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ORIGINAL ARTICLE

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SUMMARY

Trimetazidine exerts antianginal properties at the cellular level, without haemodynamic effect in clinical and experimental conditions. This cytoprotection was attributed to a decreased utilization of fatty acids for energy production, balanced by an increased incorporation in structural lipids. This study evaluated the influence of Trimetazidine on complex lipid synthesis from [2-^{3}H] glycerol, in ventricular myocytes, isolated rat hearts and in vivo in the myocardium and several other tissues. In cardiomyocytes, Trimetazidine increased the synthesis of phosphatidyl-choline (+ 80%), phosphatidyl-ethanolamine (+ 210%), phosphatidyl-inositol (+ 250%) and cardiolipid (+ 100%). The common precursor diacylglycerol was also increased (+ 40%) whereas triacylglycerol was decreased (−70%). Similar results were obtained in isolated hearts with 10 μM Trimetazidine (phosphatidyl-choline + 60%, phosphatidyl-ethanolamine + 60%, phosphatidyl-inositol + 100% and cardiolipid + 50%), the last two phospholipids containing 85% of the radioactivity. At 1 μM, Trimetazidine still stimulated the phospholipid synthesis although the difference was found significant only in phosphatidyl-inositol and cardiolipid. In vivo studies (10 mg/kg per day for 7 days and 5 mg/kg, i.p. before the experiment) revealed significant changes in the intracellular lipid biosynthesis, with increased labelling of phospholipids and reduced incorporation of glycerol in nonphosphorous lipids. Trimetazidine increased the glycerol uptake from plasma to the other tissues (liver, cochlea, retina), resulting in an altered lipid synthesis. The anti-anginal properties of Trimetazidine involve a reorganisation of the glycerol-based lipid synthesis balance in cardiomyocytes, associated with an increased uptake of plasma glycerol that may contribute to explain the pharmacological properties reported in other organs.

INTRODUCTION

Trimetazidine (TMZ, 1-[2,3,4-trimethoxy-benzyl] piperazine, 2 HCl), which was introduced as an antianginal drug 20 years ago, exerts its properties at the cellular level, without haemodynamic effects in clinical conditions [1–3]. Bricaud et al. [4] demonstrated the beneficial effect of a long-term TMZ treatment in ischemic cardiopathy without associated adverse effects. In vitro, the beneficial properties of the molecule were reported in isolated perfused heart models, which confirmed that the mechanism of the beneficial effect was not associated to any haemodynamic component [5,6]. Using a similar model, Lavanchy et al. [7] showed that during a global normothermic ischemia, TMZ prevented acidosis and inorganic phosphate accumulation and increased the recovery of adenosine triphosphate (ATP) at reperfusion. Similar results were obtained on cardiomyocyte cultures

submitted to hypoxia, in which the presence of the molecule decreased the intracellular accumulation of Ca^{2+} and Na^{+} and protected cardiac cells against accumulation of H^{+} [8,9]. The direct cytoprotective effect was confirmed by the observation that TMZ significantly reduced the hypoxia-induced enzyme leakage [10] and prevented the electro-mechanical alterations in cellular models [11]. Several studies raised the hypothesis that this cytoprotective effect could be associated with a reduction of the fatty acid utilization for energy production. The reduced palmitoyl-carnitine β-oxydation in isolated mitochondria [12], and increased glucose dependence of ATP production in isolated cardiomyocytes [13] suggested that the cytoprotective effect of TMZ might be due to reduction of the fatty acid utilization for energy production, possibly through the inhibition of β-oxydation. As a matter of fact, TMZ was described to inhibit long chain fatty acid 3-ketoacetylCoA thiolase [14].

However, such a mechanism of action addresses two questions. The first is the fate and toxicity of the excess of nonoxidized fatty acids [15]. During the past 20 years of TMZ utilization for angina treatment in humans, or in any experimental investigation no report indicated any adverse effect possibly correlated with an excess of fatty acid (or metabolite) storage. The second is the efficiency of TMZ in the noncardiac diseases, such as in the treatment of vertigo and tinnitus and ophthalmologic disorders of ischemic origin. Apart from the reduction of the β-oxidation pathway, TMZ might act at the level of phospholipid metabolism. Interestingly, in a previous paper, we reported that TMZ is able to induce in cardiac myocytes a large increase in phospholipid synthesis from inositol and ethanolamine [16]. This effect was associated with a significant increase in the incorporation of fatty acids in phospholipids [17], which could explain the absence of adverse effects due to fatty acid nonoxidation.

The present study was intended to evaluate the influence of TMZ on the synthesis of complex lipids from glycerol at the myocyte level, and in the heart in vitro and in vivo.

