Trimetazidine Counteracts the Hepatic Injury Associated with Ischemia-Reperfusion by Preserving Mitochondrial Function

AZIZ ELIMADI, ABDELLATIF SETTAF, DIDIER MORIN, ROSA SAPENA, FATIMA LAMCHOURI, YAHIA CHERRAH and JEAN-PAUL TILLEMENT

Département de Pharmacologie (A.E., D.M., R.S., J.P.T.), CNRS (D.M.), IM3, Faculté de Médecine de Paris XII, France; Département de Pharmacologie (A.S., F.L., Y.C.), Faculté de Médecine et de Pharmacie de Rabat, Morocco

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ABSTRACT

Recent studies suggest a crucial role played by mitochondria in the pathogenesis of ischemia-reperfusion injury. This study was conducted to clarify the role of trimetazidine, a cellular anti-ischemic agent, on mitochondria isolated from rat liver subjected to 120-min normothermic ischemia followed by 30-min reperfusion. Rats were divided into groups, pretreated with different doses of trimetazidine (5, 10 and 20 mg/kg/day) or saline and subjected to the ischemia-reperfusion process; another group served as the sham-operated controls. Alanine aminotransferase and aspartate aminotransferase activities and hepatocyte ATP content, bile flow and mitochondrial functions were assessed. Ischemia-reperfusion caused membrane leakage from hepatocytes and a decrease in ATP content and in bile flow. These effects were well correlated with alterations in mitochondrial function, namely, decrease in ATP synthesis, NAD(P)H level and mitochondrial membrane potential and generation of mitochondrial permeability transition. The pretreatment of rats with trimetazidine prevented these ischemia-reperfusion deleterious effects at both the cellular and mitochondrial level in a dose-dependent manner. It is concluded that trimetazidine at an optimal dosage of 10 mg/kg/day protects mitochondria against the deleterious effects of ischemia-reperfusion. This protective effect appears to be the key factor through which this drug exerts its cytoprotective activity.

Trimetazidine [1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride; Vastarel] has been described as a cellular anti-ischemic agent both in experimental conditions and in clinical trials (for review, see Harpey et al., 1989). By using isolated cardiac quiescent myocytes prepared from rat heart ventricles, Cruz et al. (1987) have shown that under anoxic conditions, trimetazidine improved the resistance of these cells to the effects of high concentrations of Ca2+. Their ATP content was maintained at almost the control value, and the K+ leakage was reduced. It has been shown using an experimental model of liver ischemia-reperfusion that pretreatment with trimetazidine limited the extent of the pathologi- cal dysfunctions, namely the increase in plasma membrane aminotransferases and the decrease in biliary flow and in hepatocyte ATP content (Tsimoyiannis et al., 1993). Moreover, Guarnieri and Muscari (1993) demonstrated that trimetazidine improved the functions of mitochondria isolated from hypertrophied perfused rat hearts. Recently, we have shown that trimetazidine protected isolated liver mitochondria against the deleterious effects of t-BH, a free radical generator, which when associated with Ca2+ overload induced the MPT (Elimadi et al., 1997). Salducci et al. (1996) have also demonstrated, using the same mitochondrial preparation, that trimetazidine restored ATP synthesis previously decreased by CsA.

In a clinical study, Detry (1993) has shown that trimetazidine administration significantly improved exercise tolerance, namely total work, duration of exercise and time to 1-mm ST-segment depression, without changing the rate-pressure product of patients with angina.

Although its effects have been demonstrated, the mechanism or mechanisms of action of this drug are not fully elucidated. Because mitochondria are the cellular organelles most affected by ischemia-reperfusion, our objectives were to check the protective effect of trimetazidine on liver functions, to define its dose dependency and to gain insight into the mechanism of action of this drug. For this purpose, an experimental protocol associating normothermic ischemia and then reperfusion of rat liver in situ was used in which rats were pretreated with increasing doses of trimetazidine. At the end of the experiment, mitochondria were extracted and checked for respiratory control, membrane potential, resistance to induced swelling and NAD(P)H level.

