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3-(2,2,2-TRIMETHYLHYDRAZINIUM)PROPIONATE (THP)—A NOVEL γ-BUTYROBETAINE HYDROXYLASE INHIBITOR WITH CARDIOPROTECTIVE PROPERTIES

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Abstract. A protein fraction containing γ-butyrobetaine hydroxylase (sp.act. 1.54 mU/mg) was isolated from the rat liver by differential precipitation with ammonium sulphate. 3-(2,2,2-Trimethylhydrazinium)propionate (THP), a noncompetitive enzyme inhibitor, when administered orally to rats for 10 days (150 mg/kg) elicited a reduction in myocardial free carnitine and long-chain acyl carnitine content by 63.7 and 74.3%, respectively. This reduction in free carnitine concentration causes a suppression of the free fatty acid oxidation, as measured by the production of $^{14}CO_2$ and ketone bodies. The inhibition of fatty acid oxidation is particularly manifest when their metabolism is stimulated by feeding a fat-rich diet to the animals or in fasting rats.The inhibition of fatty acid metabolism at the stage of activation (acyl carnitine formation) can account for the cardioprotective effect of THP, which is assessed by its ability to prevent a decrease in ATP level and myocardial energy charge as well as to prevent a rise in creatine phosphokinase and lactic dehydrogenase (myocardium-specific isozyme) activity in rat blood serum in response to isoproterenol and epinephrine. Regulation of the carnitine-dependent fatty acid metabolism in ischaemia is a pathogenetically justified approach to pharmacological treatment of ischaemic myocardium. In its biochemical mechanism, THP significally distinguishes itself from other known inhibitors of fatty acid oxidation.

Impairment of lipid metabolism occurring in myocardial cells in ischaemia belongs to the main biochemical pathogenetic manifestations of the disease. The level of free fatty acids and products of their metabolic activation—acyl carnitine and acyl-CoA—possessing some properties of detergents becomes elevated in response to adrenergic stimulation [1–3]. Inadequate supply of oxygen and the ensuing failure of the myocardium to oxidise $NADH_2$ and $FADH_2$ result in the inhibition of fatty acid oxidation and accumulation of their metabolites, e.g. β-hydroxyacyl CoA [4, 5]. The products of fatty acid activation and partially oxidised metabolites exert a destabilising effect on mitochondrial membranes and are capable of suppressing the activity of adeninenucleotide translocase [6, 7].

A rise in acyl carnitine and acyl-CoA concentration in the mitochondria reflects the inability of mycardial cells to oxidise large amounts of fatty acids in ischaemic heart, while an increase in acyl-CoA concentration in the cytoplasm can be caused by free carnitine deficiency observed in ischaemic myocardium [8–10].

After establishing the fact that ischaemic myocardium is depleted of carnitine, administration of exogenous carnitine has been proposed as a means of restituting fatty acid metabolism. Under certain conditions, treatment with exogenous carnitine can ameliorate the functioning of ischaemic myocardium and bring down the level of toxic fatty acid metabolites [11, 12]. The effectiveness of exogenous carnitine depends on the presence of proton acceptors in amounts required for β-oxidation. However, in advanced ischaemia the rate of regeneration of proton acceptors declines due to a fall in partial oxygen pressure in the tissues [13], making any further introduction of exogenous carnitine irrelevant.

Ischaemic lesions lend themselves to metabolic correction also by inhibiting the carnitine-dependent oxidation of fatty acids at the stage of activation (acyl carnitine formation). To this end, inhibitors of carnitine acyl transferases I and II can appear useful for therapeutic purposes [14, 15].

Moreover, the inhibition of carnitine-dependent metabolism of fatty acids is possible by lowering the level of intracellular free carnitine [16]. This effect can be achieved with the aid of carnitine synthesis and transport inhibitors. With the above facts in mind we examined the effects of a γ-butyrobetaine analogue 3-(2,2,2-trimethylhydrazinium)propionate (THP) on γ-butyrobetaine hydroxylase activity, on concentration of free carnitine and long-chain acyl carnitine and on the rate of free fatty acids oxidation. Catecholamine-induced lesions in the myocardium were used as a model for evaluation of cardioprotective properties of THP with respect to biochemical alterations occurring in ischaemic myocardium.

