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Anti-arrhythmic effects of (−)-carnitine chloride and its acetyl analogue on canine late ventricular arrhythmia induced by ligation of the coronary artery as related to improvement of mitochondrial function

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1 Using the two-stage coronary ligation method, first described by Harris, (1950), anti-arrhythmic effects (AAE) of (−)-carnitine chloride (LCC) and acetyl (−)-carnitine chloride (ALCC) were studied in conscious unrestrained dogs in comparison with those of disopyramide (D). Two-stage ligation of the coronary artery resulted in a significant decrease in the myocardial free carnitine content.

2 Intravenous administration of LCC (300 mg kg^{-1}) and D (5 mg kg^{-1}) suppressed the ventricular arrhythmia induced by coronary ligation after 24 hours. ALCC (300 mg kg^{-1}) was found to be less potent.

3 An improvement of the mitochondrial function (respiratory control index (RCI) and oxidative phosphorylation rate (OPR)) was noted with LCC and ALCC and there was a linear correlation between AAE expressed as reduction of arrhythmic ratio and improvement in the OPR, whereas there was no improvement in mitochondrial function after D.

4 Plasma carnitine concentration was increased after administration of LCC, attaining the value of around 8 mM at 10 min after 300 mg kg^{-1}. At 60 min, the plasma carnitine concentration was still about half as high as at 10 min. After ALCC, both acetyl carnitine and free carnitine were found in the plasma. The concentration of the former was decreased after attaining a peak value of around 0.2 mM at 10 min, while the plasma concentration of free carnitine was gradually increased.

5 The anti-arrhythmic effects of LCC and ALCC were ascribed to the improvement of mitochondrial oxidative phosphorylation, while effects other than the improvement of the mitochondrial activity were suggested as mechanisms of anti-arrhythmic effects of D.

Introduction

It is well documented that the myocardium utilizes free fatty acids (FFA) as a major exogenous energy source in the aerobic state, and (−)-carnitine, present in abundance in the myocardium as a normal constituent, plays an important role in fatty acid metabolism as a carrier for transmembrane movements of fatty acyl groups. Fatty acid metabolism can be augmented through facilitation of the transport of acyl CoA esters as a permeable carnitine intermediate from the cytoplasm to the sites of oxidation in mitochondria (for references see Opie, 1979; Shug, 1979).

Recently much attention has been focused on the arrhythmogenicity of FFA under conditions of ischaemia as a result of many clinical and experimental observations (Oliver *et al.*, 1968; Kurien *et al.*, 1969, 1971; Willebrands *et al.*, 1973; Cowan & Vaughan Williams, 1977; 1980). Loss of (−)-carnitine from the myocardium and accumulation of FFA and its intermediates such as long chain acyl CoA esters and long chain acyl (−)-carnitine in the cardiac tissue

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during ischaemia (Whitmer *et al.*, 1978) and consequent inhibition of the translocation of adenosine 5'-triphosphate (ATP) and adenosine 5'-pyrophosphate (ADP) across the inner mitochondrial membrane (for references see: Shug, 1979) have been advocated as a cause of the mitochondrial respiratory dysfunction, culminating in the deterioration of the myocardial mechanical performance and serious cardiac arrhythmias (Kjekshus & Mjøs, 1972; Folts *et al.*, 1978) and it was postulated that replenishment of (−)-carnitine would reduce the incidence of serious arrhythmias by improving fatty acid metabolism. Indeed, it was reported that carnitine exerted anti-arrhythmic effects on the cardiac dysrhythmias associated with ischaemia or excess FFA (Folts *et al.*, 1978; Suzuki *et al.*, 1981).

In the present study an attempt was made in conscious unrestrained dogs to evaluate the anti-arrhythmic effects of (−)-carnitine and acetyl (−)-carnitine, on the ventricular arrhythmias induced by two-stage coronary ligation, in comparison with those of disopyramide. To elucidate the biochemical background of the anti-arrhythmic effects, the effects of these substances on the respiratory functions of myocardial mitochondria were also studied.