METHODS

In vitro studies on myocytes

These investigations were performed on neonatal rat cardiac myocytes in culture. The hearts were removed under sterile conditions from 2 to 4 days-old Wistar rats, and the myocytes were isolated and cultured according to published procedures [18]. The ventricles were collected in sterile conditions, cut to small pieces, washed three times and minced in calcium free saline at 30 °C. The tissue was then submitted to a six step proteolytic dissociation (10 min, 30 °C) with trypsin (1 : 250; DIFCO, Paris, France). The supernatants were pooled and centrifuged (1000 g, 15 min), and the cell pellet was resuspended in culture medium. The myocyte proportion was increased through a two-step selective adhesion procedure. The isolated myocytes were suspended in the culture medium (4×10^5 cells/ml) and seeded in 60 mm plastic dishes (Falcon Primaria; Becton Dickinson, Pont de Claix, France) at a density of 2×10^6 cells per dish. The culture was grown in Ham's F 10 basal medium supplemented with 10% fetal calf serum (SEROMED, Biochrom, Berlin, Germany), 10% human serum, penicillin and streptomycin. The free calcium concentration in the medium was standardised at 1.2 mM. The medium was renewed 24 h after seeding, and every 2 days thereafter. The cells were incubated at 37 °C in a humidified atmosphere (with 5% CO_2). Confluence was reached within 2 days, and all the experiments were conducted on 5-day-old cultures. The cells were pre-treated overnight with TMZ (500 μM in Puck G saline solution or Puck G saline alone for control), and then incubated in the same solution containing [2-^{3}H] glycerol (10 μM, 1.1 μCi/mL) (NEN). After 105 min, the cells were harvested in methanol (2 mL), and homogenized with an ultrasonic processor. Chloroform was added (4 mL), and 0.73% NaCl (1.2 mL) to cause phase separation. The phospholipids (PL) were separated from nonphosphorous lipids (NPL) on silica cartridges (Waters, Touzart et Matignon, Courtaboeuf, France). The PL classes were separated by thin layer chromatography on silica plates (Polygram Sil-G; Macherey-Nagel, Hoerdt, France), in the solvent system chloroform/methanol/acetic acid/petroleum spirit 35–60/boric acid (40/20/10/30/1.8 v/v/v/v/w) [19]. The separation of the classes of NPL was achieved by thin layer chromatography [20] in the solvent system hexane/diethyl ether/methanol/acetic acid (90/20/30/2 v/v/v/v). Each experiment was carried out on three dishes per group and repeated on five culture preparations. The data were expressed as mean ± SEM, and submitted to a two-way analysis of variance (ANOVA) with TMZ and culture as fixed factors [21]. Because the culture effect and the cross-interaction were not significant, only the results of the TMZ effect are presented. When significantly different, the means were compared by the Newman–Keuls test.

In vitro studies on the isolated heart

These investigations were performed using the isolated perfused rat heart model. The hearts ($n = 6$) were

isolated from 10 week-old Wistar rats, 300–320 g (CERJ, Le Genest St Hisle, France) and perfused in Langendorff mode for 20 min with Krebs–Henseleit medium gazed with a CO_2/O_2 mixture (5/95), containing TMZ (1 or 10 μM) or free of TMZ for control. After stabilisation, the perfusion solution was turned to the same solution containing 1 mM [2-^{3}H] glycerol (1 μCi/mL) for 20 min. At the end of perfusion, the heart was washed out from the remaining radiolabelled medium by 10 mL of Krebs–Henseleit solution injected through the cannula. The hearts were homogenized in 30 mL chloroform/methanol (2/1 v/v). The homogenate was filtered and a sample (100 μL) was withdrawn for the radioactivity counting. A 0.73% NaCl solution was added to cause phase separation. The radioactivity was determined in samples of the aqueous and organic fractions, and PL and NPL were separated on silica cartridge (Waters). The PL and NPL classes were separated as described above. The data were expressed as mean ± SEM, and submitted to a one-way ANOVA [21].

In vivo studies

The experiment was performed on 180 g Long Evans rats under sodium pentobarbital anaesthesia. This strain was choosen for in vivo experiments because of the natural colouring of retina and cochlea, which warrants an exhaustive dissection of these two organs. The rats were divided into one control group and two TMZ treated groups ($n = 6$), and fed for one week a diet containing TMZ to achieve a daily intake of 10 or 50 mg/kg, or no TMZ for control. The animals were then allowed to fast overnight and received an intraperitoneal injection of half a daily dose of TMZ in 0.9% NaCl (5 or 25 mg/kg) or vehicle for control. After 10 min, the animals were given, through the femoral vein, an injection of [2-^{3}H] glycerol (50 μCi/rat, in 200 μL of 0.9% NaCl). The incorporation of [2-^{3}H] glycerol in tissue lipids was investigated 30 min after tracer injection. A blood sample was withdrawn as well as the heart, cochlea (× 2), retina (× 2) and a liver sample. The whole cochlea was isolated from the temporal bone and weighed without further microdissection. The organs were homogenized in chloroform/methanol (2/1) and fractionated as described above. The data were expressed as mean ± SEM, and submitted to a two way ANOVA with TMZ and organ as fixed factors [21]. When significantly different, the means were compared by the Newman–Keuls test.