ABBREVIATIONS: MPT, mitochondrial permeability transition; t-BH, t-butylhydroperoxide; CsA, cyclosporin A; RCR, respiratory control ratio; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; Δψ, mitochondrial membrane potential; ROS, reactive oxygen species.
Materials and Methods

Drug administration. Adult male Wistar rats, weighing 250 to 300 g (Janvier, le Genest-St-Ise, France), were used in this study. All animal procedures used are in strict accordance with the French agency’s policies (ministere de l’Agriculture et de Pa Foret, authorization N° 00768) about animal experimentation.

Animals were divided into five groups (15 rats in each). A nontreated group and three treated groups were subjected to 120 min of normoxic liver ischemia followed by a 30-min reperfusion protocol. Animals in the treated groups were randomly allocated to each trimetazidine (Servier Laboratories, Neuilly, France) pretreatment of 5 mg/kg (n = 15), 10 mg/kg (n = 15) or 20 mg/kg (n = 15), whereas the nontreated group received the same quantity of saline solution. Trimetazidine was administered by intramuscular injection each day for 7 days before the induction of ischemia. Sham-operated group (n = 15) received the same surgical procedure as the other groups without being subjected to the ischemia-reperfusion protocol.

Trimetazidine solution was prepared daily; it was dissolved in saline [0.9% NaCl (w/v)] and appropriately warmed to body temperature before injection.

Surgical procedure. The technique of liver ischemia described by Nauta et al. (1989) was used in this study. The surgical procedure was performed 1 hr after the last drug or saline administration with the animals under general anesthesia using rectified ether. After section of the ligaments of the liver, hepatic normothermic ischemia was induced for 120 min by hilum clamping of the hepatic pedicles of segments I to V. To preclude the vascular congestion of the alimentary tract, the blood supply by the portal pedicles of segments VI and VII was not interrupted. During the period of ischemia, 0.5 ml of saline was given through the dorsal vein of the penis every 30 min to maintain hemodynamic stability. Bile was collected via the cannulation of the common bile duct with a fine catheter (Biotrol, Paris, France). Reperfusion was established by removal of the clamps.

After a 30-min reperfusion, the animals were killed, and their livers were immediately removed; mitochondria were isolated according to the procedure described below.

Liver function tests. Blood samples for measurement of ASAT and ALAT activities were collected after a 30-min reperfusion. Plasma enzymes activities were determined by enzymatic technique using a Boehringer-Mannheim (Mannheim, Germany) kit. The hepatic ATP content was determined by enzymatic procedure according to the method of Jaworek et al. (1974).

Isolation of mitochondria. Rat liver mitochondria were isolated as described by Johnson and Lardy (1967). Briefly, after the rats were killed, liver was excised rapidly and placed in medium containing 250 mM sucrose, 10 mM Tris and 1 mM the chelator EGTA, pH 7.5, at 4°C. The tissue was scissors minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at 600 x g for 10 min (Servall RC 28 S). The supernatant was centrifuged for 5 min at 15,000 x g to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at 15,000 x g for 5 min. Then, the resulting mitochondrial pellet was washed with the same medium from which the EGTA was omitted and centrifuged for 5 min at 15,000 x g, resulting in a final pellet containing ~50 mg of protein/ml. The protein content was determined by the method of Lowry et al. (1951). The mitochondrial suspension was stored on ice before the assay of membrane potential, mitochondrial swelling, NAD(P)H level and mitochondrial respiration.