Methods

Arrhythmic models

Thirty three beagle dogs of either sex, weighing 6.5 to 9.5 kg (6 to 10 months of age) were fasted for 24 h before and after the operation. Just before the induction of anaesthesia, a blood sample (4 ml) was withdrawn from the brachiocephalic vein with a heparinized syringe. The animals were anaesthetized with halothane inhalation (0.8% in 95% O_2 plus 5% CO_2) using an animal respirator (Takashima Shoten TB-100 I) and a vaporizer (Cyprane, Floutec 3) after induction of anaesthesia with sodium thiopentone (20 mg kg^{-1} i.v.). After an intramuscular injection of suxamethonium chloride (40 $\mu g\ kg^{-1}$) and atropine sulphate (0.5 mg kg^{-1}), catheters were inserted into the aortic arch and superior vena cava through the left carotid artery and the jugular vein, respectively. The other free end was led subcutaneously to emerge at the back of the neck. A left thoracotomy was performed through the fourth intercostal space to expose the heart. A bipolar silver electrode was attached to the epicardial surface of the left auricle to record the atrial electrocardiogram. The lead wire was led subcutaneously to emerge behind the neck of animals. The anterior descending branch of the left coronary artery was dissected free from the surrounding connective tissue and two-stage ligation was performed according to the method originally described by Harris (1950). In some animals only the dissection of the left coronary artery was performed (sham-operated animals). During the operation, the condition of the animals was monitored by observing the aortic blood pressure, atrial electrocardiogram and standard limb lead II ECG. Halothane inhalation was stopped after the second ligation. However, artificial respiration was maintained until the resumption of spontaneous respiration. After the operation, the animals were given penicillin (100,000 i.u.) and streptomycin (0.5 g) intramuscularly.

Experiments were undertaken in the conscious, unrestrained state 24 h after coronary ligation. Each beagle dog was placed in a separate cage (42 × 60 × 39 cm) and allowed to adapt to experimental circumstances for 1 h before starting experiments. During this period the aortic blood pressure, ECG and atrial electrocardiogram were continuously monitored. Blood samples (4 ml) were withdrawn for determination of the plasma FFA and free (−)-carnitine. Throughout the experiments the aortic blood pressure was recorded on a linearly recording ink-writing oscillograph (Watanabe Instruments, WTR-281) through the aortic catheter placed in the aortic arch, using a telemeter for measurement of the blood pressure (Nishimu Telemetry System) devised by Nonaka & Ueno (1980). The three high speed (25 cm s^{-1}) recordings of the atrial electrocardiogram and ECG (Standard lead II) conducted at intervals of 10 min before administration of the drugs, using a Biomedical telemeter (Nihon Kohden, RZ5) and recorded on an ink-writing oscillograph (Nihon Kohden WI-180), provided the control data. After administration of the drugs the recordings were made at 30 s, every 1 min during the initial 10 min, every 2 min during the next 20 min and finally every 5 min for the period of 30 min. After the observation period of 1 h, the second administration of the same dose of drug was carried out and recordings of the various parameters were performed following the same protocol as in the first administration. To express the severity of arrhythmia, the arrhythmic ratio, defined as the number of ventricular ectopic beats divided by the total heart rate (Hashimoto *et al.*, 1982), was used. When the second administration of the drug resulted in an identical response to the first, the animals were immediately anaesthetized with α-chloralose and urethane (45 mg kg^{-1} and 450 mg kg^{-1}) via the venous catheter and the last sample of the venous blood (4 ml) was taken. A left thoracotomy was performed under artificial respiration again and the heart was rapidly excised. The excised heart was rinsed in ice-cold physiological saline solution and the visible infarction area was removed. The ventricular myocardium of the adjacent area was dissected out and about 1 g portion was frozen in liquid nitrogen. The frozen muscles were

stored at − 80°C until the analyses of the myocardial carnitines. Residual portions of the dissected myocardium (approximately 2 g) were used for preparation of the mitochondria. After gentle blotting they were weighed and minced using small scissors.

Blood samples for determination of plasma FFA, (−)-carnitine and acetyl (−)-carnitine were withdrawn through the implanted venous catheter at 3, 5, 10, 20, 30 and 60 min after drug administration and were centrifuged immediately at 3,000 r.p.m. at 4°C for 15 min. Separated plasma was frozen with liquid nitrogen and kept at − 80°C.

Drugs were injected through an indwelling venous catheter for 170 s for both (−)-carnitine chloride and its acetyl analogue and the control saline solution (NaCl 7.26% w/v) and for 10 s for disopyramide. After injection, 2 ml of physiological saline solution was always flushed through the catheter. The doses of (−)-carnitine chloride and its acetyl analogue were given as mg kg^{-1} of the free base.

Preparation of mitochondria

Mitochondria were prepared following the method of Sordahl *et al.* (1971). The ice-cold isolation medium containing 0.21 M D-mannitol, 0.07 M sucrose, 0.001 M EGTA and 0.01 M Tris base (pH 7.4) was added to the weighed and minced myocardial tissues (1:10, by weight). After incubation with protease (15 mg g^{-1} tissue) (Sigma type IV) for 25 min under gentle stirring, the mixture was centrifuged at 500 *g* for 5 min. The supernatant was discarded. The precipitated tissue was resuspended in the ice-cold isolation medium (1:10), and homogenized with 5 strokes of a motor-driven Potter-type loosely fitting Teflon homogenizer at 500 r.p.m. The homogenate was centrifuged at 500 *g* for 5 min. The supernatant was again centrifuged at 6,500 *g* for 10 min. The precipitated mitochondrial pellet was washed 3 times with a small volume of an ice-cold incubation medium containing 0.3 M D-mannitol, 0.01 M KCl, 0.2 mM EGTA 0.005 M potassium-phosphate buffer and 0.01 M Tris base (pH adjusted to 7.4). Finally the mitochondrial pellet was suspended in the incubation medium at a concentration of approximately 10 mg ml^{-1}. The entire procedure was carried out at 0°C. Mitochondrial protein was measured by the method of Lowry *et al.* (1951).