RESULTS

Effect of Trimetazidine on ventricular myocytes lipids

As shown in *Figure 1*, the radio-labelled glycerol was incorporated in the complex lipids of cultured ventricular myocytes. In the nonphosphorous lipids, the entire radioactivity was recovered in diacylglycerols and triacylglycerols. The most important part of the radioactivity in the phospholipid fraction was recovered in phosphatidyl-choline, although a significant amount of radio-labelling was also observed in phosphatidyl-ethanolamine, phosphatidyl-inositol and in the mixture cardiolipid + phosphatidic acid. The radio-labelling of sphingomyelin and phosphatidyl-serine was always low. When TMZ was added to the medium, the total glycerol uptake was significantly increased (approximately + 40%, $P < 0.01$). However, in spite of this enhanced uptake, the intracellular available glycerol remaining in the aqueous fraction was reduced by 30% ($P < 0.05$), suggesting that the metabolism rate is increased. This effect of TMZ was confirmed by the observation that the presence of the molecule significantly enhanced the incorporation of glycerol in most of the phospholipid classes. The synthesis of phosphatidyl-choline, phosphatidyl-inositol, phosphatidyl-ethanolamine, and cardiolipid (+ phosphatidic acid) were increased by 55%, 300%, 140%, and 100%, respectively

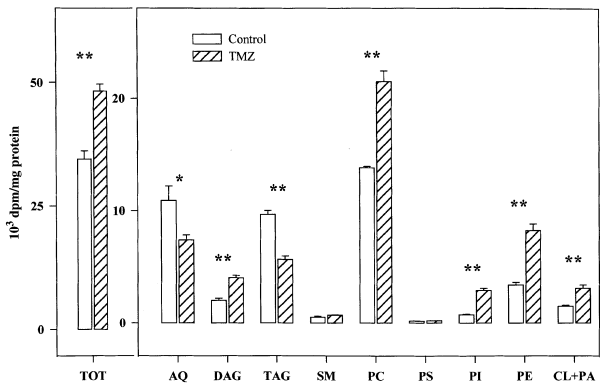

Figure 1 Influence of Trimetazidine (500 μM) on the uptake of [2-^{3}H]-glycerol by cultured ventricular myocytes (TOT) and its incorporation in the various lipid fractions. (DAG and TAG, di- and triacylglycerol; SM, sphingomyelin; PC, phosphatidyl-choline; PS, phosphatidyl-serine; PI, phosphatidyl-inositol; PE, phosphatidyl-ethanolamine; CL, cardiolipid + phosphatidic acid; AQ, aqueous fraction). Data are expressed as mean ± SEM ($n = 15$, 3 dishes per point repeated on 5 different cultures). (*$P < 0.05$, **$P < 0.01$).

($P < 0.01$). Conversely, TMZ did not influence the synthesis of sphingomyelin and phosphatidyl-serine (*Figure 1*), or the labelled amount of lysophosphatidyl-choline (data not shown). In the non-phosphorous lipid fraction, the treatment with TMZ resulted in an increased radiolabelling of diacylglycerol (+ 100%), and a significant reduction of triacylglycerol synthesis (–45%) ($P < 0.01$).

Effect of Trimetazidine on the lipids of isolated perfused rat heart

The results obtained with [2-^{3}H] glycerol perfusion in isolated perfused rat hearts are presented in *Figure 2* and *Figure 3*. After 20 min of perfusion, the amount of radioactivity was significantly higher in the hearts perfused with TMZ (10 μM) than in control (+ 90%). This result was associated with a large increase in both the labelled glycerol remaining in the aqueous fraction (+ 110%), and the labelled glycerol in the organic fraction (+ 50%) featuring the glycerol incorporated in complex lipids. However, this increase in complex lipid labelling affected the various lipid fractions differently. As shown in *Figure 2*, TMZ significantly increased the radioactivity in the phospholipid fraction (+ 70%) but decreased the radioactivity in the nonphosphorous lipid fraction (–30%). This result suggests that TMZ specifically influence the biosynthesis of the phospholipids. Another experiment was carried out in similar conditions, but with a perfusion time increased to 40 min to reach an equilibrium state. In these conditions, the differences were no longer significant, except in the aqueous fraction, which was still higher by 30% in the TMZ group (data not shown).

The dispatching of the radiolabelled glycerol in the various lipid classes after 20 min of perfusion confirmed that TMZ specifically affected the incorporation of glycerol in phospholipids (*Figure 3a*). The synthesis of phosphatidyl-choline, phosphatidyl-inositol, phosphatidyl-ethanolamine, and cardiolipid were increased by 60%, 110%, 60% and 50%, respectively ($P < 0.01$). As already observed in isolated cells, TMZ did not significantly influence the synthesis of the other PL classes (sphingomyelin, phosphatidyl-serine and lysophosphatidyl-choline). More than 80% of the radio-labelled glycerol incorporated in phospholipids was found in phosphatidyl-inositol and cardiolipid, although these two classes represent, in mass, less than 20% of the total cardiac phospholipids. In the nonphosphorous lipids, TMZ slightly reduced the incorporation of glycerol in diacylglycerol and did not alter the synthesis of triacylglycerol.

