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BENEFICIAL EFFECTS OF TRIMETAZIDINE ON MITOCHONDRIAL FUNCTION AND SUPEROXIDE PRODUCTION IN THE CARDIAC MUSCLE OF MONOCROTALINE-TREATED RATS

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Abstract—The administration of a single dose of monocrotaline (105 mg/kg) after 21 days produced in rats a reduction of cardiac mitochondrial function at the level of complexes I, II and IV of the respiratory chain, associated with the formation of heart hypertrophy, prevalently of the right ventricle. Moreover, in these rats, the submitochondrial particles produced more $O_2^{\cdot-}$ and in the cardiac tissue there was an elevation of malondialdehyde content. The repeated administration of trimetazidine (5 mg/kg/24 hr) improved the cardiac mitochondrial function, particularly in state 3 of respiration. In addition, the treatment with trimetazidine reduced, in the heart muscle, both the production of mitochondrial $O_2^{\cdot-}$ and the content of tissue malondialdehyde. Trimetazidine added alone did not significantly change either the cardiac mitochondrial activity, or the mitochondrial $O_2^{\cdot-}$ production in comparison to control rats. Also, the content of tissue malondialdehyde was not modified by the repeated administration of trimetazidine. In all the experimental conditions examined, the content of cardiac water-soluble fluorescence substrates did not significantly change in comparison to control rats.

It has been shown that the administration of the plant pyrrolizidine alkaloid monocrotaline (MCT) produces in rats a progressive pulmonary vascular injury which leads to an elevated pulmonary arterial pressure and cardiac right ventricular hypertrophy [1, 2]. Such sequelae of events, which ultimately produce a condition of pneumotoxicity and heart failure, have suggested that the treatment of rats with MCT could be a useful model for studying the biochemical mechanisms involved in the formation of heart failure in a definite interval of time [3]. Until now, relatively poor information was available on the nature of biochemical events that influence the evolution of compensated hypertrophied heart muscle to a condition of depressed cardiac pump function. In rats, these changes, which can occur according to the type of induced stress, consist mainly in reduction of myofibrillar ATPase activity, with a shift of the more abundant V_1 isoenzyme myosin form to the V_3 form [4]. In addition, there is a depression of cardiac energetics due to a reduction of the mitochondrial function which becomes particularly evident in conditions of acute prolonged hypertrophy [5]. The relatively scarce knowledge on the nature of biochemical events leading to heart failure due to a condition of heart hypertrophy has probably limited the number of possible pharmacological interventions able to improve the cardiac function in this condition. Since the drug trimetazidine [TMZ; 1-(2,3,4 trimethoxybenzyl)-piperazine dihydrochloride] showed beneficial effects on the cardiac energetics of ischemic-exposed heart muscle [6] or in rats administered with doxorubicin [7], we have studied in rats the effect of TMZ injections on cardiac mitochondrial function impaired by MCT treatment.

MATERIALS AND METHODS

Male Wistar rats weighing 250–300 g were injected with a single subcutaneous dose of MCT (105 mg/kg) dissolved in aqueous solution (pH 7.0) and the effects on cardiac muscle were examined 21 days later. The animals treated with TMZ received intraperitoneally the drug dissolved in NaCl 0.9% at the dose of 5 mg/kg/24 hr. In the MCT-treated rats, the TMZ injections started the same day as MCT treatment. Controls were injected with an equivalent volume of saline. The hearts were excised from diethylether-anesthetized animals and after atria removal, the right ventricles were separated from the left ventricles plus septum and then separately weighed. Mitochondria were isolated from both left and right ventricles following the procedure of Williams and Barrie [8]. The heart muscle was homogenized gently in 180 mM KCl, 10 mM EDTA and 0.5% bovine serum albumin, pH 7.2, using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 1000 *g* for 5 min, filtered and then recentrifuged at 5000 *g* for 10 min. The mitochondrial pellet was washed twice with a medium containing 180 mM KCl, 2 mM EDTA, 0.5% bovine serum albumin and finally suspended in 180 mM KCl, 0.5% bovine serum albumin. Mitochondrial respiratory control index (RCI), state 3-oxygen consumption (QO_2) and the ADP/O ratio were calculated from the oxygen partial pressure measured by a Clark electrode fitted in a closed water jacket at 25° (Gilson Instruments, France). The assay medium, consisting of 3 mM substrate (glutamate, or succinate, or ascor-

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Table 1. General characteristics of experimental rats

	Body weight (g)	Heart weight (g)	Right ventricular weight/left ventricular + septum weight
Control	250 ± 5.6	0.62 ± 0.02	0.30 ± 0.01
Control + TMZ	261 ± 3.2	0.63 ± 0.02	0.31 ± 0.02
MCT	239 ± 10.6*†	0.75 ± 0.03*†	0.44 ± 0.04*†
MCT + TMZ	241 ± 6.6*†	0.70 ± 0.04*†	0.42 ± 0.02*†

Rats were treated with the indicated doses of TMZ or MCT (see Materials and Methods), or with saline (control) and ratio of right ventricle weight to the weight of left ventricle plus septum was determined 21 days later. Values are expressed as means ± SE (N = 12). Significant difference ($P < 0.05$) from values in other groups; * different from control; † different from TMZ-treated rats.