Measurement of mitochondrial respiratory functions

Measurement of mitochondrial respiratory functions was accomplished polarographically by a method described by Estabrook (1967) using a Clark type oxygen electrode (Yellow Springs Instruments, Model 5331) and an Oxygen Monitor (Yellow Springs Instruments, Model 53) in an essentially closed cuvette. The mitochondria (final concentration 0.5–1 mg ml^{-1}) were incubated at 25°C in 2 ml of the incubation medium in the presence of 0.015 M succinate as a substrate and 250 μM ADP.

Measurements of free carnitine and acetyl carnitine contents in plasma and myocardium

Perchloric acid extracts were prepared at 0°C from the pulverized myocardium and the stored plasma using the method described by Pearson *et al.* (1974). (−)-Carnitine was measured in coupled enzymatic assay according to the method of Marquis & Fritz (1964). Acetyl (−)-carnitine was determined enzymatically by the method described by Pearson *et al.* (1974).

Drugs used

(−)-Carnitine and acetyl (−)-carnitine were provided as cloride by Earth Chemical Co., Ltd (Ako, Japan) and were dissolved in distilled water in a concentration of 20% w/v (as free (−)-carnitine). The pH of the solution was adjusted to 6.8 with 1N NaOH solution. Larger volumes of 1N NaOH solution were necessary to adjust the pH of the (−)-carnitine chloride solution. The final concentration of NaCl was calculated to be 7.26% w/v. Thus, 7.26% w/v NaCl solution (saline solution) (1.5 ml kg^{-1}) was used as a vehicle control. Following the pH adjustment, solutions were filtered through a sterile millipore filter with mesh of 0.45 μm (Millipore Corporation, Cathivex).

Disopyramide phosphate was provided by Roussel Laboratories.

Statistics

All the parameters were expressed as mean ± s.e. Statistical significance of differences between mean values was determined using paired or non-paired Student't *t* test and a level of $P < 0.05$ was considered significant.

Results

Evaluation of anti-arrhythmic effects

Twenty four hours after coronary ligation, the majority of the cardiac electrical activity of the conscious unrestrained dogs was multifocal and ectopic ventricular rhythms; the average arrhythmic ratio calculated as the number of the ventricular ectopic beats divided by the total heart rate was 96.7 ± 0.7% ($n = 33$) before drug application. The tissue contents of free (−)-carnitine of the coronary-ligated and