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MATERIALS AND METHODS

Animals. Wistar male rats weighing 180–200 g were used in experiments.

Reagents and isotopes. Test kits for ATP, ADP, AMP, creatine phosphokinase and lactic dehydrogenase isozymes (Boehringer, F.R.G.), L-carnitine, oleic acid, palmitic acid, palmitoyl-L-carnitine (Sigma, St Louis, MO), albumin V fraction, carnitine acetyltransferase (Sigma, St Lous, MO), β-hydroxybutyrate dehydrogenase (Boehringer, F.R.G.), γ-butyrobetaine (Sigma, St. Louis, MO), isoproterenol, epinephrine (Serva, F.R.G.), dithiobisnitrobenzoic acid (Gee Lawson Chemicals, U.K.) α-ketoglutarate, ascorbic acid (Serva, F.R.G.), oxidised glutathione (Reanal, Hungary), 1-^{14}C-acetyl-CoA (sp.act. 56 mCi/mM; NEN, U.S.A.), 1-^{14}C-palmitoyl-L-carnitine (sp.act. 55 mCi/mmol, NEN, U.S.A.), 1-^{14}C-oleic acid (sp.act. 46 mCi/mmol, Izotop, U.S.S.R.), U-^{14}C-palmitic acid, (sp. act. 800 mCi/mmol, NEN, U.S.A.), THP (synthesised in the Institute of Organic Synthesis) were used.

Isolation procedure and determination of γ-butyrobetaine hydroxylase activity. γ-Butyrobetaine hydroxylase was isolated from the supernatant of rat liver homogenates by differential precipitation with ammonium sulphate following a procedure described elsewhere [17]. The enzyme-containing fraction was salted out at 50–60% ammonium sulphate saturation. Enzyme activity measured for the 16 μM concentration of γ-butyrobetaine was equal to 1.54 mU/mg protein.

γ-Butyrobetaine hydroxylase activity was assessed by the amount of carnitine formed from γ-butyrobetaine. Carnitine was assayed radiometrically with the aid of carnitine acetyltransferase and ^{14}C-acetyl-CoA. The ^{14}C-acetyl carnitine was isolated by ion-exchange chromatography on Dowex 2 × 8 (Cl^--form, 200–400 mesh) following enzymatic reactions [18]. The incubation mixture (final volume 125 μl) consisted of γ-butyrobetaine (75 μl, 6.65–26.6 μM), THP solution (10 μl, 50–500 μM), 20 μl of ascorbate and α-ketoglutarate mixture (500 μl 37.5 mM sodium ascorbate, 375 μl 25 mM α-ketoglutarate, 125 μl 1 M potassium phosphate buffer, pH 6.7), 10 μl of γ-butyrobetaine hydroxylase and catalase mixture (150 μl of isolated γ-butyrobetaine hydroxylase solution, −77 mU, 50 μl catalase solution −2 mg, 50 μl 100 mM potassium phosphate buffer, pH 6.7, 250 μl H_2O), ferrous ammonium sulphate (10 μl, 6.25 mM). Following incubation for 30 min at 30° the samples were supplemented with 25 μl 1.6 M KOH and kept for 20 min at 56°. Then, 35 μl of the neutralising solution were added (1 ml 1 M HEPES acid, 400 μl 0.5 M glutathione, 10 μl 100 mM EDTA, 250 μl 2 M H_3PO_4, water to 1.75 ml). Following neutralisation the sample should have pH 7.6. After adding 10 μl of 1-^{14}C-acetyl-CoA (2 nm, 15 nCi) and 5 μl of carnitine acetyltransferase (21 μg protein) the mixture was incubated for 30 min at 30° and each sample was supplemented with 290 mg of dry Dowex resin 2 × 8 (Cl^--form, 200–400 mesh) and 600 μl H_2O. The samples were shaken for 10 min, spun down (2000 g × 5 min) and the supernatant (0.4 ml), was mixed with Bray's scintillation fluid (10 ml). Radioactivity was measured in an LKB-Wallac Rack-Beta-1217 liquid scintillation counter.