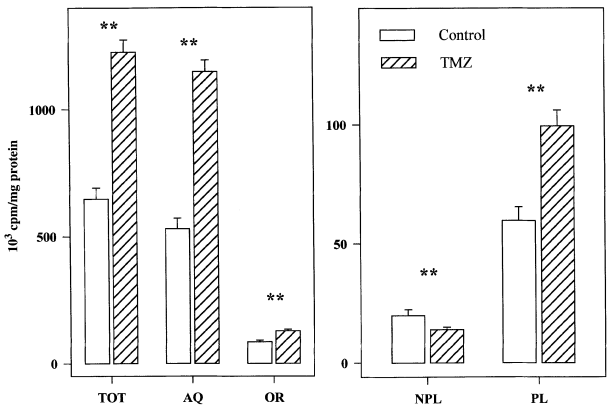

Figure 2 Effect of Trimetazidine (10 μM) on the radioactivity in the fractions collected from isolated perfused rat heart after 20 min perfusion with [2-^{3}H] glycerol ($n = 6$). (AQ, aqueous fraction; OR, organic fraction; NPL, nonphosphorous lipids; PL, phospholipids.) (*$P < 0.05$, **$P < 0.01$).

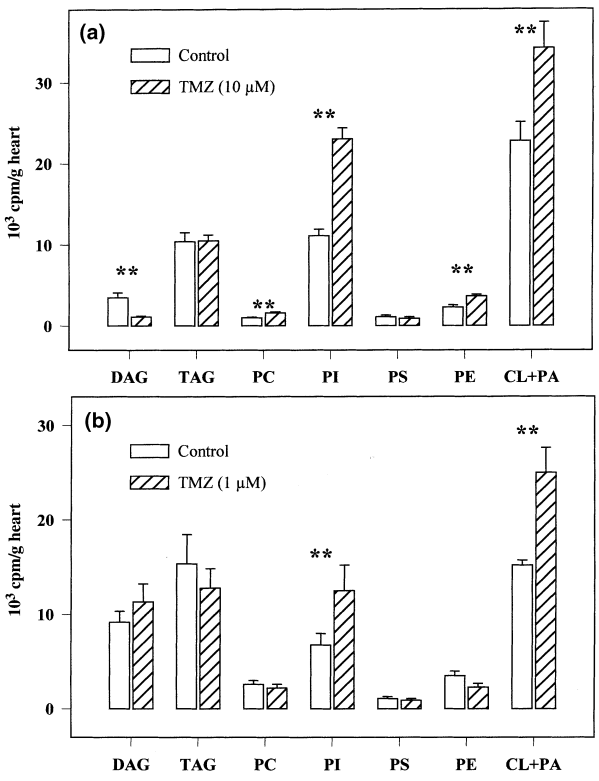

Figure 3 Influence of Trimetazidine (a) 10 μM and (b) 1 μM on the incorporation of [2-^{3}H]-glycerol in the various lipid fractions of isolated perfused rat heart. ($n = 6$). (DAG and TAG, di- and triacylglycerol; PC, phosphatidyl-choline; PS, phosphatidyl-serine; PI, phosphatidyl-inositol; PE, phosphatidyl-ethanolamine; CL + PA, cardiolipid + phosphatidic acid). (*$P < 0.05$, **$P < 0.01$).

This experiment was repeated with 20 min perfusion at a lower dose of TMZ (1 μM). In these conditions, only the incorporation of glycerol in phospholipid was stimulated by TMZ. In those classes in which the radioactivity was low at 10 μM TMZ (phosphatidyl-choline, phosphatidyl-serine and phosphatidyl-ethanolamine), no difference could be detected between the TMZ group and the control group at 1 μM TMZ. Conversely, as shown on *Figure 3b*, TMZ at 1 μM induced a significant increase in the synthesis of phosphatidyl-inositol and cardiolipid, + 80% and + 60%, respectively ($P < 0.01$).

Effect of Trimetazidine on glycerol metabolism in vivo

In the group of rats given TMZ for one week (per os 10 mg/kg per day) and half the daily dose just before experiment (5 mg/kg i.p), the radioactivity in the plasma was significantly lower after 30 min than in the control group (310 ± 9.1 vs. 388 ± 22.3 kcpm/mL, $P < 0.01$). Moreover, increasing the dose of TMZ to 50 mg/kg per day and 25 mg/kg i.p further decreased the plasma radioactivity (290 ± 6.4 kcpm/mL, $P < 0.01$). Conversely, in the organs investigated, the total radioactivity was higher in the TMZ treated groups than in the control group (*Figure 4*). All the organs were collected after 30 min, which may hide some important kinetic differences, as evidenced by the significant 'organ' effect ($P < 0.01$). Nevertheless, the two-way ANOVA revealed a significant TMZ effect ($P < 0.01$), which was mainly due to the higher dose. The absence of a significant cross interaction suggests that the increase affected all the organs investigated. Both the aqueous fraction and lipid fraction displayed a higher radioactivity in the TMZ-treated groups than in the control group (*Figure 5* and *Figure 6*, respectively). This difference was significant in the aqueous fraction for both the TMZ effect ($P < 0.01$) and the organ effect ($P < 0.01$), as well as in the lipid fraction ($P < 0.05$ and $P < 0.01$, respectively). These data indicate that TMZ increased both the glycerol uptake from the plasma and the glycerol incorporation in complex lipids. In the aqueous fraction, the significant cross interaction ($P < 0.01$) suggests that the amplitude of the glycerol-uptake increase due to TMZ may differ among the organs. Conversely, the absence of significant cross interaction in the lipid fraction outlines that the TMZ-induced increase in the incorporation of glycerol in complex lipids may affect all the organs (*Figure 6*).