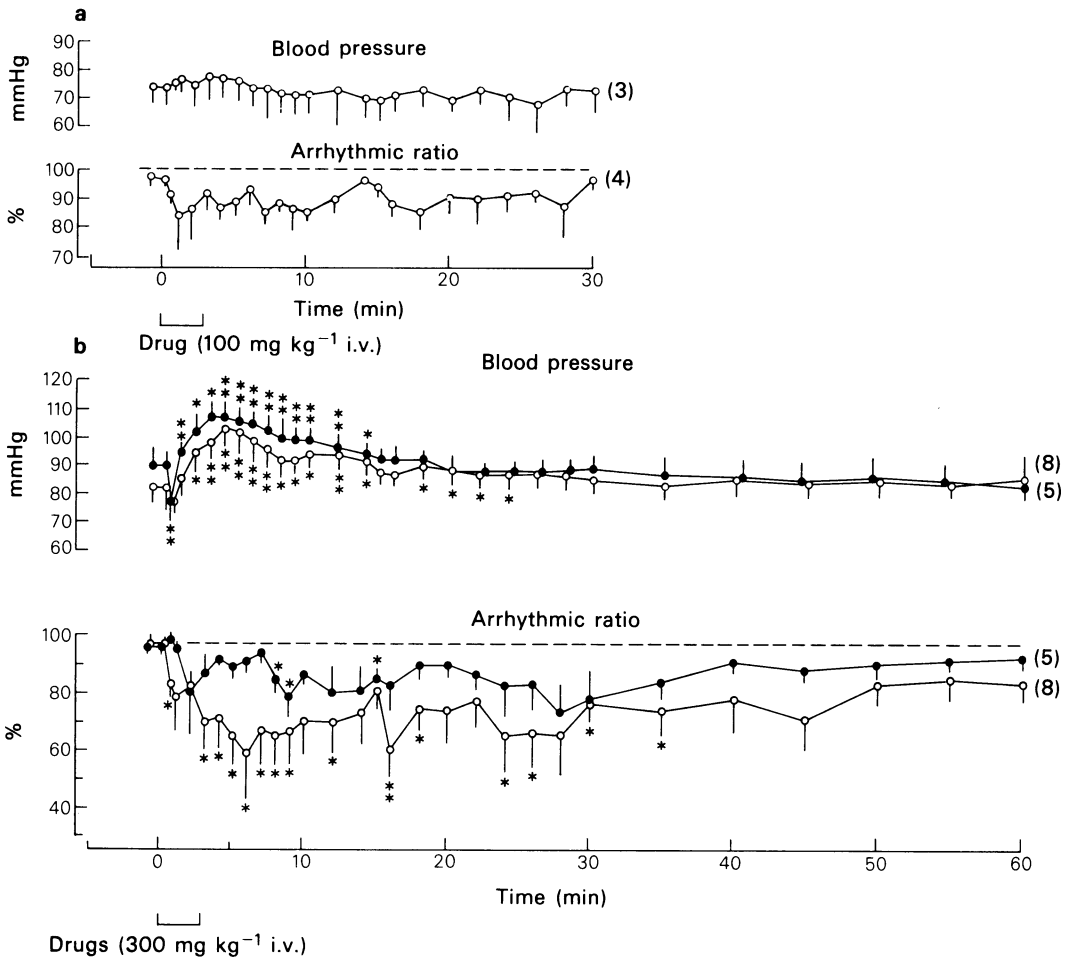

Figure 1 Effects of (−)-carnitine chloride (open circles) (a) 100 mg kg^{-1} (i.v.) and (b) 300 mg kg^{-1} (i.v.) and acetyl (−)-carnitine chloride (closed circles) (b) 300 mg kg^{-1} (i.v.) on the ventricular arrhythmias and blood pressure of the coronary-ligated dogs, 24 h after coronary ligation. Each point and the vertical line indicate the mean and the s.e., respectively. Numbers in the parentheses are the numbers of animals used. Asterisks indicate values significantly different from the values before administration of the drugs, $*P<0.05$, $**P<0.01$.

saline-administered dogs were significantly lower than those of the sham-operated dogs (791 ± 194 nmol g^{-1} ($n=4$) vs. 1398 ± 31 nmol g^{-1} ($n=4$), $P<0.05$).

Figure 1 depicts the anti-arrhythmic effects of (−)-carnitine chloride. Low doses (100 mg kg^{-1} i.v.) reduced the arrhythmic ratio without producing any obvious changes in the blood pressure, while the anti-arrhythmic effects of the higher dose (300 mg kg^{-1} i.v.) were associated with a rise in blood pressure. Although qualitatively similar to that observed with (−)-carnitine chloride, the effects of acetyl (−)-carnitine on the arrhythmias were less pronounced. Even with 300 mg kg^{-1} a significant effect was noted only transiently at around 10 min after the injection (Figure 1). There was an immediate fall in blood pressure, which was followed by a prolonged elevation lasting for 14 min. As can be seen from Figure 2, injection of the control saline solution (vehicle) induced a small but significant increase in the blood pressure. However, it had no effects on the ventricular dysrhythmias.

Intravenous administration of disopyramide (5 mg kg^{-1}) produced an immediate and pronounced anti-arrhythmic effect, the arrhythmic ratio reaching almost zero %. The anti-arrhythmic effect was associated with a significant rise in the blood pressure and lasted for about 30 min (Figure 2).