Optical monitoring of mitochondrial membrane potential. Mitochondrial membrane potential (Δψ) was evaluated from the uptake of rhodamine 123 (Interchim, Montlucon, France), which accumulates electrophoretically into energized mitochondria in response to their negative-inside membrane potential (Emaus et al., 1986). Then, 1800 μM of the phosphate buffer (250 mM sucrose, 5 mM KH₂PO₄, pH 7.2 at 25°C), 3 mM succinate and 0.3 μM rhodamine 123 were added to the cuvette, and the fluorescence scanning of the rhodamine 123 was monitored using a Perkin-Elmer SA (Courtaboeuf, France) LS 50B fluorescence spectrometer. After 30 sec, mitochondria (0.5 mg/ml) were added. The Δψ was calculated according to the Nernst equation.

Mitochondrial swelling measurements. Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 520 nm by using a Hitachi (ScienceTec, les Ulis, France) model U-3000 spectrophotometer, according to the procedure described by Halestrap and Davidson (1990), with some modifications.

Two ways were used for inducing the mitochondrial swelling. The first used energized mitochondria; in this case, mitochondria (4 mg) were added to 3.6 ml of the phosphate buffer. A quantity of 1.8 ml of this suspension was added to both sample and reference cuvettes; then, 6 mM succinate was added to the sample cuvette only, and the A₅₂₀nm scanning was started. The second used nonenergized mitochondria. It was induced by t-BH in the presence of Ca²⁺. Mitochondria (4 mg) were added to 3.6 ml of Tris buffer (150 mM sucrose, 5 mM Tris-HCl, pH 7.4, at 25°C). A quantity of 1.8 ml of this suspension and 100 μM Ca²⁺ was added to both sample and reference cuvette. After 5 min of incubation, swelling was started by the addition of 10 μM t-BH to the sample cuvette only, and the A₅₂₀nm scanning was started.

Determination of mitochondrial NAD(P)H level. Mitochondrial pyridine nucleotides [NAD(P)H] were monitored by measuring their autofluorescence at excitation and emission wavelengths of 360 and 450 nm, respectively, in a Perkin-Elmer LS 50B fluorescence spectrometer, according to the procedure described by Minezaki et al. (1994). Mitochondria (2 mg) were added to 1.8 ml of the phosphate buffer containing 6 mM succinate, and the autofluorescence of NAD(P)H was determined.

Measurement of mitochondrial respiration. O₂ consumption was measured by a Clark-type oxygen microelectrode (Eurosip Instruments, Cergy, France) in a thermostat-controlled chamber. Mitochondria (0.5 mg) were added to 1.8 ml of the phosphate buffer. Mitochondrial respiration was initiated by the addition of succinate (6 mM final concentration), and oxidative phosphorylation was started by the addition of ADP to a final concentration of 0.1 mM. O₂ consumption recordings allowed the calculation of the OCR and the P/O ratio, which is the ADP consumed divided by O₂ used in state 3 respiration.

Statistical analysis. All values are given as mean ± SEM. Statistical comparisons were made between nontreated rats and sham-operated rats or ischemia-reperfused treated rats by using Mann-Whitney test. A value of P < .05 was considered statistically significant.

Results

Macroscopic observations. The macroscopic observations of livers after ischemia-reperfusion were performed 5, 10, 15 and 20 min after reperfusion. The livers of the nontreated group were dark and presented many congested areas; both increased gradually with time. When pretreated with trimetazidine, the livers were uniformly red without congested areas. This effect of trimetazidine on the morphology of rat livers appears to be roughly dose dependent.

Effects of trimetazidine on the liver function. As shown in figure 1, ischemia-reperfusion injury increased the levels of both plasma ASAT and ALAT compared with sham-operated rats. The activities of plasma ASAT and ALAT were 58 and 70 times higher than those of sham-operated rats, respectively.

Treatment of rats for 7 days with 10 or 20 mg/kg/day trimetazidine reduced the increase in plasma activities of
The decrease in hepatic ATP content was well correlated with the decrease in bile flow after ischemia-reperfusion. Again, trimetazidine at all the doses used sustained the bile flow at a level 40% of that of sham-operated group.

