had a greater protective effect when applied during I/R, cardiomyocytes were treated with a solution of simulated ischemia supplemented with 10 µM ranolazine for 6 minutes and were then reperfused with control solution also supplemented with ranolazine for 10 minutes. As a result of this experiment, we observed a progressive and sustained increase in diastolic intracellular Ca²⁺ that did not improve at any time during reperfusion (Figure 2B). Finally, adult cardiomyocytes were subjected to 6 minutes of simulated ischemia solution containing 10 µM ranolazine, followed by 10 min of reperfusion in the absence of the drug. The increase in diastolic intracellular

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**Figure 1.** Effect of ischemia-reperfusion on intracellular Ca²⁺ transients. A: shows the mean trace of changes in intracellular Ca²⁺ transients from several isolated adult cardiomyocytes loaded with the fluorescent indicator, Fura-2AM, and subjected to a protocol of simulated I/R. The cells were stimulated at a frequency of 0.5 Hz. B: shows a representative individual [Ca²⁺], transient recorded in control solution, during ischemia, and after cell reperfusion. I/R: ischemia/reperfusion. The dashed arrow indicates the standard error of the mean, which is shown on the right in Figure A. P < .05.
Figure 2. Effect of ranolazine applied in different experimental groups. Traces show the time course of changes in the mean intracellular Ca²⁺ transients recorded in cardiomyocytes subjected to I/R (blue traces; n = 59) and in cells treated with ranolazine applied at different times (red traces). A: ranolazine (10 μM) was applied at the onset of reperfusion (n = 16). B: ranolazine (10 μM) was added during ischemia and reperfusion (n = 24). C: ranolazine (10 μM) was applied during ischemia only (n = 31). I+Ran/R, ischemia + ranolazine/reperfusion; I+Ran/R+Ran, ischemia + ranolazine/reperfusion + ranolazine; I/R, ischemia/reperfusion; IR+Ran, ischemia-reperfusion + ranolazine. The dashed arrow indicates the standard error of the mean, shown on the right.

Ca²⁺ generated during ischemia decreased significantly at reperfusion with the control solution (Figure 2C). After application of ranolazine during simulated ischemia only, the intracellular Ca²⁺ transient significantly improved at reperfusion in cardiac cells compared with the other protocols (Figure 3). Indeed, the Ca²⁺ overload generated by ischemia was significantly reduced during reperfusion in this group. In addition, the intracellular Ca²⁺ transient amplitude recovered significantly during reperfusion without ranolazine (Figure 3B).

Ranolazine Prevents Intracellular Ca²⁺ Overload by Decreasing Diastolic Intracellular Ca²⁺ in Ischemic Cardiomyocytes

A detailed analysis of diastolic intracellular Ca²⁺ showed that it increased progressively, was higher in the I/R group, and did not recover initial values at reperfusion. Meanwhile, the addition of ranolazine to the ischemic solution produced a similar intracellular Ca²⁺ increase during ischemia, but in this case it was restored to preischemic values at reperfusion without ranolazine (Figure 4A). Treating the cells with ranolazine during ischemia restored the systolic increase of intracellular Ca²⁺ to levels similar to preischemic values (Figure 4B). Diastolic intracellular Ca²⁺ decreased during reperfusion in the I+Ran/R group (105.86% ± 3.07%) and was significantly lower than in the I/R group (116.65% ± 1.83%) (Figure 4C).

Ranolazine Improves [Ca²⁺], Transient Amplitude in Cardiomyocytes Submitted to Ischemia-reperfusion. Records With Confocal Microscopy

To gain greater insight into the cardiomyocyte contraction process, we repeated the experiments by confocal imaging using
the line scan model. By scanning the long axis of the cardiomyocytes, the “rigor” of the cells can be followed as the contractures during diastole. First, we analyzed intracellular Ca\(^{2+}\) transients, which confirmed the results of Ca\(^{2+}\) measurements with microfluorimetry (Figure 5). We observed a marked recovery of intracellular [Ca\(^{2+}\)] transients after submission of cardiomyocytes to the IR protocol, using ranolazine (10 μM) during ischemia and removing it at the beginning of reperfusion (Figure 5A). The bar graph in Figure 5B indicates a significant decrease of the amplitudes of intracellular [Ca\(^{2+}\)] transients that recovered partially during reperfusion. Meanwhile, Figure 5C shows a significant recovery in the amplitude of the intracellular [Ca\(^{2+}\)] transients corresponding to the I+Ran/R protocol. Figure 5C also shows that the amplitude of intracellular [Ca\(^{2+}\)] transients during ischemia was maintained in the presence of ranolazine but not in its absence.