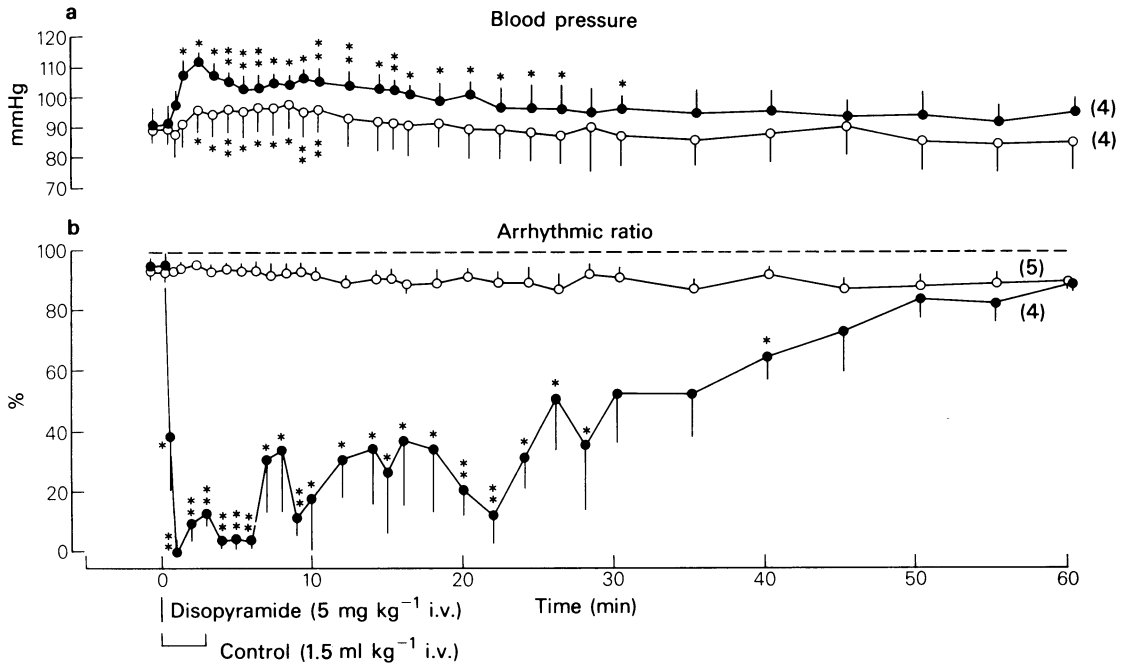

Figure 2 Effects of saline solution (1.5 ml kg^{-1}) (open circles) and disopyramide (closed circles) on the ventricular arrhythmias (b) and blood pressure (a) of the coronary-ligated dogs (24 h after coronary ligation). Asterisks indicate values significantly different from the values before drug administration, $*P<0.05$, $**P<0.01$.

After intravenous administration of acetyl (−)-carnitine chloride vomiting was invariably observed, occurring several times within 10 min, while 300 mg kg^{-1} of (−)-carnitine chloride base induced vomiting only in one of 10 dogs, and soon after the second trial. Restlessness was always observed in all dogs during the injection of (−)-carnitine chloride and its acetyl analogue at high doses.

Effects on mitochondrial respiratory function

Figure 3 illustrates the effects of (−)-carnitine chloride and its acetyl analogue, on the ADP: O ratio, respiratory control index (RCI) and oxidative phosphorylation rate (OPR) of mitochondria, in comparison with those of disopyramide. Two-stage ligation resulted in a significant decrease in RCI and OPR. However, there was no change in the ADP:O ratio. After administration of (−)-carnitine chloride and acetyl (−)-carnitine chloride a significant improvement in the RCI and OPR was noted, while there was no change in the ADP:O ratio. The anti-arrhythmic agent, disopyramide produced an elevation of the mitochondrial ADP:O ratio. However, it produced no improvement in the RCI or OPR.

Relationship between anti-arrhythmic effects and mitochondrial respiratory functions

Figure 4 shows the relationships between the maximum anti-arrhythmic effects of (−)-carnitine chloride and its acetyl analogue as well as disopyramide and the RCI or the OPR of the mitochondria isolated from the same animal. There were good correlations between the arrhythmic ratio and OPR of the mitochondria ($r=-0.789$, $P<0.001$) isolated from the sham-operated animals and from the animals treated with saline, (−)-carnitine chloride and acetyl (−)-carnitine chloride.

Changes in the plasma concentration of carnitine and acetyl carnitine

Figure 5 illustrates the changes in plasma concentration of free (−)-carnitine and acetyl (−)-carnitine after administration of (−)-carnitine chloride and acetyl (−)-carnitine chloride. Following the administration of (−)-carnitine chloride (300 mg kg^{-1}), plasma concentrations of free (−)-carnitine were increased attaining a value of approx. 8 mM at 10 min. At 60 min, plasma concentrations of the free

