Pre-treatment with trimetazidine increases sarcolemmal mechanical resistance in reoxygenated myocytes

Marisol Ruiz-Meana *, David Garcia-Dorado, Margarita Juliá, Miguel A. González, Javier Inserte, J. Soler-Soler

Servicio de Cardiología, Hospital Universitari Vall d’Hebron, Paseo Vall d’Hebron 119 – 129, Barcelona 08035, Spain

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Abstract

Objective: Cytoskeletal and sarcolemmal fragility secondary to anoxia may contribute to sarcolemmal rupture and cell death during reoxygenation of cardiomyocytes. This study investigated the influence of trimetazidine (TMZ), a drug with effects on lipid metabolism and cell membranes, on reoxygenation-induced sarcolemmal rupture. Methods: Isolated adult rat myocytes were submitted to 60 min of metabolic inhibition and 5 min of hypo-osmotic reoxygenation to simulate reperfusion edema in situ. Cells were allocated to 3 groups of treatment: in one group, TMZ 100 μmol/l was added to both the metabolic inhibition and reoxygenation buffers (group TMZ); another group was submitted to the same treatment but cells had previously been incubated with TMZ 100 μmol/l for 3 h (group TMZ-Pre); a control group underwent metabolic inhibition and hypo-osmotic reoxygenation without any treatment. Cell morphology was monitored throughout the experiment and sarcolemmal integrity was assessed by quantification of LDH activity and trypan blue exclusion test.

Results: After 60 min of metabolic inhibition most cells (83.1 ± 2%) presented rigor contracture without between-group differences. Reoxygenation resulted in hypercontracture of 84.2 ± 2.3, 91.2 ± 1.4 and 84.1 ± 2.1% of cells in TMZ, TMZ-Pre and control groups, P = NS. The trypan blue exclusion test revealed a higher proportion of cells with sarcolemmal integrity in TMZ and TMZ-Pre groups than in controls (12.7 ± 2.0, 10.0 ± 1.5 and 6.3 ± 0.8%, respectively, P = 0.002). No between-group differences in LDH activity in the extracellular medium were observed at the onset or at the end of metabolic inhibition. However, LDH release was significantly lower (P = 0.002) in the TMZ-Pre group (1.6 ± 0.1 IU/1000 cells) than in the TMZ and control groups (1.9 ± 0.2 and 2.2 ± 0.1 IU/1000 cells). Conclusion: Preincubation of cardiomyocytes with TMZ does not prevent rigor contracture induced by metabolic inhibition or hypercontracture during subsequent reoxygenation, but does improve sarcolemmal resistance to reoxygenation-induced mechanical stress. This could help to explain the beneficial effect of TMZ on infarct size.

Keywords: Sarcolemma; Cytoskeleton; Osmotic swelling; Reoxygenation; Reperfusion; Cell viability; Myocardial ischemia; Myocardial infarction

1. Introduction

Anoxia, ischemia and metabolic inhibition reduce the mechanical resistance of the sarcolemma and the cytoskeleton of myocytes [1,2]. The mechanical weakness induced by ischemia can be evidenced as an increase in osmotic fragility. During myocardial reperfusion, swelling and hypercontracture impose severe mechanical stress on the myocyte sarcolemma and cytoskeleton which may cause sarcolemmal disruption and cell death [3–5]. In a previous study we have shown that swelling and hypercontracture may cooperate to disrupt the sarcolemma during reoxygenation, but only in myocytes with anoxia-induced mechanical fragility [3]. The mechanism responsible for sarcolemmal fragility is not known. It has been suggested that changes in the phosphorylation state or hydrolysis of some cytoskeleton proteins could play a role [6,7]. Recent observations suggest that fragility could be at least in part mediated by changes in the lipid structure of the sarcolemma, reducing its mechanical resistance [8].

* Corresponding author. Tel. (+34-3) 4183400 (ext. 4948); fax: (+34-3) 4284301; e-mail: dgdorado@ar.vhebron.es

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Trimetazidine (1-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride, TMZ) has been shown to have a protective effect against ischemic injury without associated central hemodynamic changes [9], and to be able to limit myocardial necrosis after transient coronary occlusion [10]. However, the mechanism of this action is not well understood. Recent studies suggest that TMZ may preserve lipid metabolism [11], and modify the physico-chemical properties of cell membranes [12].