Oxidation of 1-^{14}C- *oleic acid and* 1-^{14}C-*palmitoyl-L-carnitine to* $^{14}CO_2$. Rat heart homogenates were prepared after the animals given a fat-rich diet (20% of margarine in the daily ration) had received a course treatment with THP and from the hearts of intact animals (20% myocardial homogenate with 0.25 M sucrose). 1-^{14}C-oleic acid or 1-^{14}C-palmitoyl-L-carnitine were used as radioactive substrates. The rate of metabolism was evaluated by measuring the amount of $^{14}CO_2$ and ^{14}C-soluble products formed [19].

Biochemical analysis of the myocardium. The hearts extracted from animals sacrificed by decapitation were frozen in liquid nitrogen between cooled steel blocks, ground to powder and homogenised in 0.6 N $HClO_4$. The supernatant was neutralised with KOH to pH 7.9. $KClO_4$ was removed by centrifugation (2000 g × 30 min). The solution obtained and the acid-insoluble homogenate fraction were stored at −30°. The acid-soluble extract was employed for the determinations of ATP [20], ADP, AMP [21] and free carnitine [22]. The acid-insoluble homogenate fraction was used in long-chain acyl carnitine assays [23].

Biochemical analysis of sera. Creatine phosphokinase, myocardium-specific lactic dehydrogenase (LDH) activity and β-hydroxybutyrate was assayed [24–26].

The Wilcoxon–Mann–Whitney nonparametrical U-criterion was employed for statistical evaluation of data. A nonparametrical method was also applied for plotting kinetic curves, elucidation of the pattern of γ-butyrobetaine hydroxylase inhibition and for the assessment of K_m and V_{max} values [27].

RESULTS

The study of γ-butyrobetaine hydroxylase in present experimental conditions (enzyme concentration in the sample was equal to 1.54 mU, γ-butyrobetaine concentration 4, 8, 12 and 16 μM) revealed that the reaction rate was linearly related to substrate concentration. THP (16 and 40 μM) appreciably decreases the reaction rate (Fig. 1).

A nonparametrical method was applied to assess the K_m and V_{max} values. The constant found for the γ-butyrobetaine hydroxylase reaction in the absence/presence of THP suggests a noncompetitive inhibition of enzyme activity. For example, V_{max} drops 1.73-fold and 3.25-fold in the presence of 16 μM and 40 μM, THP, respectively, whereas K_m virtually shows no variation (Table 1, Fig. 2).

Oral administration of THP (150 mg/kg) in intact rats for 10 days elicited a decrease in the concentration of free carnitine and its acylated form by 63.7 and 74.3%, respectively (Table 2). This decrease was accompanied by the inhibition of 1-^{14}C-oleic acid oxidation to $^{14}CO_2$ in rat myocardium homogenates, the process being dose-dependent. The suppression of 1-^{14}C-oleic acid oxidation is more pronounced upon stimulation of fatty acid metabolism by feeding a fat-rich diet to the animals. Administration of THP (100 mg/kg) for 10 days to