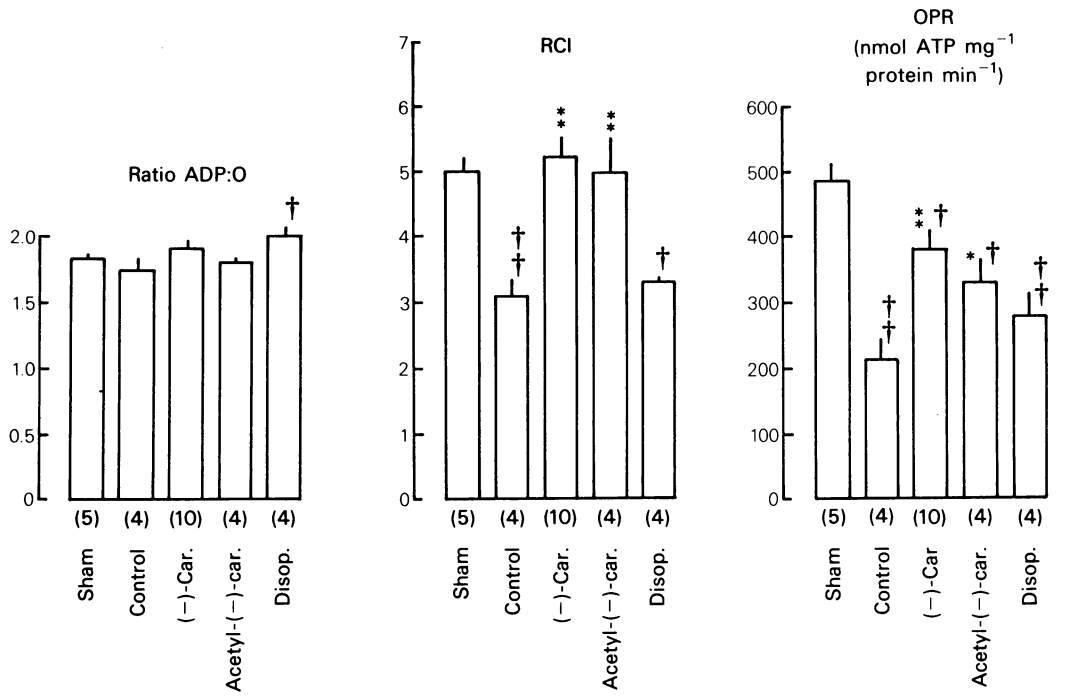

Figure 3 Effects of (−)-carnitine chloride ((−)-Car.), its acetyl analogue (Acetyl-(−)-car.) on the mitochondrial function as compared with the effects of sham-operation (Sham) and saline solution (Control) and disopyramide (Disop.). Numbers in parentheses are the numbers of animals tested. RCI: respiratory control index. OPR: oxidative phosphorylation rate. Asterisks indicate that values are significantly different from the values of the saline-administered animals $^{*}P<0.05$,$^{**}<0.01$. The daggers indicate values significantly different from the values of the sham-operated animals $\dagger P<0.05$, $\dagger\dagger P<0.01$.

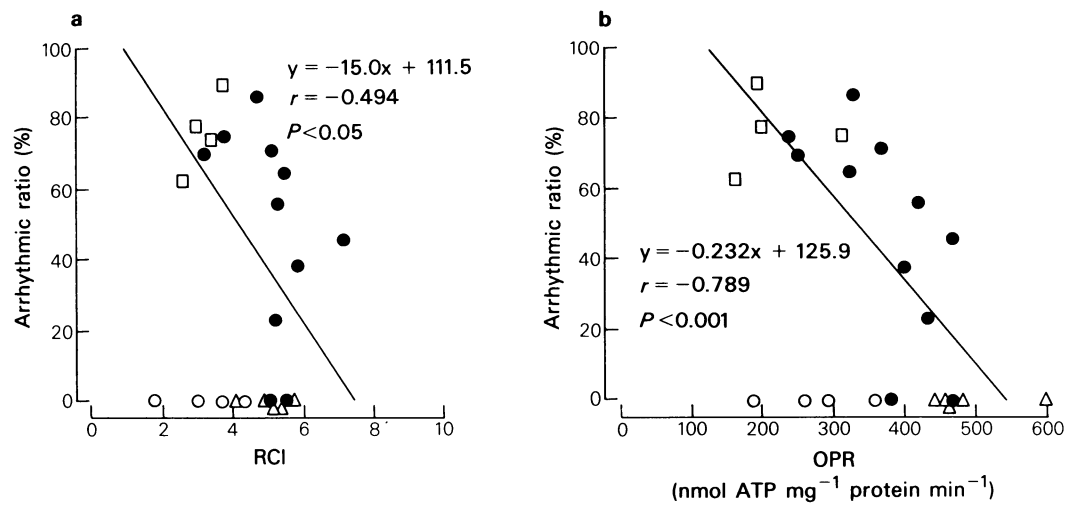

Figure 4 Relation between the arrhythmic ratio and the mitochondrial function, (a) respiratory control index (RCI) and (b) the oxidative phosphorylation rate (OPR). The closed circles represent the data from the animals treated with (−)-carnitine chloride and its acetyl analogue and the open circles animals treated with disopyramide. Open squares: saline-treated animals. Open triangles: sham-operated animals.

