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Trimetazidine protects isolated rat hearts against ischemia-reperfusion injury in an experimental timing – dependent manner

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Abstract The present study investigated the tolerance of the isolated rat heart to ischemia–reperfusion after administration of trimetazidine (TMZ) at different experimental phases, as well as the possible involvement of p38 MAPK and JNKs in this response. Isolated rat hearts were perfused in Langendorff mode. Untreated hearts after stabilization (S) were subjected to 20 min of zero-flow global ischemia (I) and 45 min of reperfusion (R), (NORM), n = 9. TMZ (10^{-5} M) was administered (in the perfusate): a) only at S phase, (TMZ-STAB), n = 8, b) only at R, (TMZ-REP), n = 8 and c) during both S and R, (TMZ-STAB+REP), n = 8. Recovery of left ventricular developed pressure at 45 min of R (Rec) was significantly higher in TMZ-STAB and TMZ-STAB+REP and LDH release was lower in TMZ-STAB+REP and TMZ-STAB than NORM, [1153.2 (121.0) and 1152.1 (86.8) vs 1573.5 (138.2), $P < 0.05$]. TMZ induced cardioprotection did not involve p38 MAPK and JNKs. Phospho-p38 MAPK and JNKs levels after I/R were not changed with TMZ treatment. In TMZ-REP, Rec and LDH release were similar to NORM, but the rate of functional recovery (ratio of Rec at 10 min of R to Rec) was 86.7% (13.3) for TMZ-REP vs 53.8% (7.7) for NORM, $P < 0.05$. This effect was associated with decreased myocardial lactate content early at reperfusion. In conclusion, preischemic administration of TMZ protects against I/R injury while TMZ given only at reperfusion accelerates recovery of function without reducing the extent of injury.

Key words Trimetazidine – ischemia-reperfusion injury – metabolism – MAPK kinases

Introduction

Over the past years, the better understanding of the pathophysiology of ischemia-reperfusion injury has allowed the development of effective therapeutic strategies in limiting myocardial necrosis. Various interventions that target the preischemic period and/or reperfusion have been investigated for their efficacy in preventing myocardial injury after an ischemic insult [21, 32]. Among the most effective experimental strategies are included interventions such as ischemic preconditioning and its pharmacological mimicking [32]. Furthermore, since myocardial ischemia is essentially a metabolic event, recent research has focused on the efficacy of interventions that target metabolism. A number of dif-

ferent approaches have been used to manipulate energy metabolism [5, 30]. Interventions targeting metabolic substrates, such as the preischemic myocardial glycogen content or various steps of cardiac metabolism, have been shown to increase tolerance of the myocardium to ischemia [22, 23, 26]. In fact, optimizing energy metabolism by stimulating glucose metabolism seems to be a potential strategy to protect the heart from ischemia-reperfusion [14]. This can be achieved by direct stimulation of the rate limiting enzyme for glucose oxidation – the pyruvate dehydrogenase complex or indirectly by switching metabolism from fatty acid to glucose metabolism [14]. The latter seems to be very important particularly in the setting of ischemia-reperfusion since glucose metabolism requires less oxygen than fatty acids in order to produce an equivalent amount of ATP. A number of pharmacologic agents that can be cardioprotective by optimizing energy metabolism are now available [14].

Trimetazidine is one of the first, well-studied metabolic agents. Its action on cardiac metabolism is thought to be mediated by partial inhibition of long-chain 3-keto acyl-CoA thiolase (3-KAT), shifting fatty acid beta-oxidation to glucose oxidation. The latter provides a better energy yield since it consumes less oxygen than beta-oxidation for a given amount of ATP produced. Its anti-ischemic efficacy has been demonstrated in experimental studies in the presence or absence of additional exogenous free fatty acids in the perfusate [29].