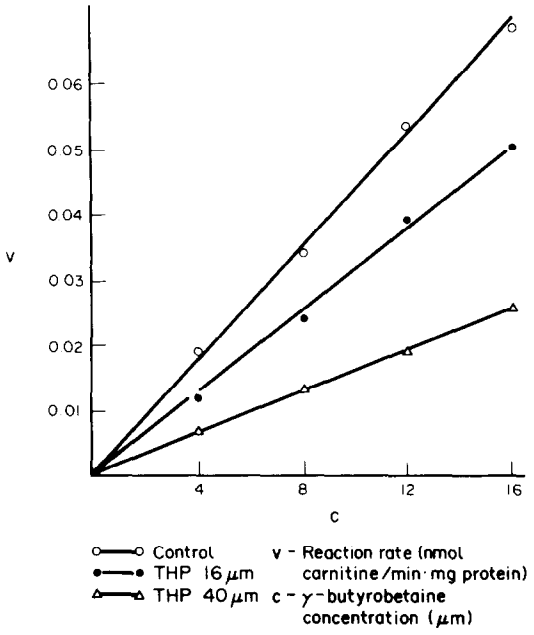

Fig. 1. Influence of THP on γ-butyrobetaine hydroxylase activity. Each sample contained 1.54 mU of γ-butyrobetaine hydroxylase, γ-butyrobetaine concentration amounted to 4, 8, 12 and 16 μM; THP was used in 16 and 40 μM concentrations. Under the present conditions the γ-butyrobetaine hydroxylase reaction rate is linearly related to substrate concentration. Each point is an average of three measurements.

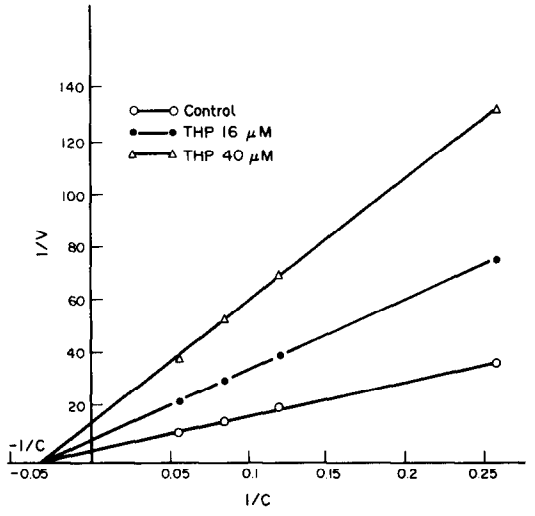

Fig. 2. Influence of THP on γ-butyrobetaine hydroxylase activity. The primary data used for obtaining constants by a non-parametrical method and for the plotting of curves are presented in Fig. 1. Each point is an average of three measurements.

rats fed on a standard diet inhibited 1-^{14}C-oleic acid oxidation in the myocardium by 25.7%, as compared with 48.7% in the animals given a fat-rich diet (Table 3). Continuous administration of THP had no effect on the oxidation of 1-^{14}C-palmitoyl-L-carnitine (Table 3).

An increase in the concentration of L-carnitine in the incubation medium causes a dose-dependent intensification of U-^{14}C-palmitic acid oxidation to $^{14}CO_2$ and ^{14}C-soluble products by rat heart homogenates. For instance, L-carnitine in the concentration of 1 μM enhances the formation of $^{14}CO_2$ and ^{14}C-soluble products by 58.4 and 42.7%, respectively. When the concentration of L-carnitine is raised to 10 μM, the appropriate values are 233% and 137.6% (Table 4), indicating the sensitivity of

Table 1. The values of K_m and V_{max} for γ-butyrobetaine hydroxylase in the presence of various THP concentrations

No.	THP (μM)	K_m (γ-Butyrobetaine, μM)	V_{max} (Carnitine, nmol/min · mg protein)
1	—	47.2 (29.6–55.2)	0.26 (0.19–0.30)
2	16	32.8 (32.2–33.6)	0.15 (0.10–0.16)*
3	40	36.8 (26.4–46.4)	0.08 (0.06–0.09)*, **

K_m and V_{max} values for each experiment were calculated using a nonparametrical method [17]. The K_m values show no appreciable variation. The double reciprocal plot was obtained using the mean value K_m for three experiments. K_m was equal to 39.0 μM.

* Statistically significant variation with respect to group 1 ($P < 0.05$).

