PHARMACOLOGICAL PRECONDITIONING WITH L-CARNITINE: RELEVANCE TO MYOCARDIAL HEMODYNAMIC FUNCTION AND GLYCOGEN AND LACTATE CONTENT

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ABSTRACT

Carnitine is a vital biologic substance facilitating fatty acids transport into mitochondria for ATP production. This study was to investigate the effects of pre-ischemic pharmacological preconditioning (PC) with L-carnitine (L-Car) on myocardial infarct size and cardiac functions in ischemic and reperfused isolated rat heart and meanwhile on left ventricular glycogen and lactate content. Isolated rat hearts were subjected to 30 min coronary artery occlusion followed by 120 min reperfusion. The hearts (n= 8-12) were perfused with L-Car (0.5-5 mM) only for 15 min before to 10 min after induction of ischemia. Preconditioning of the hearts with L-Car provided concentration-dependent cardioprotection as evidenced by improved postischemic ventricular functional recovery (developed pressure, left ventricular end diastolic pressure and coronary flow rate) and reduced myocardial infarct size ($p<0.001$). L-Car (2.5 mM) decreased both glycogen ($p<0.001$) and lactate ($p<0.05$) content in left ventricle during ischemia compared with the control. The results of this study demonstrate that L-Car pharmacologically precondition the hearts against ischemic and reperfusion injury in part by recovery of postischemic ventricular hemodynamic functions, depletion of glycogen and therefore reduction of lactate accumulation.

Keywords: L-carnitine, preconditioning, glycogen, lactate, isolated rat heart.

INTRODUCTION

Ischemic preconditioning (IPC) mediated by one or more brief periods of ischemia and reperfusion is considered to be the most powerful cardioprotective technique of recent years (Murry *et al.*, 1996). IPC has two cardioprotective phases, early, lasting up to 3-4 h and delayed ischemic preconditioning lasting from 1 to 4 days. Attempts to increase these phases have met with limited success. There is an essential need for developing a pharmacological preconditioning approach that can significantly increase these phases of cardioprotection. Carnitine is an essential cofactor under physiological conditions in the intermediary metabolism and transport of long-chain fatty acids from the cytoplasm of cells to the mitochondrial matrix for ATP production. This pathway within the mitochondria is the major source of energy for the heart (Neely and Morgan, 1974). Many studies have shown that L-carnitine (L-Car) exerts a protective effect against ischemic/reperfusion injury, and clinical trials confirm these beneficial effects although controversial results are observed (Ferrari *et al.*, 2004; Arsenian, 1997). Several studies have shown that L-carnitine reduces ischemic/reperfusion injury in heart by counteracting the toxic effect of free fatty acids, which increases during ischemia, and by improving glucose metabolism (Neely and Morgan, 1974). In addition to increase the rate of fatty acid transport into mitochondria, L-Car reduces the intramitochondrial ration of Acetyl-CoA to free CoA, thus stimulating the activity of pyruvate dehydrogenase and increasing the oxidation of pyruvate (Lopaschuk, 2000). It has also been shown that propionyl-L-carnitine which penetrates faster than L-Car into myocytes is effective in inhibiting of reperfusion ventricular fibrillation and production of free radicals presumably by accelerating pyruvate dehydrogenase pathway (Cui *et al.*, 2003; Lango *et al.*, 2001).

Myocardial glycogen stores are an essential endogenous source of ATP formation, enabling the myocytes to maintain ion pump activity and cellular integrity during no-flow ischemia (Stanley *et al.*, 1992; Eberli *et al.*, 1991; Weiss and Hiltbrand, 1985). However, uncontrolled glycogenolysis during ischemia may lead to lactate accumulation, intracellular acidosis, and cell death (Neely and Grotyohann, 1984). Several mechanisms are suggested for cardioprotective effects of IPC, including preischemic myocardial glycogen depletion (Bradamante *et al.*, 2000). The glycogen hypothesis states that glycogen depletion occurs during the preconditioning episodes and that the resultant lower glycogen levels during prolonged ischemia contribute to the attenuation of ischemic acidosis. This in turn results in less ischemic injury and contributes to improved post ischemic

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metabolic and contractile recovery. The aim of the present study was to study the effects of pre-ischemic administration of L-Car on infarct size and postischemic cardiac hemodynamic functions. Furthermore, because myocardial glycogen content is an important source of energy during ischemia (Stanley *et al.,* 1992), we investigated the effects of L-Car on left ventricle glycogen and lactate content after ischemia.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (270-330 g) were used in this study. Animals were given food and water ad libitum. They were housed in the Animal House of Tabriz University of Medical Sciences at a controlled ambient temperature of 25 ± 2°C with 50 ± 10% relative humidity and with a 12-h light/12-h dark cycle (lights on at 7:00 a.m.). This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No 85-23, revised 1985).