Trimetazidine has been shown to have several other cellular actions. In fact, trimetazidine is shown to limit changes in intracellular sodium and pH during ischemia-reperfusion, it prevents the intracellular accumulation of Na^+ and Ca^{2+} in the myocyte, and has anti-inflammatory and antioxidant effects [17]. A potential modulatory effect on intracellular cardioprotective signaling could also be involved in trimetazidine-induced cardioprotection against ischemia-reperfusion. This issue has not been previously addressed. Several pharmacological agents are now shown to be cardioprotective either by inhibition of the activation of proapoptotic kinases, such as p38 mitogen activated protein kinase (p38 MAPK) and c-jun N-terminal kinases (JNKs) or upregulation of anti-apoptotic kinases, such as ERK or PI3K-Akt kinases [2, 7]. On the basis of this evidence the present study investigated whether trimetazidine-induced cardioprotection can be associated with changes in ischemia-reperfusion induced activation of p38 MAPK and JNKs. In addition, this study has been extended to explore possible cardioprotective effects of trimetazidine administration during the reperfusion phase. Numerous studies now emphasize the importance of reperfusion interventions in limiting ischemic injury [7], but this issue has been poorly addressed with regard to trimetazidine.

Methods

Animals

Thirty-three Wistar male rats (280–330 g) were used for this study. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996).

Isolated heart preparation

Rats were anesthetized with intraperitoneal injection of ketamine hydrochloric acid (150 mg/kg) and heparin (1000 IU/kg) was given intravenously before thoracotomy. Hearts were rapidly excised, placed in ice-cold Krebs-Henseleit buffer (composition in mM: sodium chloride 118, potassium chloride 4.7, potassium phosphate monobasic 1.2, magnesium sulfate 1.2, calcium chloride 1.4, sodium bicarbonate 25, and glucose 11) and then were perfused in a Langendorff mode. Perfusion with oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer was established within 60 s after thoracotomy. The perfusion apparatus was heated to ensure a temperature of 37 °C throughout the experiments. Hearts were paced at 320 beats per min with a Harvard pacemaker. The pacemaker was turned off during the period of ischemia. A water-filled balloon, connected to a pressure transducer, was advanced into the left ventricle through an incision in the left atrium.

During the stabilization period, flow was adjusted to attain perfusion pressure of 70 ± 2 mmHg and thereafter constant-flow perfusion was applied. Left ventricular balloon volume was adjusted to produce an average initial left ventricular end-diastolic pressure of 6 mmHg in all groups and was held constant thereafter throughout the experiment. Since the balloon was not compressible, left ventricular contraction was isovolumic. As intraventricular volume was maintained at a constant value, diastolic fiber length, which represented preload, did not change. Thus, the left ventricular peak systolic pressure and the left ventricular developed pressure (LVDP), defined as the difference between left ventricular peak systolic pressure and left ventricular end-diastolic pressure, represent contractility indices obtained under isometric conditions rather than just hemodynamic parameters [22–24].

Trimetazidine administration

Trimetazidine (1-[2,3,4-trimethoxybenzyl] piperazine dihydrochloride) was diluted in Krebs-Henseleit buffer to a concentration of 10^{-5} M. Pilot studies showed that, at

this particular dose, trimetazidine had an effect on ischemia-reperfusion, whereas at a lower dose of 10^{-6} M, although a trend towards an improved postischemic functional recovery was observed, this did not reach statistical significance.

Experimental protocols

Isolated hearts were subjected to 20 min of stabilization, which was followed by 20 min of global, zero-flow ischemia and 45 min of reperfusion. The following experimental groups were formed based on the time of trimetazidine administration.

- Trimetazidine was not administered (NORM), n = 9.
- Trimetazidine was administered only during stabilization (TMZ-STAB), n = 8.
- Trimetazidine was administered during both stabilization and reperfusion (TMZ-STAB+REP), n = 8.
- Trimetazidine was administered only during the reperfusion period (TMZ-REP), n = 8.

The experimental protocols are shown in Fig. 1.

At the end of the reperfusion period, hearts were frozen in liquid nitrogen and were kept at –80 °C in order to be used for determination of p38 MAPK and JNKs activation.

Total protein preparation, SDS-PAGE and immunoblotting

Protein analysis was performed as previously described. Briefly, 0.2 g of frozen tissue was homogenized in ice-cold Tris-sucrose buffer (0.35 M sucrose, 10 mM Tris-HCl pH = 7.5, 1 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride) and the resulting homogenate was centrifuged at 15000 g for 20 min at 4 °C. Protein concentrations were determined by the bicinchoninic acid (BCA) method using Bovine Serum Albumin (BSA) as a standard.