** Statistically significant variation with respect to group 2 ($P < 0.05$).

Table 2. Free carnitine and long-chain acyl carnitine content in rat myocardium

No.	Experimental group	Free carnitine (nmol/g tissue)	Long-chain acyl carnitine (nmol/g tissue)
1	Control	510 (420–626)	39 (19–56)
2	THP	185 (105–260)*	10 (3–15)*

THP (150 mg/kg) was given orally to rats for 10 days as an aqueous solution. On the 10th day, one hour following the last administration the animals were decapitated and their hearts extracted. The extracted organs were frozen in liquid nitrogen between steel blocks, ground to powder in liquid nitrogen and further handled as described in Materials and Methods.

* Statistically significant variation with respect to group 1 ($P = 0.001$).

Table 3. Oxidation of 1-^{14}C-oleic acid and 1-^{14}C-palmitoyl-L-carnitine in rat heart homogenates

Labelled compound	Diet	Number of experiment	THP dose (mg/kg)	$^{14}CO_2$ dpm/100 mg tissue · hr Control	THP	% of control
1-^{14}C-Oleic acid	Standard laboratory diet	1	100	2456 (2121–2684)	1828 (1404–1560)*	74.35 (61.2–81.7)*
		2	200	3958 (3423–4653)	2339 (2012–2543)*	59.1 (50.8–64.5)*
		3	200	4063 (3508–4728)	1695 (1409–1918)*	41.7 (34.0–47.5)*
	Fat-rich diet	4	100	7551 (6508–9024)	3871 (2952–4613)*	51.3 (39.1–61.1)*
1-^{14}C-Palmitoyl-L-carnitine	Fat-rich diet	5	200	6700 (5300–9070)	5950 (5200–6600)	88.8 (77.6–98.5)

The animals were given a standard and fat-rich (20% of margarine by weight) diet. An aqueous solution of THP (100 and 200 mg/kg) was given orally for 10 days. On the 10th day, one hour following the last administration the animals were decapitated and their hearts extracted. The extracted organs were washed in cold 8 mM Na phosphate buffer (pH 7.4) and used to prepare 20% myocardial homogenates in 0.25 M sucrose containing 0.4 mM EDTA. The incubation mixture (4 ml) contained myocardial tissue (100 mg), 1 mM $MgCl_2$, 80 mM KCl, 1 mM ATP, 8 mM Na phosphate buffer (pH 7.4), 0.1 mM 1-^{14}C-oleic acid in combination with albumin fraction V (final concentration 6 mg/ml) or 0.1 mM 1-^{14}C-palmitoyl-L-carnitine. The samples were incubated at 37° for 60 min with continuous shaking. A gelatine capsule containing glass wool moistened with 0.3 ml hyamine hydroxide dissolved in methanol (1:1) was then added to each sample followed by the addition of 30% $HClO_4$ (0.5 ml). The samples were sealed and kept for another 30 min at 37°. Following the incubation the glass wool was transferred to scintillation vials containing 10 ml of scintillation fluid (9 ml toluene scintillator + 1 ml absolute alcohol). Sample radioactivity was measured with an LKB-Wallac Rack-Beta-1217 liquid scintillation counter.

* Statistically significant variation with respect to control ($P < 0.05$).

Table 4. Effect of L-carnitine on U-^{14}C-palmitic acid oxidation to $^{14}CO_2$ and ^{14}C-soluble products by rat heart homogenates *in vitro*

No.	L-Carnitine concentration (μM)	dpm/100 mg tissue · hr $^{14}CO_2$	dpm/100 mg tissue · hr ^{14}C-soluble products
1	—	7,697 (6,994–8,399)	20,544 (20,325–20,950)
2	1	12,195 (10,826–12,939)*	29,325 (26,522–31,760)*
3	10	25,632 (24,116–26,540)*, **	48,821 (46,630–52,615)*, **

The hearts of intact animals were used to prepare the homogenate. Composition of the incubation medium is specified in the footnote to Table 3. U-^{14}C-palmitic acid (0.1 mM) in combination with fraction V albumin (the final concentration 6 mg/ml) served as radioactive substrate. Incubation time: 60 min at 37°. $^{14}CO_2$ and ^{14}C-soluble products were measured as described in the footnote to Table 3 and in Materials and Methods.