In this study we investigated the hypothesis that the beneficial effect of TMZ on infarct size is due, at least in part, to a protective effect against the sarcolemmal mechanical fragility induced by anoxia. To test this hypothesis, sarcolemmal integrity was assessed in isolated myocytes submitted to transient metabolic inhibition followed by hypo-osmotic reoxygenation in the presence or the absence of the drug.

2. Methods

Animals were handled according to the recommendations of the Declaration of Helsinki and the experimental procedures were approved by the Research Commission of the Hospital General Universitari Vall d'Hebron. Ventricular heart muscle cells were isolated from adult male Sprague-Dawley rats (250–300 g) as previously described [13]. The animals were deeply anesthetized with barbiturates (thiopental 100 mg/kg i.p.). Whole hearts were perfused in a Langendorff system with a modified Krebs buffer (NaCl 110 mM, KCl 2.6 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25 mM, glucose 11 mM) nominally free of calcium and with collagenase 0.03% (type II, SERVA, Germany) to promote cell dissociation. After filtering dissociated tissue, cells were subjected to two centrifugation steps and to a progressive normalization of calcium levels to a final concentration of 1 mM. Rod-shaped cells were selected by means of a 4% bovine serum albumin gradient (fraction V, Boehringer Mannheim, Germany), and plated in medium 199/HEPES (Sigma, St Louis, USA) with 4% fetal calf serum (Gibco, USA) in Falcon dishes preincubated overnight as described previously [13]. Three hours after plating, the cells became attached to the surface of the dishes and a population of 75% rod-shaped cells was selected by changing the medium and washing out broken cells.

2.1. Experimental protocol

Isolated myocytes were incubated for 60 min in a metabolic inhibition buffer (NaCl 140 mM, KCl 3.6 mM, MgSO₄ 1.2 mM, CaCl₂ 1 mM and HEPES 20 mM, pH 7.4). Three study groups were used (Fig. 1). In a control group, 5 min of hypo-osmotic reoxygenation was applied at the end of the metabolic inhibition period by changing to a 80 mOsm NaCN-free buffer (NaCl 24.2 mM, KCl 3.6 mM, MgSO₄ 1.2 mM, CaCl₂ 1 mM and HEPES 20 mM). In TMZ-treated cells, the drug was added to the medium at two different timepoints of the experimental protocol: in one group, TMZ 100 μmol/l in the metabolic inhibition and reoxygenation buffers; TMZ-Pre = the same as TMZ plus preincubation with TMZ 100 μmol/l for 3 h before metabolic inhibition; LDH = lactate dehydrogenase determination.

![Fig. 1. Study design. MI = metabolic inhibition; Reox = hypo-osmotic reoxygenation; TMZ = TMZ 100 μmol/l in the metabolic inhibition and reoxygenation buffers; TMZ-Pre = the same as TMZ plus preincubation with TMZ 100 μmol/l for 3 h before metabolic inhibition; LDH = lactate dehydrogenase determination.](https://academic.oup.com/cardiovascres/article-abstract/32/3/587/353054/15)
and TMZ-Pre protocols, respectively. In these experiments, the length of the rod-shaped cells, square-shaped cells and hypercontracted cells, respectively, was measured at the onset of metabolic inhibition, after 60 min of metabolic inhibition and after reoxygenation.

2.3. Enzyme release

Lactate dehydrogenase (LDH) release was used as an index of sarcolemmal integrity [16]. LDH assay was performed by spectrophotometric analysis (SLT Spectra Vision, Austria) of supernatant samples (10 µl) taken before the onset of metabolic inhibition, at the end of metabolic inhibition and after 5 min of reoxygenation. Results on LDH release were normalized as IU of LDH activity/1000 cells.

2.4. Statistical analysis

A total of 168 culture dishes were used in this study. The number of dishes per group of treatment ranged from 7 to 64. Data were analyzed by using commercially available software (SPSS/PC + V4.0, Chicago, IL). Comparisons involving multiple groups were performed by means of analysis of variance (ANOVA). Statistical comparisons between groups were performed by means of the Duncan test only if homogeneity was rejected at the ANOVA test. A critical P-value of 0.05 was used for all tests. All values are expressed as mean ± s.e.m.