After boiling for 5 min in Laemmli sample buffer, protein aliquots (40 µg) were loaded onto 10% or 12% (w/v) acrylamide gels and subjected to SDS-PAGE. After Western blotting, filters were probed with specific antibodies against dual phospho-p38 MAPK, total p38 MAPK (1:1000, New England Biolabs), phospho-JNKs, total JNKs (1:1000, New England Biolabs) and immunoreactivity was detected by enhanced chemiluminescence. Immunoreactivity for phospho-p38 and phospho-JNK was expressed as the ratio of their optical densities to total p38 MAP kinase and JNK optical densities, respectively. Immunoblots were quantified using the Alpha Scan Imaging Densitometer (Alpha Innotech Corporation, 14743, Catalina Street, San Leandro, CA). Five samples from each group were used for phosphorylated p38 MAPK and JNKs determination.

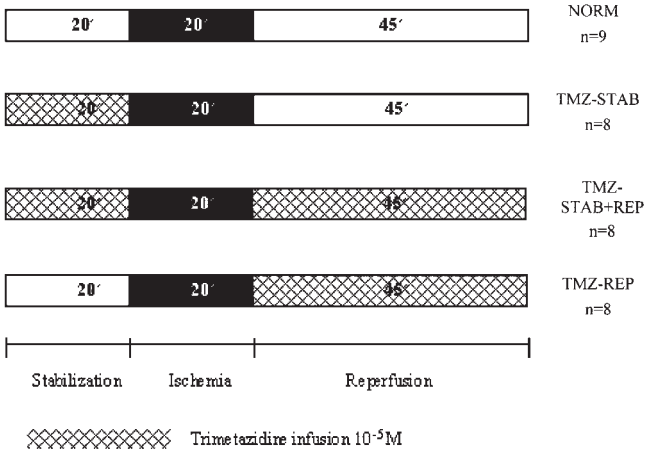

Fig. 1 Experimental protocols

Measurement of mechanical function

Left ventricular systolic function was assessed by recording LVDP (mmHg) which was measured at the end of the stabilization period and at 10, 15, 20, 25, 30, 35, 40 and 45 min of reperfusion. At baseline, the positive and negative first derivative of left ventricular developed pressure (+dp/dt and –dp/dt in mmHg/s) were also measured. Recovery of LVDP at 45 min of reperfusion was expressed as % of the initial value (Rec). The rate of postischemic functional recovery was assessed as the % ratio of postischemic recovery at 10 min of reperfusion to that of 45 min (Rec10/Rec).

Lactate dehydrogenase (LDH) activity measurement

Coronary effluent was collected at 3, 5, 10, 20, 30 and 45 min of reperfusion and was used for the measurement of LDH activity in IU/l (LDH UV Fluid, Rolf Greiner Biochemica, Germany). The total amount of LDH activity (LDHint, in IU/l min] was assessed by integrating the area under the LDH-time curve during reperfusion and was used as an index of necrosis.

Myocardial lactate measurement

Isolated hearts were subjected to 20 min of stabilization, which was followed by 20 min of global, zero-flow ischemia and 10 min of reperfusion either with or without trimetazidine administration (10^{-5} M). Left ventricular tissue was extracted in 40 mM potassium acetate (pH 4.8). Homogenates were centrifuged at 12000 g for 10 min at 4 °C. Myocardial lactate content (in mg/g left

ventricular weight) was measured in the supernatant spectrophotometrically at 340 nm, using the L-lactic acid assay kit (Boehringer Mannheim, Enzymatic Bioanalysis, Darmstadt).

Statistics

Values are presented as mean (s.e.m.). One-way analysis of variance with Bonferroni correction was used when multiple comparisons were carried out. Unpaired t-test was used to test for differences between groups. A two-tailed test with a P value less than 0.05 was considered significant.

Results

Cardiac function at baseline

Left ventricular developed pressure (LVDP) and the positive and negative first derivative of LVDP (+dp/dt, –dp/dt) at the end of the stabilization period for the four groups are shown in Table 1. Trimetazidine administration did not alter the baseline functional parameters. LVDP, +dp/dt and –dp/dt were similar in all groups, $P > 0.05$.