* Statistically significant variation with respect to group 1 ($P < 0.05$).

** Statistically significant variation with respect to group 2 ($P < 0.05$).

myocardial homogenates to variations in the concentration of L-carnitine in the incubation medium.

Continuous administration of oral THP in the dose 200 and 400 mg/kg for 10 days brings about a dose-dependent reduction in the production of β-hydroxybutyrate in rats after 48-hr fasting (Table 5).

Isoproterenol administration to rats brings about a fall in the free carnitine content in the myocardium by 56.8% concurrent with a 4.2-fold elevation of the long-chain acyl carnitine concentration. Following THP treatment (100 mg/kg orally for 10 days) subsequent administration of isoproterenol results in a reduction in free carnitine concentration by 77.8%, as compared with control animals, and by 48.7% in comparison with the rats receiving isoproterenol. The level of long-chain acyl carnitine observed in response to isoproterenol, given after THP pretreatment for several days, exceeds 1.74-fold the control level, i.e. THP effectively reverses the isoproterenol-induced elevation in the content of this metabolite in the myocardium (Table 6).

Exposure to isoproterenol brings down the ATP content and enhances the amount of AMP by 50.7 and 47.8%, respectively. Such a redistribution in the levels of adenine nucleotides causes a reduction in energy charge by 1.33-fold. A prior administration of THP effectively protects the myocardium from isoproterenol-induced variations in the content of energy substrates (Table 7).

The protective effect of THP capable of reversing

Table 5. β-Hydroxybutyrate content in rat blood serum

Diet	No.	Group	β-Hydroxybutyrate (μmole/ml)
Fed rats	1	Control	0.070 (0.039–0.147)
48-hr fasting rats	2	Control	1.775 (1.434–2.165)*
	3	THP 200 mg/kg	0.936 (0.805–1.036)*, **
48-hr fasting rats	4	Control	2.473 (1.871–3.150)*
	5	THP 400 mg/kg	0.269 (0.146–0.478)*, **

THP in the dose 200 and 400 mg/kg was given orally to rats for 10 days as aqueous solution. On the 10th day, one hour following the last administration the animals were sacrificed by decapitation. β-Hydroxybutyrate content was assayed in the serum [26].

* Statistically significant variation with respect to group 1 ($P < 0.01$).

** Statistically significant variation with respect to group 2 ($P < 0.01$).

Table 6. Free carnitine and long-chain acyl carnitine content in rat myocardium

No.	Experimental group	Free carnitine (nmole/g tissue)	Long-chain acyl carnitine (nmole/g tissue)
1	Control	447 (260–870)	43 (16–78)
2	Isoproterenol	193 (93–302)*	182 (117–214)*
3	THP + isoproterenol	99 (60–140)*, **	75 (68–110)*, **

An aqueous solution of THP (100 mg/kg) was administrated orally to rats for 10 days. An aqueous solution of isoproterenol (50 mg/kg) was injected s.c. on the 10th day, 1 hr later the animals were sacrificed by decapitation. The hearts were extracted, frozen between steel blocks cooled in liquid nitrogen,ground to powder in liquid nitrogen and treated as described in Materials and Methods. Animals from group 2 received only s.c. injections of isoproterenol (50 mg/kg). Animals in group one served as controls.

* Statistically significant variation with respect to group 1 ($P = 0.005$).