**Effect of trimetazidine on RCR and ATP synthesis of isolated mitochondria.** Ischemia-reperfusion drastically affected both the mitochondrial coupling and ATP synthesis efficiency as demonstrated by the decrease in RCR and P/O, respectively (table 1). Indeed, RCR of mitochondria isolated from hepatocytes of sham-operated group was $3.94 \pm 0.20$, and this value decreased to $1.51 \pm 0.13$ ($P < .001$) when the rats were subjected to 120-min ischemia followed by 30-min reperfusion. Similarly, P/O decreased from $1.20 \pm 0.06$ to $0.41 \pm 0.10$ ($P < .001$).

Trimetazidine treatment protected mitochondria from the deleterious effects of ischemia-reperfusion on both the RCR and P/O. This protection is clearly seen with trimetazidine doses of 10 and 20 mg/kg (table 1). Indeed, when rats were treated with 10 or 20 mg/kg/day trimetazidine, RCR and P/O returned almost to sham-operated group values (i.e., to normal range).

**Prevention by trimetazidine of mitochondrial membrane potential dissipation after ischemia-reperfusion.** Mitochondria isolated from sham-operated group have a $\Delta \psi$ value of $-169 \pm 5.3$ mV (table 1). When the rats were subjected to 120-min ischemia followed by 30-min reperfusion, the mitochondrial $\Delta \psi$ of these rats dropped to $-129 \pm 3.9$ mV ($P < .001$). Trimetazidine prevented the decrease in mitochondrial $\Delta \psi$ induced by ischemia-reperfusion. Indeed 5, 10 or 20 mg/kg trimetazidine increased the $\Delta \psi$ to $-149 \pm 12$ ($P < .05$), $-162 \pm 5.0$ ($P < .001$) and $-154 \pm 6.2$ ($P < .001$) compared with the nontreated group, respectively.

**Trimetazidine protective effect on NAD(P)H level decreased by ischemia-reperfusion.** It is well recognized that the ischemia-reperfusion phenomenon is characterized...
by an increase in ROS generation (Rao et al., 1983), which will directly or indirectly enhance the oxidation of NAD(P)H. As shown in Table 1, the levels of NAD(P)H of mitochondria isolated from hepatocytes of rats subjected to ischemia followed by reperfusion was decreased compared with the values of sham-operated group. When rats were pretreated with 10 or 20 mg/kg trimetazidine, the NAD(P)H level was not affected. However, pretreatment of rats with 5 mg/kg trimetazidine did not significantly prevent the decrease in mitochondrial NAD(P)H level.

**Effect of trimetazidine on the rate of swelling of isolated mitochondria.** MPT occurrence was assessed by the resulting large-amplitude swelling. Figure 4 shows the values of initial rates of swelling of sham-operated, nontreated and treated groups. Once energized with succinate, mitochondria isolated from all groups swelled. Ischemia followed by reperfusion exacerbated the swelling rate of mitochondria isolated from the nontreated group compared with that of sham-operated group. The administration of trimetazidine at doses of 10 or 20 mg/kg/day to the rats completely prevented the ischemia-reperfusion effect on mitochondrial volume.

The similar observation has been made when the swelling was induced by t-BH in the presence of Ca\(^{2+}\) (Fig. 4). Interestingly, the extent of this latter swelling was less important than that of the former.

**Discussion**

These data show that 120-min normothermic ischemia followed by 30-min reperfusion of rat liver caused aminotransferase leakage, a good index of hepatocyte structural membrane damage. Corresponding functional alterations are demonstrated by a decrease in both hepatocellular ATP content and in bile flow. These data are in accordance with those of others and attest that exposition of rat liver to this protocol of ischemia-reperfusion led to extensive damage of hepatocytes (Okuaki et al., 1996; Yamamoto et al., 1996).