Cardiac function after ischemia and reperfusion

Recovery of LVDP at several time points during reperfusion are shown in Fig. 2. The recovery at 45 min of reperfusion (Rec) was higher in TMZ-STAB+REP and TMZ-STAB hearts compared to NORM hearts, [48.2 (4.9) and 48.4 (4.0) vs 35.29 (3.4) respectively, $P < 0.05$]. Rec was similar between TMZ-REP and NORM hearts [37.34 (6.97) vs 35.29 (3.4) respectively, $P > 0.05$]. However, the rate Rec10/Rec was higher in TMZ-REP as compared to NORM hearts [86.7% (13.3) vs 53.8% (7.7), $P < 0.05$)].

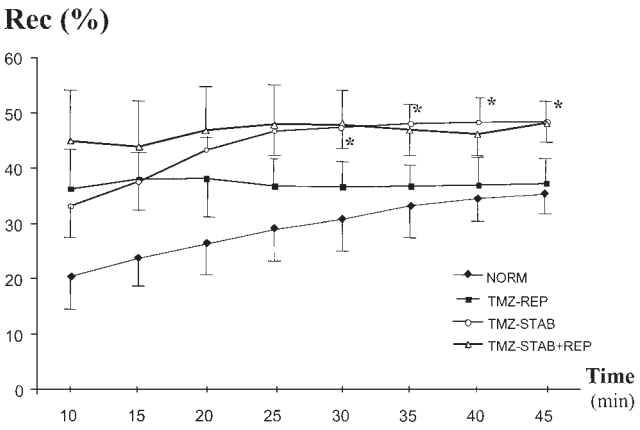

Fig. 2 Recovery of left ventricular developed pressure (Rec) at 10, 15, 20, 25, 30, 35, 40 and 45 min of reperfusion in normal hearts (NORM), trimetazidine-treated hearts only during stabilization (TMZ-STAB), trimetazidine-treated hearts during stabilization and reperfusion (TMZ-STAB+REP) and trimetazidine-treated hearts only during reperfusion (TMZ-REP). *P < 0.05 TMZ-STAB vs NORM, †P < 0.05 TMZ-STAB+REP vs. NORM

Table 1 Left ventricular developed pressure at baseline (LVDP in mmHg), at 10 min and 45 min of reperfusion (LVDP10, LVDP45 in mmHg) and the positive and negative derivative of LVDP (+dp/dt and –dp/dt in mmHg/s) at baseline in normal hearts (NORM), trimetazidine-treated hearts only during stabilization (TMZ-STAB), trimetazidine-treated hearts during stabilization and reperfusion (TMZ-STAB+REP) and trimetazidine-treated hearts only during reperfusion (TMZ-REP). The values are mean (s.e.m)

Group	LVDP	+dp/dt	–dp/dt	LVDP10	LVDP45
NORM	122.2 (2.4)	4965 (188.0)	2398 (81.4)	25.2 (5.8)	43.2 (4.3)
TMZ-STAB	122.1 (3.4)	5022 (112.2)	2443 (66.1)	39.1 (7.8)	58.6 (4.1)*
TMZ-STAB+ REP	120.5 (1.9)	5141 (86.7)	2509 (59.4)	54.6 (13.7)*	58.1 (6.0)*
TMZ-REP	126.2 (4.1)	5370 (226.9)	2587 (74.2)	47.4 (13.8)	47.6 (9.0)

*P < 0.05 vs NORM

LDH release

LDHint was lower in TMZ-STAB+REP and TMZ-STAB hearts compared to NORM hearts [1153.2 (121.0) and 1152.1 (86.8) vs 1573.5 (138.2) respectively, $P < 0.05$]. However, trimetazidine administration during reperfusion did not diminish LDH release as compared with non-treated hearts, [1545.2 (277.1) vs 1573.5 (138.2), $P > 0.05$].