** Statistically significant variation with respect to group 2 ($P < 0.01$).

Table 7. ATP, ADP, AMP content and energy charge in rat myocardium

No.	Experimental group	ATP (μmol/g tissue)	ADP (μmol/g tissue)	AMP (μmol/g tissue)	E
1	Control	2.84 (2.40–3.50)	1.16 (0.76–1.47)	0.71 (0.38–0.92)	0.770 (0.720–0.850)
2	Isoproterenol	1.40 (0.64–1.89)*	1.10 (0.93–1.44)	1.05 (0.71–1.28)*	0.580 (0.390–0.760)*
3	THP + isoproterenol	2.15 (1.25–2.70)*, **	1.04 (0.70–1.45)	0.60 (0.39–0.84)**	0.700 (0.570–0.800)**

The experiment was designed as specified in the footnote to Table 6.
* Statistically significant variation with respect to group 1 ($P < 0.01$).
** Statistically significant variation with respect to group 2 ($P < 0.05$).

E is energy charge: $\frac{2ATP + ADP}{2(ATP + ADP + AMP)}$.

adrenomimetic-mediated lesions in the myocardium was also confirmed by measuring the activity of serum creatine phosphokinase and that of cardiac LDH isozyme. Epinephrine raises serum creatine phosphokinase activity by 1.98-fold and stimulates the emergence of the myocardium-specific LDH isozyme. THP (200 mg/kg), given orally for 10 days, reverses the increase in creatine phosphokinase activity and decreases the release of cardiac LDH isozyme into the bloodstream (Table 8).

DISCUSSION

The reaction catalysed by γ-butyrobetaine hydroxylase terminates the chain of reactions involved in carnitine biosynthesis [28]; it occurs in the liver and kidneys, wherefrom the synthesised carnitine is transferred to other organs and tissues including the myocardium [29, 30].The ability of THP to suppress γ-butyrobetaine hydroxylase (Figs 1 and 2, Table 1) is a crucial stage in the molecular mechanism of action of the drug. Depressed carnitine biosynthesis subsequently leads to a drop in the concentration of its free and acylated forms in the myocardium (Table 2).

The oxidation of 1-^{14}C-oleic acid to $^{14}CO_2$ is inhibited concurrently with the free carnitine concentration decline in the myocardium (Table 3). In aminals fed a fat-rich diet, which are characterised by enhanced fatty acid metabolism, the THP-induced decrease in the free carnitine level causes a more marked inhibition of fatty acid metabolism in the myocardium. The effects of the fat-rich diet resemble, to a certain extent, the metabolic changes observed in ischaemic myocardium. In this case, accumulation of FFA activation products (acyl-CoA, acyl carnitine) and partially oxidised intermediates thereof is possible.

THP did not exert any effect on $^{14}CO_2$ formation from 1-^{14}C-palmitoyl-L-carnitine (Table 3). Provided the observed changes in $^{14}CO_2$ production from 1-^{14}C-oleic acid were due to the dilution of the CO_2 pool or its fixation in the carboxylation reaction, equally directed alterations would be also expected with 1-^{14}C-palmitoyl-L-carnitine used as a radioactive substrate. The lack of effect on the oxidation of this substrate suggests that the decreased formation of $^{14}CO_2$ from oleic acid is most probably explained by the ability of THP to inhibit carnitine-dependent oxidation of fatty acids. The capacity of THP to lower the concentration of free carnitine underlies the mechanism of this type of effect.

Experimental evidence obtained with L-carnitine indicates that myocardium homogenates are sensitive to changes in L-carnitine concentration in the incubation medium (Table 4). That THP inhibits the oxidation of fatty acids is confirmed by ketogenesis data in fasting rats. Continuous administration of THP, depending on the drug dose, prevents β-hydroxybutyrate accumulation in rat serum stimulated by fasting (Table 5).

The inhibitory effect of THP on FFA metabolism is more manifest under conditions conducive to its stimulation. In this case, the THP-elicited decrease in carnitine concentration can reach absolute values leading to a dramatic inhibition of FFA oxidation.