In the heart samples, the phospholipids were separated from the nonphosphorous lipids, and the radioactivity was determined in these two lipid fractions (*Figure 7*). TMZ induced an alteration of intracellular lipid biosynthesis. The synthesis of phospholipids was significantly increased + 25% and + 45% at the lower and higher TMZ doses, respectively. Conversely, the synthesis of nonphosphorous lipids (mainly TAG) was significantly decreased (–20% approximately). The analysis of the heart revealed that TMZ significantly influenced the incorporation of glycerol in cardiac lipids (after 5 mg/kg i.p). As in all the in vitro investigations, this effect resulted in a significant increase in phospholipid turnover, which

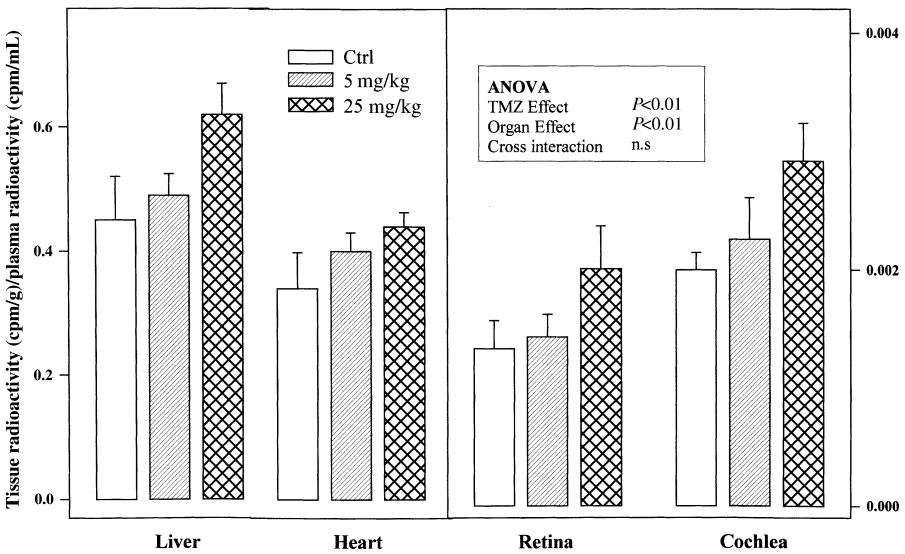

Figure 4 In vivo influence of Trimetazidine (0, 10 or 50 mg/kg per day for 7 days and 0, 5, and 25 mg/kg i.p just before the experiment, respectively) on the total uptake of [2-^{3}H]-glycerol in heart, liver, retina and cochlea ($n = 9$).

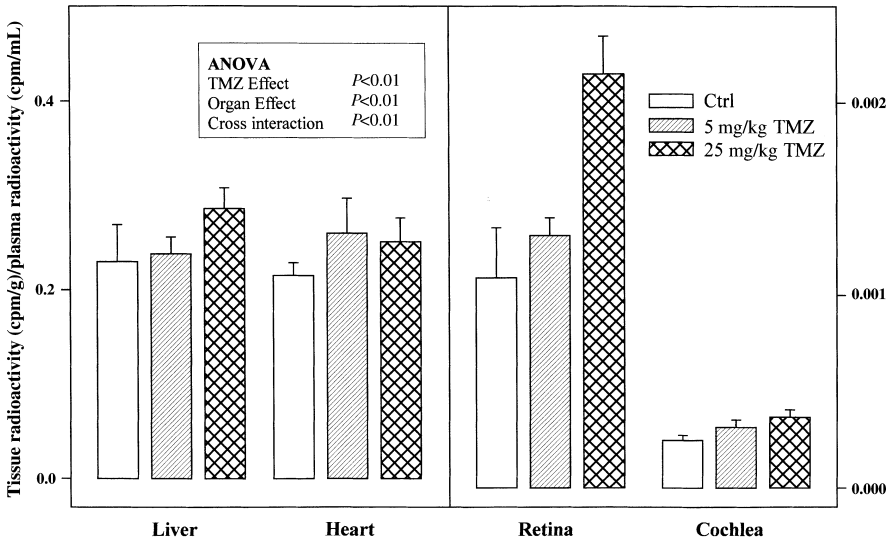

Figure 5 In vivo influence of Trimetazidine (0, 10 or 50 mg/kg per day for 7 days and 0, 5, and 25 mg/kg i.p just before the experiment, respectively) on the radioactivity recovered in the aqueous fraction of the heart, liver, retina and cochlea after injection of [2-^{3}H]-glycerol ($n = 9$).