3. Results

3.1. Cell morphology

Immediately before metabolic inhibition, the total number of cells per well averaged 52797 ± 2261, with a high proportion of rods (74.9%), without between-group differences. Metabolic inhibition induced rigor contracture of virtually all rod-shaped myocytes. This process was however somewhat slower when TMZ was present in the metabolic inhibition buffer than in controls, as shown in Fig. 2. This delaying effect exerted by TMZ on rigor contracture development was not statistically significant. After 60 min of metabolic inhibition, the number of cells preserving the rod shape was the same in TMZ-treated cells as in controls (3.6 ± 0.5 and 3.2 ± 0.5%, respectively, P = NS). The magnitude of the reduction in cell length imposed by rigor contracture was also identical in TMZ-treated cells and controls (Fig. 3).

Reoxygenation induced hypercontracture of virtually all myocytes presenting rigor at the end of the metabolic inhibition period. The percent of hypercontracted cells after reoxygenation increased from 12.9 ± 1.5, 12.1 ± 1.3 and 8.4 ± 1.1 (P = 0.2) to 84.1 ± 2.1, 84.2 ± 2.3 and 91.2 ± 1.4 (P = 0.16) in the control, TMZ and TMZ-Pre groups, respectively. The reduction in cell length associated with hypercontracture was also the same in TMZ-treated cells as in controls (Fig. 3). The trypan blue exclusion test showed that the proportion of hypercontracted cells which excluded the dye (i.e., which retained sarcolemmal integrity), although low in all 3 groups, was almost twice as large in the TMZ and TMZ-Pre groups as in control cells (P = 0.0016, Fig. 4).

3.2. Enzyme release

Results on LDH measurements are summarized in Fig. 5. LDH concentration was minimal in samples obtained at the onset of metabolic inhibition (0.43 ± 0.04 IU/1000 cells).
cells) without between group differences (P = 0.95). A small (0.87 ± 0.08 IU/1000 cells) and homogeneous (P = 0.15) LDH release occurred during the 60 min of metabolic inhibition. Hypo-osmotic reoxygenation, however, elicited a rapid and marked release of LDH, which was significantly smaller in cells pretreated with TMZ than in controls (1.6 ± 0.1 vs. 2.2 ± 0.1 IU/1000 cells, respectively, P = 0.002), while an intermediate effect was observed in cells exposed to TMZ during metabolic inhibition and reoxygenation only (Fig. 5). In additional experiments using 90 min of metabolic inhibition there were no differences in LDH release between control and TMZ-Pre cells at the onset of metabolic inhibition (0.6 ± 0.1 vs. 0.6 ± 0.1, P = NS) or after 90 min of metabolic inhibition (1.2 ± 0.3 vs. 1.5 ± 0.3, P = NS). After 5 min of reoxygenation a non-significant trend towards a reduction in LDH release in the TMZ-Pre group was observed.

4. Discussion

This study investigated the effect of TMZ on the mechanical resistance of the sarcolemma of cardiomyocytes submitted to osmotic stress during reoxygenation. Preincubation with TMZ did not modify the number of myocytes hypercontracting during reoxygenation, but reduced the number of myocytes presenting sarcolemmal disruption upon hypercontraction, as indicated by the trypan blue test and enzyme release measurements. These results are in agreement with the hypothesis that TMZ exerts a direct protective action against mechanical fragility induced by metabolic inhibition.

Several studies have suggested a direct anti-ischemic effect of TMZ on myocardial cells, but the mechanism of this action is not well characterized. The drug has no effect under normoxic conditions [9] and its beneficial effect during ischemia or hypoxia has been related to protection against the reduction of energetic compounds [17], the accumulation of protons [18] and the generation of oxygen-derived free radicals [19]. Perfusion of isolated rat hearts submitted to partial global ischemia with TMZ 6 × 10⁻⁷ mol/l resulted in acceleration of reconstitution of energy pools during subsequent reperfusion [17]. The protective effect against acidosis was also observed in experiments carried out by Renaud [18] using primary cultures of cells from chick embryonic heart and rat heart. Boucher et al. [20] observed that TMZ 10⁻⁶ mol/l reduced ischemic contracture in isolated perfused rat hearts submitted to 30 min subtotal ischemia.