ROS production occurring during the ischemia-reperfusion process seems to be a major determinant of tissue injury (Rao et al., 1983). These ROS are generated from both intracellular and extracellular sources (Jaeschke and Mitchel, 1989). Within the liver cells, mitochondria appear to be the major source of these toxic species as well as the organelle the most affected by them (Gonzalez-Flecha et al., 1993). Indeed, our data show that ischemia-reperfusion produced acute alterations of mitochondrial functions compared with that of mitochondria isolated from sham-operated rats. The damage involved mainly mitochondrial uncoupling leading to decrease in ATP synthesis. We also observed that mitochondria from liver of rats subjected to ischemia-reperfusion undergone extensive swelling regardless of the swelling process used. Interestingly, once these mitochondria are energized with succinate, they swell; furthermore, the extent of their swelling is much greater than when t-BH and Ca\(^{2+}\) were used as inducing agents. A likely explanation is that MPT occurrence is directly linked to ROS generation, so the amount of produced ROS is much more important when the mitochondria are energized than when they are deenergized. Furthermore, the membrane potential and the NAD(P)H level of these mitochondria are very low compared with those of mitochondria isolated from liver of sham-operated rats. Our data show

### TABLE 1

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<thead>
<tr>
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<th>Sham-operated rats</th>
<th>Ischemia-reperfused rats</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Trimetazidine mg/kg</td>
</tr>
<tr>
<td>Membrane potential (mV)</td>
<td>-189 ± 5.30</td>
<td>-129 ± 3.90a, 149 ± 12b, 162 ± 5.0c, -154 ± 6.20d</td>
</tr>
<tr>
<td>RCR</td>
<td>3.94 ± 0.20</td>
<td>1.51 ± 0.13a, 1.99 ± 0.22b, 3.44 ± 0.18c, 2.87 ± 0.23d</td>
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<tr>
<td>P/O</td>
<td>1.20 ± 0.06</td>
<td>0.41 ± 0.10a, 0.78 ± 0.12b, 1.13 ± 0.05c, 1.15 ± 0.07d</td>
</tr>
<tr>
<td>NAD(P)H level (arbitrary unit)</td>
<td>445 ± 25</td>
<td>198 ± 14a, 214 ± 24b, 433 ± 31c, 325 ± 32d</td>
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</tbody>
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* P < .001, statistically different from sham-operated rats.  † † † P < .001, statistically different from nontreated (no trimetazidine) ischemia-reperfused rats.  † † † † P < .001, statistically different from treated groups.
that a decrease in ATP synthesis, MPT generation, mitochondrial membrane potential collapse and decrease in the mitochondrial NAD(P)H content, which are correlated with hepatocyte membrane damage, contributed to injury of the liver subjected to ischemia-reperfusion. Regardless of the underlying mechanism, irreversible loss of mitochondrial function appears to be the key factor.

The pretreatment of rats with trimetazidine for 7 days before the induction of liver ischemia followed by reperfusion decreased the leakage of hepatocyte enzymes, prevented the decrease in their ATP content and restored the bile flow. This latter finding confirms and extends the results of Tsimoyianis et al. (1993), who used the same liver ischemia-reperfusion model. Interestingly, trimetazidine protection of hepatocytes against ischemia-reperfusion damage correlates well with its mitochondrial protection. Indeed, mitochondrial membrane potential, NAD(P)H level and rate of swelling of mitochondria isolated from liver of rats when treated with 10 or 20 mg/kg trimetazidine are maintained almost at the level of values of mitochondria isolated from liver of sham-operated rats. Taken together, all these data suggest that mitochondria might be an important target through which trimetazidine exerts its cytoprotective effect. It is noteworthy to emphasize that although trimetazidine treatment only partially prevented hepatocyte membrane leakage, decrease in hepatocyte ATP content and bile flow, it protected almost entirely their mitochondrial function. This suggests that ischemia-reperfusion injury possesses at least two components; one is intramitochondrial, and the other is extramitochondrial. Our data clearly show that trimetazidine affects mainly the intramitochondrial one. It is important to note that 10 mg/kg/day is the optimal trimetazidine dosage that gave the maximal protective effects of this drug at both the cellular and mitochondrial level.