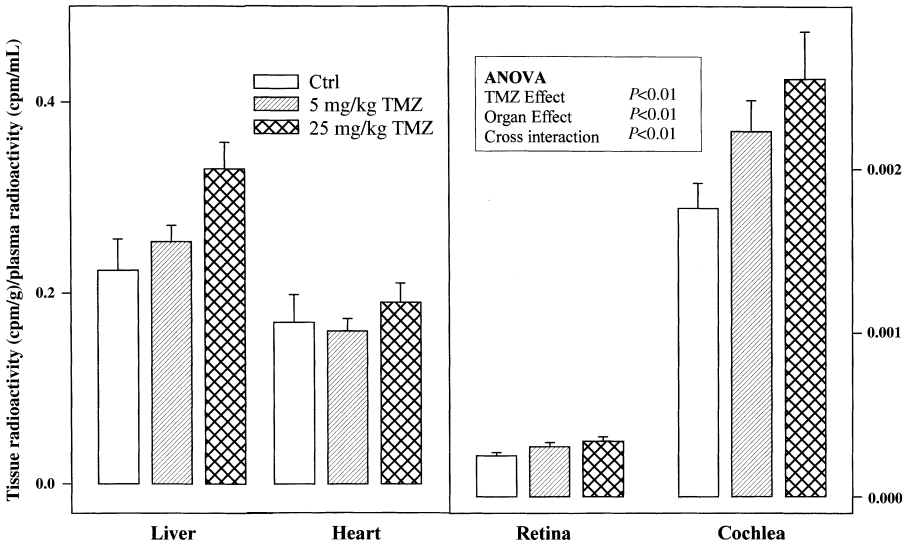

Figure 6 In vivo influence of Trimetazidine (0, 10 or 50 mg/kg per day for 7 days plus 0, 5, and 25 mg/kg i.p just before the experiment, respectively) on the radioactivity recovered in the lipid fraction of the heart, liver, retina and cochlea, after injection of [2-^{3}H]-glycerol ($n = 9$).

was, in vivo, balanced by a slightly reduced incorporation of glycerol in nonphosphorous lipids.

DISCUSSION

This study investigated the influence of TMZ on the fate of glycerol in lipid metabolism in the myocardium, at various organization levels. In the mammalian heart, phospholipids are synthesized de novo via the intracellular acylation of glycerol-3-phosphate produced from the glycerol transported across the membrane [22]. The glycero-3-phosphate is further metabolized to phosphatidic acid and then to diacylglycerol by dephosphorylation of phosphatidic acid. The results of the present study

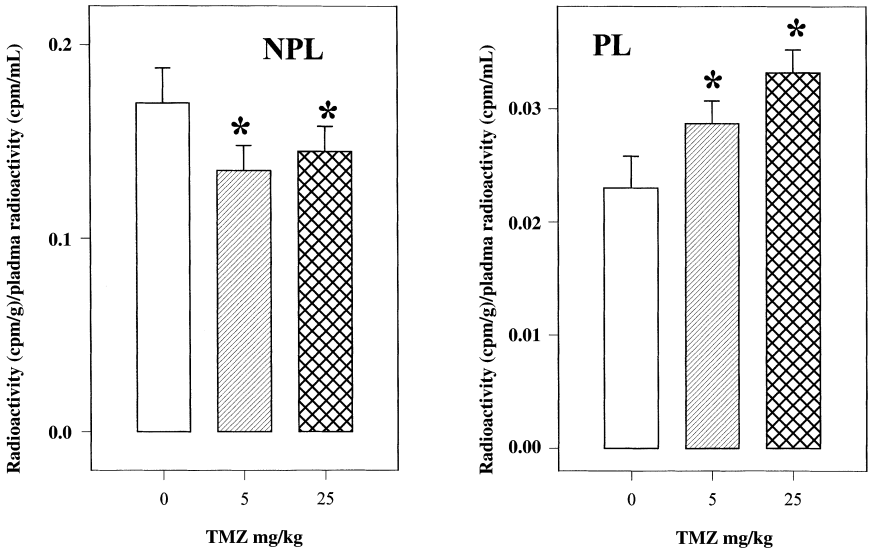

Figure 7 In vivo influence of Trimetazidine (0, 10 or 50 mg/kg per day for 7 days and 0, 5, and 25 mg/kg i.p just before the experiment, respectively) on the incorporation of radioactivity in the two main classes of cardiac lipids, after injection of [2-^{3}H]-glycerol ($n = 9$). (NPL, nonphosphorous lipids; PL, phospholipids). (*$P < 0.05$, **$P < 0.01$).