In the present study, TMZ produced a small delay in the rate of development of rigor contracture during the first minutes of metabolic inhibition. This observation is consistent with some effect of TMZ on ATP levels [21]. However, at the end of the metabolic inhibition period virtually all cells had developed rigor contracture independently of treatment allocation. This obviates a potential role of ATP preservation in the beneficial effect of TMZ on sarcolemmal integrity during subsequent reoxygenation. TMZ had no beneficial effect on LDH release during metabolic inhibition. A trend towards a larger LDH release after 60 min of metabolic inhibition was found in the TMZ-Pre group as compared to the TMZ group, and a similar trend was observed after 90 min of metabolic inhibition in the TMZ-Pre as compared to the control group.

The rate and magnitude of reoxygenation-induced hypercontracture were not affected by TMZ. The observed beneficial effect of TMZ on sarcolemmal integrity cannot be explained, therefore, by a reduction in mechanical stress, but by a direct effect on the mechanical resistance of the sarcolemma. This is in agreement with previous observations showing that TMZ may modify lipid
metabolism during hypoxia [11]. Lipidic catabolites produced during ischemia may modify the physico-chemical properties of cell membranes [22]. TMZ has been shown to selectively increase the anisotropy (i.e., to reduce the fluidity) of the outer layer of the plasma membrane in normoxic human platelets [12]. The increase in sarcolemmal resistance observed in the present study could therefore be related to a protective effect of TMZ on membrane dynamics during energy deprivation. The less marked protective effect of TMZ observed when metabolic inhibition was prolonged to 90 min suggests that this drug delays, but does not prevent, the development of sarcolemmal fragility during energy deprivation.

Alternatively, TMZ could have protected the sarcolemma against reoxygenation-induced fragility. Previous studies in the present model of hypo-osmotic reoxygenation have shown that addition of the anti-lipid peroxidant, diphenylphenylenediamine (DPPD), to the reoxygenation buffer resulted in less sarcolemmal rupture and enzyme release during reoxygenation-induced hypercontracture [8]. Some studies suggest that TMZ reduces oxygen free radical generation [23] and oxidative damage [24] during myocardial anoxia-reoxygenation. Previous unpublished observations showing that TMZ when added only during reoxygenation has no protective effect against sarcolemmal rupture refute this hypothesis. Under these conditions, LDH release during metabolic inhibition and during hypoosmotic reoxygenation was 1.2 ± 0.22 and 1.94 ± 0.44 IU/1000 cells, respectively, in the control group, and 0.98 ± 0.16 and 1.94 ± 0.08 IU/1000 cells, respectively, in the group in which TMZ (at the same concentration used in this study) was added to the reoxygenation buffer (P = NS). Altogether these observations and those from the present study suggest a protective effect of TMZ against the development of sarcolemmal fragility during metabolic inhibition. This protective effect is consistent with the described actions of TMZ on lipid metabolism [11] and cell membranes [12].

4.1. Methodological considerations

In the isolated myocyte model, cells can undergo hypercontracture without losing sarcolemmal integrity unless osmotic stress is applied during reoxygenation [3,25]. Thus, in the present studies osmotic stress was applied to all groups of treatment during reoxygenation to evaluate the effect of TMZ on the mechanical resistance of the sarcolemma. In the in situ heart, swelling and cell-to-cell mechanical interaction may impose severe stress and cause sarcolemmal rupture in non-hypercontracted myocytes [26]. The favorable effects of reducing reperfusion edema by means of hyperosmotic reperfusion is in agreement with this hypothesis [27,28]. Preservation of sarcolemmal mechanical resistance during ischemia-reperfusion could thus have a favorable effect on myocardial necrosis secondary to transient coronary occlusion and reperfusion.

5. Conclusion

This study suggests that TMZ has a protective effect against sarcolemmal fragility induced by anoxia-reoxygenation. This effect could help to explain the favorable action of TMZ on myocardial necrosis secondary to transient coronary occlusion and reperfusion.

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