Table 2. Oxidative phosphorylation of cardiac mitochondria after monocrotaline and trimetazidine treatment

		RCI	QO_2	ADP/O
Control	Glutamate	13.60 ± 0.2	65.5 ± 1.8	2.97 ± 0.2
	Succinate	3.51 ± 0.1	108.2 ± 3.0	1.94 ± 0.1
	Ascorbate	1.38 ± 0.01	161.3 ± 2.1	0.97 ± 0.01
TMZ	Glutamate	13.40 ± 0.4	68.4 ± 0.9	2.88 ± 0.03
	Succinate	3.44 ± 0.3	104.2 ± 2.3	1.88 ± 0.02
	Ascorbate + TM	1.40 ± 0.09	173.4 + 1.8	0.98 ± 0.02
MCT	Glutamate	6.80 ± 0.6*†	48.2 ± 1.9*	2.90 ± 0.06
	Succinate	2.45 ± 0.8*†	86.0 ± 4.4*†	1.80 ± 0.04
	Ascorbate + TM	1.01 ± 0.02	111.0 ± 3.8*†	0.96 ± 0.03
MCT + TMZ	Glutamate	10.90 ± 0.4‡	60.8 ± 2.1‡	2.92 ± 0.04
	Succinate	3.00 ± 0.3‡	96.8 ± 5.0‡	1.82 ± 0.05
	Ascorbate + TM	1.24 ± 0.08‡	162.4 ± 6.4‡	0.95 ± 0.06

Values are expressed as means ± SE (N = 12). Significant difference ($P < 0.05$) from values in other groups; * different from control; † different from TMZ-treated rats; ‡ different from MCT-treated rats. QO_2, oxygen consumption during state 3 respiration expressed in natom O_2/min·mg prot.

TM = tetramethylphenylenediamine.

bate plus tetramethyl phenylenediamine) 250 mM sucrose, 0.5 mM EDTA, 3 mM KH_2PO_4 at pH 7.4. ADP (250 μM), was added to the incubation mixture to initiate state 3 mitochondrial respiration.

Submitochondrial particles were prepared by sonicating the mitochondrial pellets suspended in 2 mM EDTA, pH 8.5, at 40 W (4 times for 15 sec with a 15-sec interval) using a Labsonic Sonifer cell disrupter [9]. The suspensions were centrifuged at 6000 *g* for 10 min and the resulting supernatants recentrifuged at 105,000 *g* for 30 min. The submitochondrial particles were washed twice with 250 mM sucrose, 10 mM Tris, pH 7.4, and suspended in the same buffer. Superoxide production was measured recording the superoxide-dismutase-sensitive conversion of adrenaline to adrenochrome [10] by submitochondrial particles supplemented with 0.1 mM NADH. The biochemical analyses were carried out by using frozen tissue homogenized in cold 100 mM KCl, pH 7.2 by an Ultra-Turrax homogenizer (full speed, two bursts for 5 sec each). The content of malondialdehyde (MDA) was measured according to the procedure of Jackson *et al.* [11] which used 0.8% thiobarbituric acid in presence of 2 mM EDTA. The content of water-soluble fluorescence substances was determined as described by Tsuchida *et al.* [12]. The tissues were extracted with ethanol/ether (3:1, v/v) and the resultant residues were dissolved with water. The fluorescence was measured at an excitation wavelength of 350 nm and an emission of 460 nm, after calibration of the fluorimeter (Perkin–Elmer LS5) with a quinine sulphate solution (4 μg/ml 0.1 NH_2SO_4). The proteins were estimated by the method of Bradford [13] using bovine serum albumin as the standard.

The results are the mean ± SE. The variables reported were treated firstly by single classification analysis of the variance (ANOVA), and secondly, only if a difference was detected in the first case, by multiple pairwise comparison according to Bonferoni test [14]. Differences were considered significant when $P < 0.05$.

RESULTS

Table 1 shows that the treatment of rats with TMZ did not modify the body and the heart weights. On the contrary, MCT administration decreased the body weight and increased both the heart weight and the ratio right ventricular weight/left ventricular + septum weight. In comparison to MCT-

Table 3. Effect of trimetazidine treatment on the O_2^- mitochondrial generation in control and monocrotaline-administered rats

Treatment	nmol O_2^-/min·mg prot.
Control	4.27 ± 0.56
TMZ	3.93 ± 0.49
MCT	7.12 ± 0.82*†
MCT + TMZ	5.86 ± 0.52†

Values are expressed as means ± SE (N = 12). Significant difference ($P < 0.05$) from values in other groups; * different from control; † different from TMZ-treated rats.

treated rats the general characteristics of experimental rats were not changed when the animals received TMZ together with MCT. On the contrary, in the MCT + TMZ-treated animals, no signs of congestive syndrome were observed, differently from the MCT group, where occasional ascites and pleural effusion were registered.