The net rapid blood reflow observed macroscopically on reoxygenation of liver of trimetazidine treated rats could reflect the protective effects afforded by trimetazidine to endothelial cells against free radical injury. Hepatocytes will undergo reoxygenation injury if they have had sufficient previous ischemic or anoxic injury (Caraceni et al., 1994). In contrast to hepatocytes, endothelial cells are more sensitive to reoxygenation injury (Fujii et al., 1994). Therefore, protection against endothelial cell damage is especially important because endothelial cell injury can cause disruption of the microcirculation, leading to a decrease in blood flow and, ultimately, ischemic tissue necrosis, the no-reflow phenomenon (Koo et al., 1992).

In the light of the above results, we tried to gain insight into the mechanism of action of trimetazidine. The ischemia-reperfusion injury is closely related to an excessive ROS production. In in vitro studies, trimetazidine did not show any antioxidant effect (personal data). However, when rats were pretreated with trimetazidine for 7 days, the corresponding liver mitochondria show strong resistance to the effects of ROS produced by the intramitochondrial metabolism of t-BH. In accordance with these results, Harpey et al. (1987) used rat cultured mesangial cells to show that trimetazidine decreased H$_2$O$_2$ production, an index of intracellular oxygen-derived free radicals. Taken together, both data show that trimetazidine possesses an intracellular antioxidant effect. Because hepatocytes are the major sites of drug metabolism, it is possible that once trimetazidine is metabolized by the liver, it generates some metabolites, which might possess antioxidant activity. The fact that pretreatment with trimetazidine (10 mg/kg/day for 7 days) did not protect mitochondria isolated from rat liver that are not subjected to ischemia-reperfusion against the deleterious effect of t-BH plus Ca$^{2+}$ (data not shown), however, could rule out that trimetazidine metabolites possess a direct antioxidant effect. Thus, it is clear that trimetazidine has a protective effect only when toxic or pathological conditions occurred afterwards. Therefore, it is conceivable that trimetazidine stabilizes the activity of a system that is otherwise decreased under ischemia-reperfusion conditions or antagonizes a process induced by the experimental protocol.

Barnard et al. (1993) have shown that after the ischemia-reperfusion process, GSH peroxidase activity is decreased. It is interesting to stress that GSH peroxidase is accountable for the endogenous detoxification of both H$_2$O$_2$ and the xenobiotics, for instance, t-BH (Rosser and Gores, 1995), so it is reasonable to hypothesize that trimetazidine protects mitochondria from both the swelling induced by the endogenously generated ROS (energized swelling) and that produced by exogenous generated free radicals (t-BH plus Ca$^{2+}$) by restoring the activity of GSH peroxidase. Because under physiological conditions, GSH peroxidase is at its normal activity, no effect of trimetazidine was observed. However, additional experiments are needed to verify this hypothesis.

In conclusion, 120 min of liver normothermic ischemia followed by a 30-min reperfusion has a deleterious effect on mitochondrial integrity and functions. At an optimal dosage of 10 mg/kg/day, by inhibiting mitochondrial swelling, the decrease in NAD(P)H level and in ATP synthesis, trimetazidine stabilizes the activity of GSH peroxidase. A protective effect of trimetazidine on mitochondria appears to be the key factor through which this drug exerts its cytoprotective activity.

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References


Halestrap P and Davidson M (1990) Inhibition of Ca$^{2+}$-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin A is probably caused by the


Send reprint requests to: Dr. Aziz Elimadi, Département de Pharmacologie, Faculté de Médecine de Paris XII, 8 rue du Général Sarrail, F-94010, Créteil, France. E-mail: elimadi@univ-paris12.fr