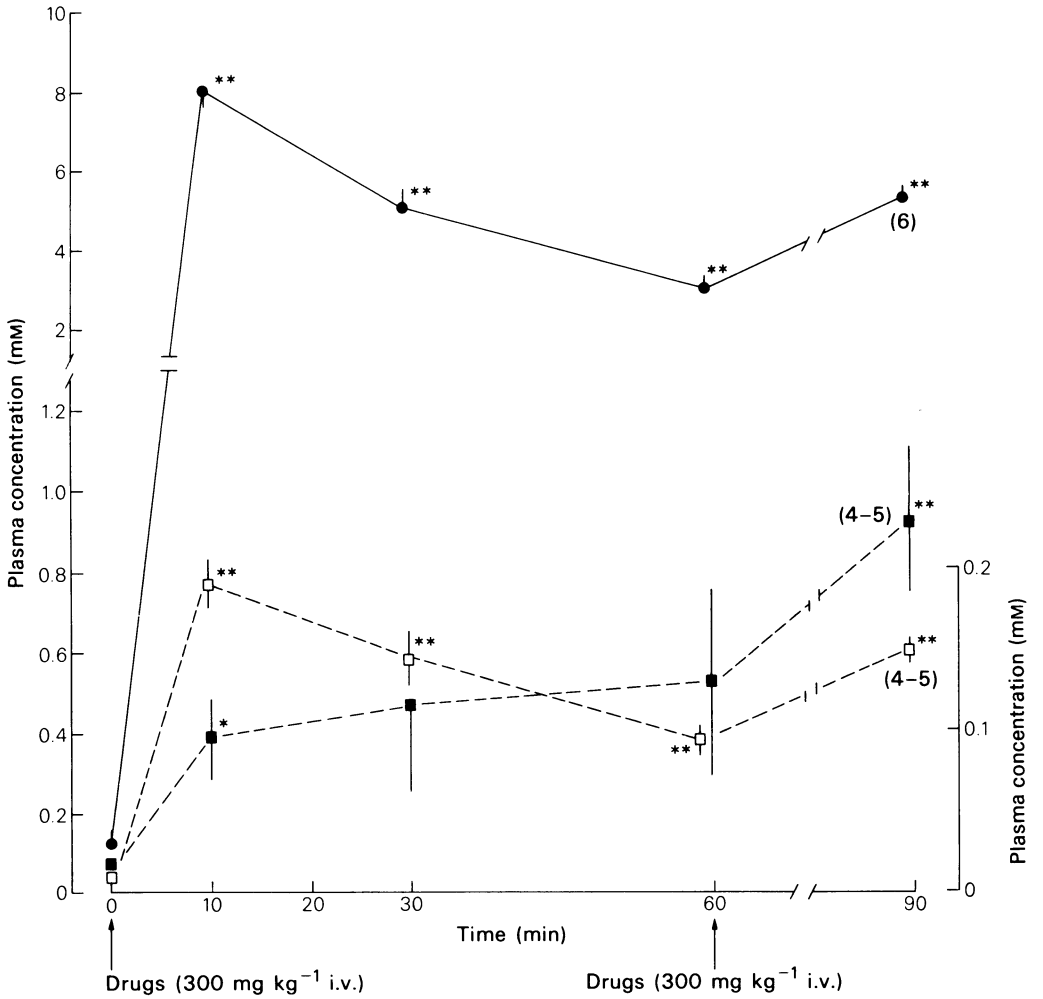

Figure 5 Changes in the plasma concentrations of (−)-carnitine and acetyl (−)-carnitine. Closed circles and squares represent the plasma concentrations of (−)-carnitine after the administration of (−)-carnitine chloride and its acetyl analogues (300 mg kg^{-1} i.v.), respectively; open squares represent the plasma concentration of acetyl (−)-carnitine after the administration of acetyl (−)-carnitine. Numbers in the parentheses are the numbers of animals used. The vertical scale on the left is for free carnitine, while that on the right, for acetyl carnitine. Asterisks indicate values significantly different from the values before drug administration $*P<0.05$, $**P<0.01$.

carnitine remained still about half as high as at 10 min. After intravenous administration of acetyl (−)-carnitine chloride (300 mg kg^{-1}), both acetyl (−)-carnitine and free (−)-carnitine were demonstrated in the plasma. The plasma concentration of (−)-carnitine was gradually increased, while that of acetyl (−)-carnitine was decreased exponentially after attaining a peak value of around 0.2 mM at 10 min. Neither the administration of the control saline solution nor of disopyramide (5 mg kg^{-1}) affected the plasma levels of free (−)-carnitine and acetyl (−)-carnitine (data not shown).