Table 8. Creatine phosphokinase (CK) and myocardium-specific lactic dehydrogenase (LDH) in rat blood serum

No.	Experimental group	Enzyme activity, U/l CK	LDH (myocardium-specific)
1	Control	2,868 (2,014–5,313)	0
2	Epinephrine	5,700 (4,900–8,000)	480 (250–640)*
3	THP (25 mg/kg) + epinephrine	6,280 (3,700–10,700)*	310 (140–545)*
4	THP (100 mg/kg) + epinephrine	4,500 (3,500–6,100)*	360 (0–780)*
5	THP (200 mg/kg) + epinephrine	3,300 (2,177–6,000)**	70 (0–90)*, **

An aqueous solution of THP (25, 100 and 200 mg/kg) was administered orally to animals in groups 3, 4 and 5, respectively. An aqueous solution of epinephrine (3 mg/kg) was injected s.c. on the 10th day, 1 hr following the last administration. The animals were sacrificed by decapitation 24 hr after epinephrine administration and enzyme activity was assayed in blood serum. Animals in group 2 received epinephrine only. Animals in group 1 served as controls.

* Statistically significant variation with respect to group 1 ($P < 0.05$).

** Statistically significant variation with respect to group 2 ($P < 0.05$).

Adrenergic stimuli are among the pathogenic factors contributory to stress-induced and ischaemic lesions in the myocardium [31]. The loss of intracellular free carnitine in ischaemia is accompanied by acyl carnitine accumulation in the myocardium [1–3, 8–10]. Similar changes have been observed after exposure to isoproterenol (Table 6). The low level of free carnitine in the myocardium may be due to the loss of carnitine from the cells with damaged plasma membranes. Such an abrupt drop in carnitine content can lead to undesirable changes in myocardial energy metabolism because of inhibited FFA metabolism. Coupled with the inability of myocardial cells to maintain the required level of proton acceptors this results in a more dramatic suppression of aerobic metabolism accompanied by the accumulation of metabolic intermediates of FFA (long-chain acyl carnitine).

These alterations lead to a lowering of ATP content, impaired energy charge and to lesions in the cellular plasma membranes in the myocardium (Table 7 and 8).

The drop in carnitine concentration elicited by γ-butyrobetaine hydroxylase inhibition upon THP administration appears to occur gradually and can lead to the release of considerable amounts of NAD participating in glycolysis and Krebs' cycle reactions. The total energy output may remain constant possibly due to the enhancement of the alternative pathways of energy production, e.g. via glycolysis. Accelerated utilisation of glycose can be regarded as a factor contributory to the preservation of cell integrity in ischaemic myocardium [32, 33].

The above findings are supported by data obtained by studying ATP content and energy supply in the myocardium as well as enzyme activity in blood serum following repeated THP administration (Table 5 and 6).

The cardioprotective effect of THP may be associated with its ability to inhibit γ-butyrobetaine hydroxylase and, consequently, to reduce myocardial free carnitine, resulting in the prevention of long-chain acylcarnitine accumulation and depressed FFA oxidation.

Reportedly, the inhibition of FFA oxidation can be used to reverse metabolic changes in ischaemic myocardium and in diabetes [34, 35]. However, the discovered mechanism of action allows a distinction to be drawn between THP and other known FFA oxidation inhibitors also active as carnitine cycle modulators.

2-[5-(4-Chlorophenyl)pentyl]oxirane and (*S*)-4-hydroxyphenylglycine (Oxfenicine) possessing cardioprotective properties act as carnitine acyl transferase inhibitors [14, 15]. Among compounds with hypoglycaemic and antidiabetic properties 2-tetradecylglycydic acid and emeriamin-(*R*)-3-amino-4-trimethylaminobutyrate are inhibitors of the carnitine acyltransferase I, but 2-(3-methylcynnamylhydrazono)propionate is carnitine acylcarnitine translocase inhibitor [36–38].

Examination of the biochemical mechanism of action of these compounds affecting carnitine-dependent oxidation of FFA reveals that the effect of THP, in contrast to that of other inhibitors of carnitine-dependent fatty acid oxidation, is manifest one step earlier, i.e. it is associated with the inhibition of carnitine biosynthesis from γ-butyrobetaine.

Regulation of the carnitine cycle is a pathogenetically justified approach to rational amelioration of impaired myocardial metabolism in ischaemia.

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