show that TMZ increases the incorporation of glycerol in complex lipids of the heart. Moreover, this increase is specific to the phospholipids, which confirms the results in our previous paper [16]. In the cardiac myocyte, TMZ stimulated the incorporation of radio-labelled glycerol in the four main phospholipid classes (phosphatidyl-choline, phosphatidyl-inositol, phosphatidyl-ethanolamine, cardiolipid). Two main pathways were described for the synthesis of phospholipids in the heart [23]. Phosphatidyl-inositol (and cardiolipid in a parallel pathway) is synthesized through the transfer of cytidine-diphosphate (CDP) on phosphatidic acid by a cytidylyl-transferase (or CDP-diacylglycerol synthase). In a second step, the CDP moiety is withdrawn from CDP-diacylglycerol and replaced by inositol in the phosphatidyl-inositol synthase-catalyzed reaction. The main precursor of this pathway is phosphatidic acid, which appears to be upstream of the diacylglycerol and then the synthesis of phosphatidyl-inositol and cardiolipid does not require diacylglycerol. In cultured cardiac myocytes, TMZ also induced a significant increase of phosphatidyl-choline and phosphatidyl-ethanolamine synthesis. Phosphatidyl-choline is synthesized through the so-called CDP-choline pathway, characterized by the transfer of CDP to the hydrophilic moiety (phosphocholine) by CTP : phosphocholine cytidylyltransferase, the key enzyme of the pathway [24,25]. Then, the cytidylyl moiety is exchanged with diacylglycerol by a diacylglycerol : CDP-phosphocholine transferase to build phosphatidyl-choline. The CDP-ethanolamine pathway parallels the CDP-choline pathway, but involves ethanolamine-specific enzymes, except for the initial kinase, because choline kinase was shown to act also on ethanolamine [26]. In the data presented here, the amount of radio-labelled diacylglycerol was increased in cultured cardiomyocytes and that of triacylglycerol was decreased. This lowered triacylglycerol synthesis may be attributed to the strong increase in phosphatidyl-choline and phosphatidyl-ethanolamine synthesis, which may redirect the utilization of diacylglycerol from triacylglycerol to phospholipids. Using inositol as precursor for phosphatidyl-inositol [17], it was shown that the increase in phospholipid turnover in isolated cardiomyocytes could be observed from 10 μM TMZ. In this study, we used glycerol as precursor, with the advantage of labelling all the complex lipids, and the disadvantage of a reduced sensitivity of the response. For this reason, the investigations in cultures cardiomyocytes were carried out at 500 μM TMZ, the most efficient concentration of the dose–response curve with inositol [17]. However, the requirement of high doses is specific to the cell culture models and lower concentrations, close to therapeutic conditions, were used on isolated heart model and in vivo.

As in cultured myocytes, TMZ (10 μM) stimulated the incorporation of glycerol in the four main PL classes in the isolated perfused heart (phosphatidyl-inositol, phosphatidyl-ethanolamine, phosphatidyl-choline and cardiolipid).

However, the radioactivity incorporated in phosphatidyl-inositol and cardiolipid represented more than 85% of the total radioactivity recovered in the lipid fraction. This is probably the reason why only these two phospholipid classes appeared significantly affected with the lower concentration of TMZ (1 μM). Although the isolated heart responded at a much lower concentration of TMZ than the cultured cells, these experiments qualitatively gave similar results in the two preparations and showed a significant increase in PL synthesis, concerning mainly the phosphatidyl-inositol/cardiolipid pathway. At high TMZ concentration (500 μM in cells and 10 μM in isolated heart) the CDP-choline and CDP-ethanolamine pathways seem to be also increased. The qualitative differences observed between cardiomyocytes and isolated heart could be due to the high content of nonmuscle cells in the heart. Moreover, cultured cardiomyocytes are extracted from neonatal rat hearts, although perfused rat heart or in vivo experiments are performed with adult cells. Such developmental differences may explain the small differences observed in lipid metabolism. The treatment with TMZ resulted in an increased radiolabelling of diacylglycerol in the cardiomyocytes that was not observed in the isolated heart. This difference may account for the differences observed in the PL pathways. In the myocytes, the increase in diacylglycerol synthesis is consistent with the large increase in CDP-choline and CDP-ethanolamine pathways, and the lower increase in phosphatidyl-inositol + cardiolipid + phosphatidic acid synthesis. In the heart, the high increase in phosphatidyl-inositol + cardiolipid + phosphatidic acid synthesis outlines the central role played by diacylglycerol. At 10 μM TMZ, the phosphatidyl-inositol/cardiolipid synthesis from phosphatidic acid is favoured. The dephosphorylation of phosphatidic acid to diacylglycerol is then reduced and the increase in phosphatidyl-choline and phosphatidyl-ethanolamine is balanced by the decrease in diacylglycerol. At 1 μM TMZ, only the main effect is significant. The synthesis of phosphatidyl-choline and phosphatidyl-ethanolamine, as well as the dephosphorylation of phosphatidic acid to diacylglycerol were not significantly altered. These data suggest that the main effect of TMZ on the phospholipid metabolism may concern the biosynthesis pathway of phosphatidyl-inositol and cardiolipid through their activated intermediate CDP-diacylglycerol. Such a mechanism confers to TMZ some common trends with several other amphiphilic compounds [27]. Chlorpromazine was reported to inhibit PC synthesis [28], methyl-lidocaine to favor phosphatidyl-inositol synthesis in the heart [29], and lidocaine to increase both phosphatidyl-inositol and phosphatidyl-ethanolamine synthesis in the hamster heart [30].