Activation of p38 MAPK and JNKs after ischemia and reperfusion

In normal hearts ischemia-reperfusion resulted in 2.0-fold phosphorylation of p38 MAPK and 3.2-fold and 3.8-fold phosphorylation of p46 and p54 JNK, respectively, as compared to the baseline values (data not shown). The levels of phosphorylated p38 MAPK and JNKs in all experimental groups after ischemia-reperfusion are shown in Figs. 3 and 4. The activation of p38 MAPK and JNKs in response to ischemia and reperfusion was similar in all experimental groups, $P > 0.05$.

Myocardial lactate content

After 10 min of reperfusion, myocardial lactate content was 0.35 (0.03) mg/g left ventricular weight for normal

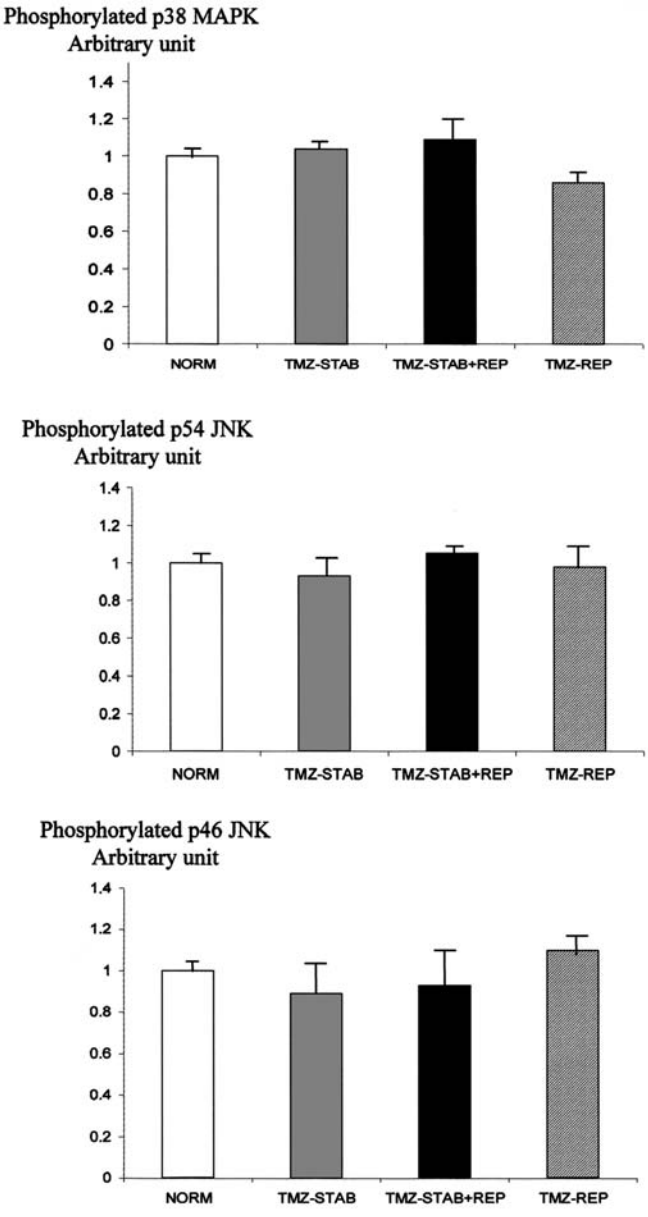

Fig. 3 Densitometric assessment of phospho-p38 MAPK (upper panel), phospho-p54 (middle panel) and phospho-p46 (lower panel) JNKs after ischemia and 45 min reperfusion in normal hearts (NORM, n = 5), trimetazidine-treated hearts only during stabilization (TMZ-STAB, n = 5), trimetazidine-treated hearts during stabilization and reperfusion (TMZ-STAB+REP, n = 5) and trimetazidine-treated hearts only during reperfusion (TMZ-REP, n = 5). (Columns are means of optical ratios, bar = s.e.m.)