Surgical procedure

Animals were pretreated with i.p. injection of 300 IU heparin and anaesthetized by sodium pentobarbital (60mg/kg, i.p.). The hearts were excised rapidly and mounted on a non-recirculating Langendorff apparatus under 100 mmHg pressure at 37.5 °C and perfused with modified Krebs–Henseleit (K/H) solution that previously equilibrated with 95% O_2–5% CO_2 (Hausenloy *et al.,* 2003). A fluid filled latex balloon was introduced into the left ventricle through incision in the left atrium and inflated to give a pre-load of 8–10 mmHg. A 6/0 braided silk suture was placed around the left descending coronary artery. Following 20 min stabilization, coronary occlusion (30min) was achieved by threading the loose ends of the ligature through a polyethylene occluder and clamping in place. Release of the clamp allowed reperfusion (120 min) of the previously ischemic tissue (Hausenloy *et al.,* 2003).

Evaluation of left ventricular function

Left ventricular functions including left ventricular end diastolic pressure (LVEDP) and left ventricular developed pressure (LVDP) were measured at regular intervals by a pressure transducer connected to the latex balloon (Powerlab system, ADInstruments, Australia). Coronary flow rate (CFR) was measured by a time collection of the coronary perfusate that dripped from the heart. The heart rate (HR) was calculated from the ECG. Rate pressure product (RPP) was calculated by multiplying left ventricular developed pressure by HR.

Measurement of infarct size

To determine infarct size, at the end of the 120 min reperfusion period, the ligature around the LAD artery was re-tied and the heart was slowly perfused with 2-3 ml of saline solution containing 0.25% Evans blue dye (w/v) via the side arm of the aortic cannula. Perfusion of the hearts by Evans blue dye delineates the non-ischemic zone of the myocardium as a dark blue area. The hearts were freezed at –20°C then the ventricle of the frozen hearts were sliced transversely in a plane perpendicular to the apico-basal axis into 2 mm-thick sections. The slices then incubated by 1% (w/v) triphenyltetrazolium (TTZ) solution in phosphate buffer ($NaHPO_4$, 88 mM; NaH_2PO_4 1.8 mM, pH= 7.4) for 15 min at 37°C to dye the non-infracted region (Hausenloy *et al.,* 2003). This procedure resulted in the normally perfused tissue being stained blue, non-infracted, non-perfused tissue stained brick red and infracted tissue remaining unstained and appeared pale. TTZ stains the non-infracted myocardium a brick red color, indicating the presence of a formazin precipitate that results from the reduction of TTZ by dehydrogenase enzymes present in viable tissue (Zacharowski *et al.,* 2001). The tissue slices were then fixed in 10% formalin for 24 hr and then placed between two glass cover sheets. The sheets cause that the tissue color can clearly be seen also makes a convenient flat surface for directly tracing the dimensions of the infarct and risk zone on a transparent sheet. The slices were drawn onto transparent sheets then by using a computerized planimetry package; the percentage of infracted tissue within the volume of myocardium at risk was calculated (Hausenloy *et al.,* 2003).

Assessment of left ventricular glycogen and lactate content

Total glycogen was measured in triplicate from homogenates of frozen biopsies after degradation to glucose using the filter-paper technique and spectrophotometric detection at 340 nm (Solling and Esmann, 1975; Botker *et al.,* 1995). Lactate was measured from the same homogenates using enzymatic analyses and spectrophotometric detection as described by Hohorst (Hohorst, 1962).

Protocols

Hearts (n=8-12) were allocated randomly to (a) Drug free control; (b) the hearts which were perfused with 0.5, 2.5 and 5mM of L-Car-enriched K/H solution from 15 min before ischemia to 10 min after ischemia. In two other groups, one drug free control (Ischemic group) and the other treated with 2.5 mM L-Car-enriched Krebs only for 15 min before ischemia (Pre-ischemic L-Car group), at the end of ischemia the hearts were frozen within 2 second in liquid nitrogen and kept in -80°C until used for glycogen and lactate measurement. In this series of experiments, two groups of hearts were also used as sham operated, non-ischemic groups, the hearts treated exactly the same as above without ligation of the coronary artery in the presence (Sham+L-Car group) or absence (Sham) of L-Car.

STATISTICS

Except for glycogen and lactate contents that are presented as mean±SD, all results are presented as mean±SEM. A one-way ANOVA with LSD post hoc test was carried out to test any differences between the mean values of different groups. The differences between groups were considered significant at a level of $p<0.05$.

RESULTS

Effects of L-Car on cardiac functions during ischemia and reperfusion

Perfusion of 0.5, 2.5 and 5mM of L-Car-enriched K/H solution had no significant effect on LVDP, HR, RPP and CFR during ischemia (data was not shown). In all L-Car treated groups LVEDP was lower than control value at the end of ischemia (pre-reperfusion point; fig.1) which attained significance only by 0.5 mM of L-Car ($p<0.05$). Re-opening of the ligated coronary artery in controls resulted in an increase in LVEDP in early reperfusion time, which continued to 60 min after reperfusion (fig.1).