The results of the investigations presented in this study also demonstrate that in addition to a strong effect on phospholipid metabolism, TMZ affected glycerol uptake. In the cardiomyocytes, TMZ increased the total radioactivity uptake by 40%. In these conditions, the acceleration of all the phospholipid pathways increased the demand in phosphatidic acid and diacylglycerol, and thus increased the acylation of phosphoglycerol. This may explain the decreased available radioactivity in the aqueous fraction, associated with a higher labelling of diacylglycerol. In the isolated heart, the total radioactivity uptake increase by TMZ was close to + 100%, much higher than in the isolated cells. Moreover, the metabolic demand focused more on phosphatidic acid than on diacylglycerol, and the flux from glycerol to diacylglycerol was reduced as compared with the cellular model. This may explain why in the isolated heart, TMZ increased the radioactivity in the aqueous fraction and reduced that of the diacylglycerol. Similar trends were observed in the in vivo investigations. TMZ reduced significantly the radioactivity in plasma and increased globally the total radioactivity uptake in the organs investigated, heart, liver, retina and cochlea. This increase significantly affected both the aqueous fraction and the lipid fraction. In retina and cochlea, phospholipids represent the major part of the total lipid fraction and the effect of TMZ on lipid fraction can largely be attributed to an effect on phospholipid synthesis.

The results collected here support the hypothesis of a membrane-related mechanism of action for TMZ, in agreement with several reports in cardiac as well as noncardiac effects. It is not possible to estimate which membranes are concerned (plasma, mitochondria) because of the weak specificity of the PLs involved. However it can be hypothesized that most of the membrane type may be affected. Increasing the turnover of both phosphatidyl-inositol and cardiolipid may influence the plasma membrane, which contains a large part of the cell phosphatidyl-inositol, and the mitochondrial membrane, which is rich in cardiolipid. Another interesting question raised by these results is the mechanism of this effect. Interestingly the phospholipids responding to the lower TMZ concentration (phosphatidyl-inositol and cardiolipid) are those displaying a synthesis pathway that largely occurs in the membrane (particularly mitochondrial). On the contrary, for those PLs responding only to higher TMZ concentrations (phosphatidyl-choline and phosphatidyl-ethanolamine) numerous

synthesis steps occur within the cytoplasm. Due to its amphiphilic properties, TMZ may be trapped in the membrane, and thus influence membrane-bound enzymes and other proteins. Moreover, recent data reported the occurrence of high affinity TMZ binding sites in rat brain and liver mitochondria membranes consistent with the suggestion that mitochondria is the major target responsible for the anti-ischemic effect of the drug [31]. But the alteration by TMZ of the other membrane dynamic properties, including fluidity, was also suggested in the outer leaflet of the plasma membrane in human platelets and erythrocyte ghosts [32], and the plasma membrane of cardiomyocytes [11].

More recently Gil-Loyzaga et al. [33] reported a protective effect of TMZ on cochlear electric activity in kainic acid-induced neurotoxicity. The membrane effect of TMZ observed here in cochlea may outline the studies showing the clinical efficiency of TMZ in vertigo [34], cochleo-vestibular disorders associated with Menière's disease [35] and tinnitus [36]. Interestingly, phosphatidyl-inositol was reported to be the phospholipid displaying the fastest turnover in the inner ear [37], and increasing PI turnover by TMZ in inner ear may represent a beneficial event in various pathological situations.

Recent studies reported the protective effect of TMZ on retinal ischemia. This effect could be correlated to the TMZ blockade of excitatory amino acid-mediated neurotoxicity as previously described by Delbarre et al. [38], where changes in phospholipid turnover might be of influence. Moreover, by altering electric activity and accelerating the phospholipids turnover, TMZ might be of potential therapeutic benefit for combating retinal ischemia and particularly in age-related macular degeneration (AMD), a heterogeneous group of disorders, which is the leading cause of blindness in the elderly. The cellular and molecular mechanisms underlying the death of photoreceptors and other retinal cells remain poorly understood. Nevertheless, ischemic events seem to be a major factor for the development of the severe form of AMD and particularly for the development of choroidal neovascularization. Interestingly, the effect of TMZ on the membrane may influence the natural course of AMD.

However, the influence of TMZ on the total glycerol uptake cannot only be attributed to the increased flux of glycerol acylation for phospholipid synthesis, since all the situations (except the cultured cardiac cells) showed an increase of radioactivity in the aqueous fraction. This suggests that in addition to the increased phospholipid synthesis, TMZ may affect glycerol uptake in the heart and the other organs considered in this study. The entry of glycerol in the inner ear fluids, endolymph and perilymph, is limited by two barrier, an endothelial blood–perilymph barrier which shares similarities with the blood brain barrier, and an epithelial barrier between perilymph and endolymph compartments. The transfer rate constant of glycerol into perilymph, calculated after intravenous injection of radioactive glycerol, is rapid ($k_{in} = 0.025$/min), similar to those observed for electrolytes [39]. Nevertheless, to our knowledge, the cellular uptake and turnover of glycerol has not been studied in this structure.

CONCLUSION

TMZ was shown in this study to increase both the uptake of glycerol and the metabolic flux from phosphoglycerol to several phospholipids, and mainly phosphatidyl-inositol. The efficiency of the drug in angina pectoris is attributed to a decreased β-oxidation, which results either from a direct effect on β-oxidation enzymes [14] or from the redirection of fatty acids to phospholipid biosynthesis [16,17], or both. This study demonstrates that the membrane effect of TMZ, which can also be observed in noncardiac tissues, is a significant contribution to the mechanism of action of this drug.

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