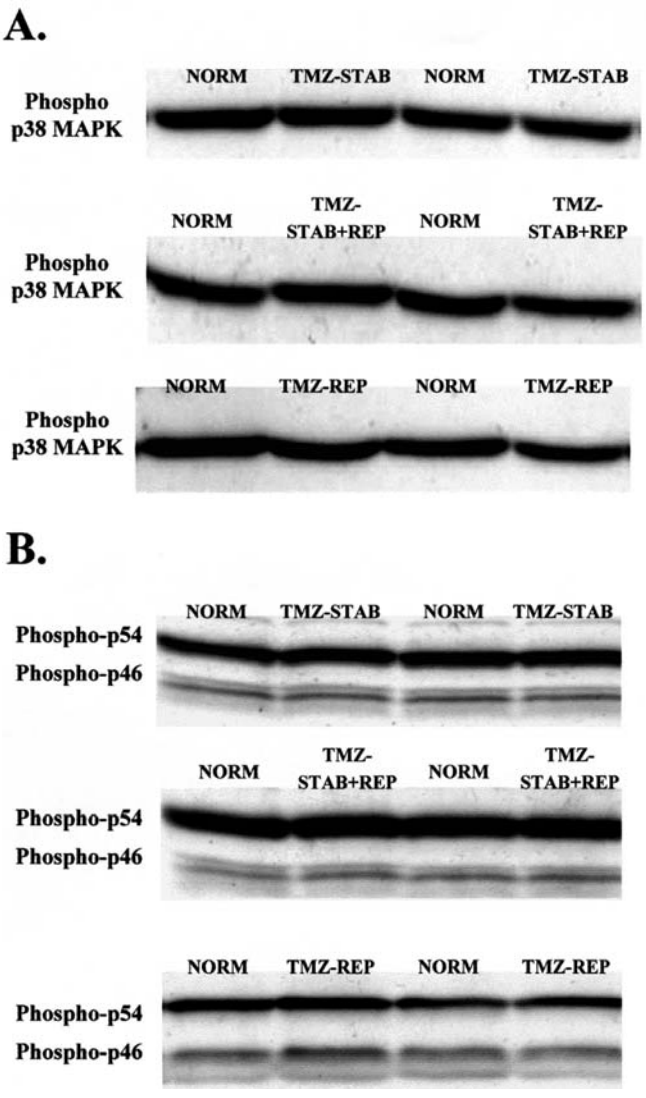

Fig. 4 Representative western blotting of phospho-p38 MAPK (A), phospho-p54 and phospho-p46 JNKs (B) after ischemia and 45 min reperfusion in normal hearts (NORM), trimetazidine-treated hearts only during stabilization (TMZ-STAB), trimetazidine-treated hearts during stabilization and reperfusion (TMZ-STAB+REP) and trimetazidine-treated hearts only during reperfusion (TMZ-REP). (Columns are means of optical ratios, bar = s.e.m.)

hearts and 0.06 (0.02) mg/g for hearts treated with trimetazidine only during reperfusion, $P < 0.05$.

Discussion

Various experimental and clinical studies have demonstrated the beneficial effect of trimetazidine on the tolerance of the heart to ischemia. In the present study, we assessed the efficacy of trimetazidine after its administration at different phases of an experimental model of zero-flow global ischemia followed by reperfusion.

Our data provide evidence that trimetazidine can protect the heart from ischemia-reperfusion injury when it is administered pre-ischemically regardless of its concomitant administration at reperfusion. In fact, post-ischemic recovery of left ventricular function was increased while LDH release was decreased both in hearts treated with trimetazidine before ischemia and hearts treated before and after ischemia. These findings are con-

sistent with previous reports from studies showing a cardioprotective effect of trimetazidine administration in models of subtotal or global ischemia with varying perfusate compositions [1, 4, 6].

The mechanisms through which trimetazidine has a cardioprotective effect when administered preischemically are not fully understood. Evidence from in vitro and ex vivo studies show that trimetazidine prevents intracellular ATP decrease, increases glucose metabolism, limits intracellular acidosis, protects against oxygen – free radical induced injury and inhibits neutrophil infiltration [9, 12, 18, 28]. Interestingly, El Banani et al. have shown that trimetazidine exerts protective effects against ionic disturbances and this is dependent on the severity of ischemia. In fact, during low flow ischemia the major effect is a significant reduction on intracellular acidosis, whereas during zero flow ischemia the main effect is found to be a significant reduction in Na_i^+ gain [6].