Discussion

The present study using the method of Harris (1950) clearly demonstrates that intravenous (−)-carnitine chloride at doses of 100 and 300 mg kg^{-1} can produce dose-dependent anti-arrhythmic effects. Although

much less potent in relation to the dose administered, acetyl (−)-carnitine seems to be more potent than (−)-carnitine at comparable plasma levels, for the plasma level (inclusive of the protein-bound form) attained after injection of 300 mg kg^{-1} of this compound was only 1/40th of that of the (−)-carnitine achieved after the injection of (−)-carnitine. The lower plasma level and the higher potency of the acetyl analogue could not be explained by a better penetrability of this compound into the tissues, for the uptake into human heart cells in culture was not different between these two compounds (Böhmer *et al.*, 1977; Mølstad *et al.*, 1977). The anti-arrhythmic effect observed after the acetyl analogue cannot be attributed to the (−)-carnitine produced; (−)-carnitine levels rose 90 min after injection of the acetyl analogue, but the anti-arrhythmic effect fell to an insignificant level at 60 min. Furthermore, the (−)-carnitine level attained after the acetyl analogue was quite low.

The impairment of the mitochondrial function was observed in the two-stage coronary-ligated dogs in association with a decrease in the myocardial free carnitine content. Administration of (−)-carnitine chloride and acetyl (−)-carnitine chloride resulted in an improvement of mitochondrial respiratory function as evidenced by a significant improvement in the RCI. ADP:O ratios of the mitochondria isolated from the myocardium of the coronary-ligated dogs were not different from those of the mitochondria from the sham-operated animals, and (−)-carnitine chloride and its acetyl analogue did not produce any effects on this parameter. However, as Edoute *et al.* (1979) indicated, the measurement of only ADP:O ratios could give erroneous information on the adequacy of myocardial oxidative phosphorylation in myocardial ischaemia or hypoxia. According to them, the mitochondrial oxidative phosphorylation rate (OPR) might be a more meaningful index to assess mitochondrial oxidative phosphorylation capacity in ischaemic or hypoxic tissue. Therefore, we also calculated the OPR and found that there was an improvement in the OPR after administration of (−)-carnitine chloride and acetyl (−)-carnitine chloride.

When the arrhythmic ratios were plotted against the OPR, a good correlation was found in the animals treated with saline, (−)-carnitine and acetyl (−)-carnitine and in the sham-operated animals. This suggests that the incidence of two-stage coronary ligation arrhythmia is connected to the impairment of the myocardial oxidative phosphorylation capacity, and that the anti-arrhythmic effects of (−)-carnitine and acetyl (−)-carnitine may probably be explained by the improvement in the mitochondrial oxidative phosphorylation capacity they produce. In contrast, there was no improvement of mitochondrial function after disopyramide. The reason why an improvement in the ADP:O ratio occurred with this compound is not clear at present. Anyway, mechanisms other than the improvement of mitochondrial function must be invoked to explain the marked anti-arrhythmic effects of this compound. The membrane stabilizing action, lengthening of the refractory period and depression of the conduction in the re-entry pathway, which have been reported by many investigators using isolated and *in vivo* heart preparations with arrhythmias (Mokler & Van Arman, 1962; Kus & Sasyniuk, 1975; 1976; Danilo *et al.*, 1977; Matsuda *et al.*, 1982; Yamada, 1982; Hashimoto *et al.*, 1982; 1983), could be the cause of the anti-arrhythmic effects of this compound.

Simultaneous with the cessation of the arrhythmia a pressor effect was observed with all the agents used. Although (−)-carnitine and its analogue can produce an improvement in the myocardial mechanical performance without producing any significant increase in the cardiac oxygen consumption and the coronary flow in the anaerobic and aerobic heart (Brooks *et al.*, 1977; Liedtke *et al.*, 1979), synchronization of the myocardial contraction consequent to a reduction of the ventricular ectopic rhythms may better explain the blood pressure rise produced by (−)-carnitine chloride and its acetyl derivative in our experiments, as the blood pressure rise was also noted with disopyramide. In addition, a high sodium load may have contributed partly to the hypertensive effect; the saline vehicle also produced an obvious pressor effect.

With 300 mg kg^{-1} acetyl (−)-carnitine restlessness and vomiting were observed in almost all the dogs. Restlessness was also observed during the intravenous injection of (−)-carnitine chloride (300 mg kg^{-1}). It may be due to a parasympathomimetic action in the gastrointestinal tracts; carnitine is a derivative of choline and acetyl carnitine is a derivative of acetylcholine (Blum *et al.*, 1971; Bongrani *et al.*, 1980). The parasympathomimetic action of these compounds are about 700 times less potent than acetylcholine. However, we should take such an effect into consideration because of the high doses used in the present study and much slower elimination from plasma of these compounds than acetylcholine (Yue & Fritz, 1962; Welling *et al.*, 1979). The actual plasma concentrations were found to be high. Therefore, a transient hypotension produced by these two carnitines could also be explained by parasympathomimetic effects on the blood vessels.

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