Table 2 reports that the cardiac mitochondrial functional parameters RCI, QO_2 and ADP/O did not change in the animals treated with TMZ, when glutamate, succinate or ascorbate + tetramethyl phenylenediamine were used as substrates. The same table shows that in the MCT-treated rats there was a significant decrease of the RCI and QO_2 values determined with all the substrates employed, while the ADP/O ratio remained close to the control values. Moreover, their values were not significantly different from the control values.

Table 3 reports that the formation of cardiac O_2^- radicals in the submitochondrial particles supplemented with NADH was strongly enhanced in the animals treated with MCT only. When the rats received MCT and TMZ together, the mitochondrial O_2^- production did not significantly change in comparison to MCT-treated rats or control rats, while it was significantly more elevated with respect to the rats treated with TMZ alone.

Table 4 shows that the formation of MDA did not change with TMZ treatment. On the contrary, the rats treated with MCT showed a higher level of MDA, which was reduced if these animals also received TMZ. The same table shows that the content of tissue water-soluble fluorescence substances had similar values in all the experimental conditions considered.

DISCUSSION

The present study indicates that the injection of MCT in rats produces a condition of heart hypertrophy, prevalently in the right ventricle, accompanied by a decrease of mitochondrial function. This deterioration of the mitochondrial respiratory activity involves the mechanisms of electron transport in general, because there is an impairment of the activities of the complexes I, II and IV of the respiratory chain. These results agree with previous research which demonstrated that MCT treatment in rats reduced the cardiac mitochondrial function and produced ventricle hypertrophy and heart failure [15, 16]. The biochemical events responsible for the reduction of mitochondrial activity induced by MCT treatment are not known.

In this experimental condition there is an increased generation of mitochondrial O_2^- radicals and of lipoperoxidative damage. It is possible that the formation of cell oxidative injuries can be considered as important determinants leading to heart failure. In fact, our previous research has indicated that the administration of oxy-radical scavengers, such as α-tocopherol derivatives, improves the mitochondrial activity depressed in MCT-treated rats [16]. In the MCT-treated rats, the repeated administration of TMZ improved the cardiac mitochondrial activity, with a particularly evident effect at the level of state 3 mitochondrial respiration induced by ADP (QO_2). This beneficial effect is very likely due to some biochemical events produced by TMZ *in vivo*, since the addition *in vitro* of the drug (0.1–1 mM) to isolated heart mitochondria did not change the values of mitochondrial respiratory parameters (data not shown). It could be interesting to note that TMZ may protect *in vivo* the cardiac energetics level, for example, in isolated rat hearts during ischemia [6] or in isolated cardiomyocytes exposed to anoxic and calcium-induced injuries [17]. This last beneficial effect may be very important in explaining our results, because TMZ seems capable of protecting the mitochondrial function by reducing the calcium-induced ATP hydrolysis in mitochondria [17]. Since TMZ has shown a non-specific calcium antagonist effect on cardiac cells [18], it is possible that TMZ exerting this effect in our experimental conditions may preserve both the mitochondrial activity and the cardiac ATP level.

Previous reports described how TMZ or its metabolites show some antioxidant effect, as has been shown

Table 4. Effect of trimetazidine and monocrotaline treatment on oxidative-derived metabolites

	MDA (nmol/mg prot.)	Water-soluble fluorescence substances (% fluorescence/mg prot.)
Control	0.84 ± 0.10	1.37 ± 0.9
TMZ	0.63 ± 0.08	1.47 ± 0.8
MCT	2.24 ± 0.12*†	1.52 ± 0.9
MCT + TMZ	1.13 ± 0.10†‡	1.61 ± 0.8

Values are expressed as means ± SE (N = 8). Significant difference ($P < 0.05$) from values in other groups; * different from control; † different from TMZ-treated rats; ‡ different from MCT-treated rats.

in several systems as generating O_2^- radicals [19, 20]. Our results (Table 3) indicate that the cardiac submitochondrial particles in the rats treated with TMZ and MCT produced less O_2^- than in those treated with MCT alone. Nevertheless, the fact that by adding TMZ *in vitro* (0.1–1 mM) to control submitochondrial particles producing O_2^-, in which we measured only a slight scavenger effect (data not shown) suggests that TMZ probably produced its maximal beneficial effect preserving the mitochondrial activity and successively reducing mitochondrial O_2^- generation. It is known, in fact, that conditions which produce mitochondrial damage may be responsible for the augmented rate of O_2^- production [21, 22].

However, the protective effect shown by TMZ on O_2^- generation could explain the reduced level of MDA measured in the cardiac tissue of rats treated with MCT + TMZ. The fact that the water-soluble fluorescence substance levels are not changed suggests that the formation of derivatives formed between amino groups and aldehydes derived from peroxidation reactions is probably not modified in our experimental conditions.

In conclusion, TMZ administered to rats injected with MCT efficiently protected the cardiac mitochondrial function and reduced the formation of O_2^- induced damage. Considering that the drug has a coronary vasodilating effect [23] and possesses a kind of non-specific calcium channel-blocker action on the vasculature [24], it remains to be seen whether the TMZ protects the cardiac muscle not only at mitochondrial level, but even by reducing some of the deleterious effects induced by MCT on the cardiovasculature.

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