Furthermore, the pattern of diastolic intracellular [Ca\(^{2+}\)] using confocal microscopy showed a significant increase in diastolic intracellular [Ca\(^{2+}\)] during ischemia and a minimum recovery on reperfusion in cells subjected to I/R (Figure 6A). However, compared with the I+Ran/R protocol, the increase in diastolic intracellular [Ca\(^{2+}\)] during ischemia and at reperfusion was lower than values in the I/R group. Finally, ranolazine did not improve cell shortening recovery in cells submitted to the I+Ran/R protocol compared with those subjected to I/R (Figure 6B).

**Ranolazine Increased Sarcoplasmic Reticulum Ca\(^{2+}\) Load but did not Stimulate Na\(^+/\)Ca\(^{2+}\) Exchanger**

Because NCX is very important for Ca\(^{2+}\) homeostasis in adult cardiomyocyte, we tested its involvement in Ca\(^{2+}\) handling during

![Figure 3](http://www.revespcardiol.org:80/)

**Figure 3.** Ranolazine addition during ischemia restored the amplitude of intracellular [Ca\(^{2+}\)] transients. A: traces show the mean for recording of intracellular Ca\(^{2+}\) transients in cardiomyocytes treated with ranolazine applied during ischemia (grey). B: representative recording of an individual intracellular Ca\(^{2+}\) transient showing significant recovery at reperfusion. The dashed arrow indicates the standard error of the mean, shown on the right. I+Ran, ischemia + ranolazine; I+Ran/R, ischemia + ranolazine/reperfusion. *P < .05.

![Figure 4](http://www.revespcardiol.org:80/)

**Figure 4.** Ranolazine restored the diastolic intracellular Ca\(^{2+}\) transients at reperfusion after ischemia. A: graphic representation of changes in diastolic intracellular Ca\(^{2+}\) during the experiment in cardiomyocytes submitted to I/R and cardiomyocytes submitted to I+Ran/R. The concentration of diastolic Ca\(^{2+}\) recovered significantly in cells treated with ranolazine. B: the bar graph summarizes the amplitude of end diastolic intracellular Ca\(^{2+}\) in control solution, I/R (n = 59), and I+Ran/R (n = 30). C: the bar graph summarizes the amplitude of end diastolic intracellular Ca\(^{2+}\), in control solution, I/R (n = 59), and I+Ran/R (n = 30). I+Ran/R, ischemia + ranolazine/reperfusion; I/R, ischemia/reperfusion; NS, not significant. *P < .05.
I+Ran/R. For this purpose, we used I+Ran/R and, 3 minutes after reperfusion, we applied 10 mM of caffeine. In the presence of caffeine, we studied the tau of recovery to baseline Ca²⁺, which was mainly due to Ca²⁺ extrusion through the NCX. Ranolazine did not alter this parameter, suggesting that the NCX exchanger is not involved in the intracellular Ca²⁺ enhancement by ranolazine during ischemia (Figure 7A). However, the analysis of sarcoplasmic reticulum Ca²⁺ load, estimated by caffeine-evoked intracellular Ca²⁺ transients, indicates that it was higher in cells treated with ranolazine compared with untreated cells, indicating that ranolazine promoted sarcoplasmic reticulum load in cardiomyocytes (Figure 7B).