It is now realized that other mechanisms such as complex signaling transduction pathways can be involved in myocardial protection against ischemic injury with various preischemic interventions. Such signaling includes p38 mitogen activated protein kinase (p38 MAPK) and c-jun N-terminal kinases (JNKs) dependent intracellular cascades. In fact, attenuation of p38 MAPK and JNKs activation during ischemia has been associated with increased tolerance of the cell to ischemic stress [3, 10, 15], while p38 MAPK and JNKs activation is found to be decreased after ischemia-reperfusion following preconditioning protocol or heat shock pretreatment [16, 24, 25]. Both of these interventions are now thought to be powerful means of cardioprotection [25, 32]. It is likely that similar intracellular signaling is also involved in the protection conferred by metabolic anti-ischemic agents such as trimetazidine. This issue though has not been previously addressed and remains largely unknown. Thus, the present study explored whether in trimetazidine-induced cardioprotection, modulation of intracellular signaling events can occur.

According to our data, ischemia-reperfusion induced activation of stress kinases was not abolished by trimetazidine administration at any experimental phase. In fact, phosphorylated levels of p38 MAPK and JNKs after ischemia-reperfusion were similar between treated and untreated hearts. This finding probably indicates that MAPK-dependent signaling pathways might not be involved in trimetazidine mediated cardioprotection, as this is found to occur in other phenotypes of cardioprotection, such as ischemic preconditioning or heat stress [16, 24, 25]. This is consistent with the notion that prevention of ischemia and protection by preconditioning seem to be differently mediated so that anti-ischemic agents may not precondition, whereas pro-ischemic agents may precondition [21]. The latter is also supported by the fact that trimetazidine is not shown to have additive effects with ischemic preconditioning. Instead, in a recent study, trimetazidine was shown to block the cardioprotective effect of this intervention [20].

Administration of trimetazidine only during reperfusion was not found to significantly increase the postischemic recovery of function or to reduce myocardial injury as this was assessed by LDH release. However, the rate of recovery of function in those hearts was significantly higher than in nontreated hearts. Since the rate of recovery of function is an index of reversible postischemic dysfunction, a phenomenon known as stunning, this finding could be indicative of trimetazidine's antistunning effect that seems to occur with its use at reperfusion. Such an effect might be due to trimetazidine's action on glucose oxidation [9] and is consistent with recent data showing that an increase in carbohydrate oxidation by dichloroacetate administration at reperfusion can reverse myocardial stunning [11]. Potential mechanisms that have been suggested include reduction of H^+ production from glycolysis uncoupled from glucose oxidation and, consequently, maintenance of cellular ion homeostasis and/or regulation of cellular redox state [8, 13, 31]. Increased lactate content is found immediately after ischemia and this has been suggested to impair recovery of contractile function [27]. Interestingly, dichloroacetate, an activator of PDH, reduces lactate and increases recovery of function, when administered during reperfusion [11, 27]. In fact, lactate levels are shown to be increased after ischemia and return to baseline levels within the first 20 min of reperfusion, while they are found to be significantly reduced with dichloroacetate within this period of time [27]. In the present study, we measured tissue lactate content at 10 min of reperfusion coinciding with the measurement of the early functional recovery. Lactate was shown to be significantly reduced in trimetazidine-treated hearts as compared to nontreated hearts while the rate of recovery of function was increased in those hearts.

An important difference seems to exist between the cardioprotective effect of dichloroacetate and trimetazidine. In fact, dichloroacetate given before ischemia does not result in cardioprotection against ischemia-reperfusion as this occurs with trimetazidine. This is probably due to the fact that acceleration of glycolysis preischemically carries over into ischemia with subsequent accumulation of injurious glycolytic products due to both absence of oxidative metabolism and the absence of flow [19]. This difference probably indicates that trimetazidine-mediated cardioprotection against ischemia-reperfusion injury also involves other mechanisms apart from its action on metabolism. Further studies are needed to clarify this issue.

In conclusion, trimetazidine is shown to protect the isolated rat heart against ischemia-reperfusion injury when it is administered preischemically. This cardioprotective effect of trimetazidine is not associated with significant changes in p38 MAPK and JNKs activation in response to ischemia-reperfusion. Administration of

trimetazidine only at reperfusion does not reduce the extent of injury but results in acceleration of recovery of function accompanied by a decreased myocardial lactate content at early reperfusion.

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