**DISCUSSION**

Since the early 1950s, intensive research has been conducted in the field of myocardial protection, the main objective being to characterize the cellular and molecular mechanisms involved in myocardial protection after the myocardium has undergone a process of I/R. First, in a previous study, we confirmed the feasibility of our experimental I/R model for isolated cardiomyocytes, in which we were able to analyze in vivo structural and intracellular Ca²⁺ changes that occur in the process of I/R. Next, in the present study, we provide new evidence that ranolazine, which is widely used for antianginal therapies, can protect cardiomyocytes against I/R injury through regulation of intracellular Ca²⁺ handling independently of its effect on I₅₅₅. Using 2 different approaches to analyze intracellular Ca²⁺ changes, we studied changes in intracellular Ca²⁺ in cardiomyocytes subjected to ischemia and/or reperfusion and the role of ranolazine, which is able to reduce the Ca²⁺ overload generated in I/R processes. In this study, we confirm that application of ranolazine during myocardial ischemia significantly restores the amplitude of intracellular Ca²⁺ transients in reperfusion. Moreover, we not only noticed improvement in the amplitude of the transients but also observed that diastolic intracellular Ca²⁺, which remained elevated throughout ischemia, returned to baseline levels after treatment with...
ranolazine during ischemia. These data are consistent with studies conducted by Hwang et al.\textsuperscript{17} in Langendorff-perfused rat hearts, in which ranolazine was applied prior to ischemia. These authors observed that intracellular Ca\textsuperscript{2+} transients, abolished by the interruption of left ventricular function after a certain period of ischemia, were rapidly restored during posts ischemic reperfusion with ranolazine.\textsuperscript{17} However, in this study, residual intracellular Ca\textsuperscript{2+} remained significantly high compared with baseline values prior to ischemia induction.\textsuperscript{17} Furthermore, in this study, the analysis of caffeine-evoked intracellular Ca\textsuperscript{2+} transients suggested that ranolazine increased the efficiency of the sarcoplasmic reticulum Ca\textsuperscript{2+} load, which is essential to preserve efficient excitation-contraction coupling in cardiomyocytes after an ischemic period. Moreover, the analysis of the decay time constant of the intracellular Ca\textsuperscript{2+} transient (tau) showed that ranolazine treatment during ischemia did not enhance intracellular Ca\textsuperscript{2+} extrusion via the NCX. Thus, NCX seemed not to be implicated in the diastolic intracellular Ca\textsuperscript{2+} increase in reperfusion when cells were treated with ranolazine during ischemia. Therefore, alternative mechanisms must be involved in this effect of ranolazine on intracellular Ca\textsuperscript{2+}, which are worth investigating.

Most previous studies have applied ranolazine prior to ischemia.\textsuperscript{17,18} Here, we applied the drug once ischemia had been started and/or at reperfusion. We aimed to reproduce what might happen in the clinical scenario when drugs should be administered at the onset of reperfusion. Significant beneficial effects on intracellular Ca\textsuperscript{2+} regulation were only observed when ranolazine was applied during ischemia, which might limit its use for clinical application in patients with ST-segment elevation myocardial infarction. However, we believe that it is still important to understand these effects on the Ca\textsuperscript{2+} homeostasis of cardiomyocytes in I/R.

In recent decades, there has been an explosion of information regarding changes in contractility following I/R myocardial syndrome, as in the case of processes known as “stunned myocardium” (stunning) and hibernating heart.\textsuperscript{19,20} Ca\textsuperscript{2+} plays a central role in the regulation of contraction and heart rate and has
been associated with prevailing heart disease, directly or indirectly, and to changes in the behavior of intracellular Ca2+ 21-23. The intracellular Ca2+ overload causes an increase in diastolic interaction of the myofilaments actin/myosin and increased left ventricular diastolic pressure (eg, “stiffness”, inability to relax normally). This diastolic disorder occurs due to prolongation of action potentials and slowing of biochemical pumps, which are necessary for the reuptake of intracellular calcium. 24,25 As a result, contractile myocardial work, oxygen consumption, and the compression of the vascular space during diastole may be abnormally high. The compression of the vascular space leads to a reduction of myocardial blood flow that decreases oxygen supply, especially in the subendocardial region of the left ventricle, while increasing the demand for oxygen to support contractile work. This pattern of cause and effect has the characteristics of a deleterious positive “feedback” system, in which ischemia generates further ischemia. 26,27 With the experiments performed in our research, we demonstrated that intracellular diastolic intracellular Ca2+ recovered significantly in adult rat cardiomyocytes subjected to a protocol of I/R when we applied ranolazine during ischemia. These data are in line with the results of other studies, 18,28 in which the effect of ranolazine occurred with the inhibition of the bK, using a model of a full heart. Ranolazine reduced the overload of intracellular Ca2+, returning postischemic diastolic intracellular Ca2+ values close to baseline preischemic levels. Sossella et al29 studied the potential effect of ranolazine on diastolic intracellular Na+ and Ca2+ overload and its ability to improve diastolic function in the muscle fibers of ventricles of human hearts from patients with end-stage heart failure undergoing heart transplantation. 29, Furthermore, to investigate the beneficial effect of ranolazine on diastolic pressure, they used Anemona sulcata toxin II (40 nM) to increase intracellular Na+ concentration in rabbit ventricular myocytes. In the presence of ranolazine, bK current, as well as diastolic intracellular Na+ and intracellular Ca2+, decreased in all pacing rates. In addition, ranolazine significantly accelerated the decline of the Ca2+ transient, which was initially slowed by A. sulcata toxin II. 30

CONCLUSIONS

In conclusion, our results clearly show that, when administered during ischemia, ranolazine protects isolated adult rat cardiomyocytes from damage by I/R, improving the excitation-contraction coupling process and decreasing Ca2+ overload in reperfusion, which is of major interest to preserve heart contractility from I/R injuries.

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CONFLICTS OF INTEREST

None declared.

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