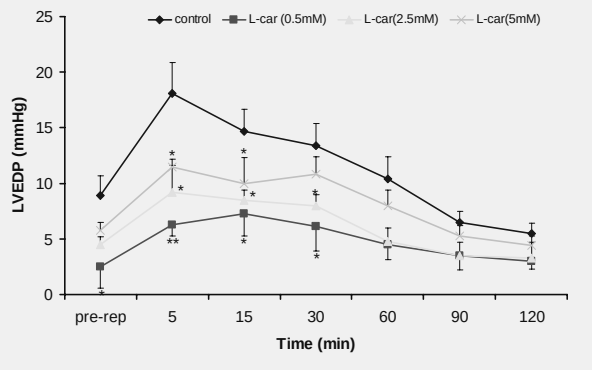

Fig. 1: The effects of pre-ischemic perfusion of isolated rat hearts with 0.5-5 mM L-Car on Left Ventricular End Diastolic Pressure (LVEDP) in the control and treated groups immediately before reperfusion and during 120 min reperfusion time. **p<0.01, * p<0.05 *vs* control. Pre-rep: Pre-reperfusion. Data are expressed as mean ±SEM.

Compared to the control group, pre-ischemic application of L-Car by all concentrations lowered the extent of LVEDP elevation (fig. 1) and prevented LVDP reduction (fig. 2) throughout the reperfusion time. As shown in fig. 1, LVEDP was significantly ($p<0.001$; $p<0.05$) lower than control value at 5, 15 and 30 min after reperfusion in the L-Car treated groups. Furthermore, compared to pre-reperfusion point, L-Car (2.5 and 5 mM) reserved RPP during the reperfusion period (table 1). Improvement of RPP and LVDP by pre-ischemic perfusion of 2.5 and 5 mM L-Car-enriched K/H solution increased CFR throughout the reperfusion phase (table 1). The effect was significant during the first 30 min of reperfusion time and 5 mM of the drug showed greater and longer effect.

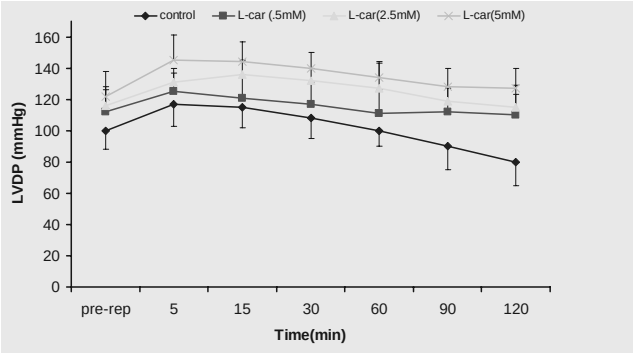

Fig. 2: The effects of pre-ischemic perfusion of isolated rat hearts with 0.5-5 mM L-Car on Left Ventricular Developed Pressure (LVDP) in the control and treated groups immediately before reperfusion (pre-rep) and during 120 min reperfusion time. Data are expressed as mean ±SEM.

Effects of L-Car on infarct size

In control group, the infarct size was 46.3 ± 3.5% while pre-ischemic perfusion of 0.5, 2.5, and 5mM of L-Car significantly reduced it to 25.9 ± 3, 17.3 ± 1.3 and 20.8 ± 1.5%, respectively (Fig.3; $p<0.001$). Risk zone volume (area at risk) in all groups was similar and did not show significant differences between groups.

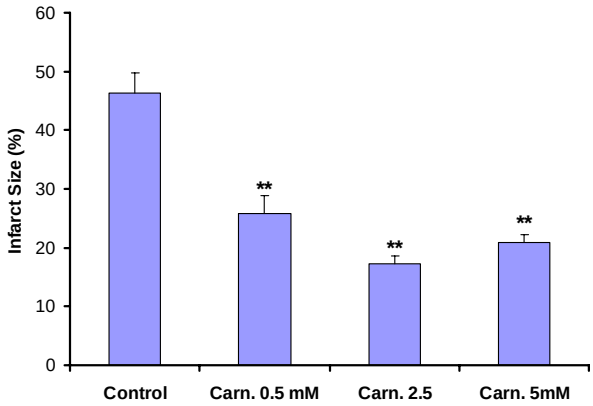

Fig. 3: The effects of pre-ischemic perfusion of isolated rat hearts with 0.5-5 mM L-Car (Carn.) on myocardial infarct size as a percentage of risk zone volume. **p<0.001 *vs*. control. Data are expressed as mean ±SEM.

Effects of L-Car on glycogen and lactate content in nonischemic and ischemic hearts

Perfusion of 2.5 mM of L-Car-enriched K/H solution of L-carntine for 15 min in sham operated, non ischemic isolated rat hearts, did not influence left ventricular either glycogen or lactate content (fig. 4). Compared to non-ischemic myocardium (sham group), after 30 min regional ischemia (ischemic group), left ventricular glycogen content declined slightly but not significantly, while the

lactate content increased significantly ($p<0.05$) by 62% (fig. 4). In comparison with the ischemic hearts, pre-ischemic perfusion of L-Car (2.5 mM; pre-ischemic L-Car group) produced a considerable reduction both in glycogen ($p<0.01$) and lactate ($p<0.05$) content of left ventricle (fig. 4).

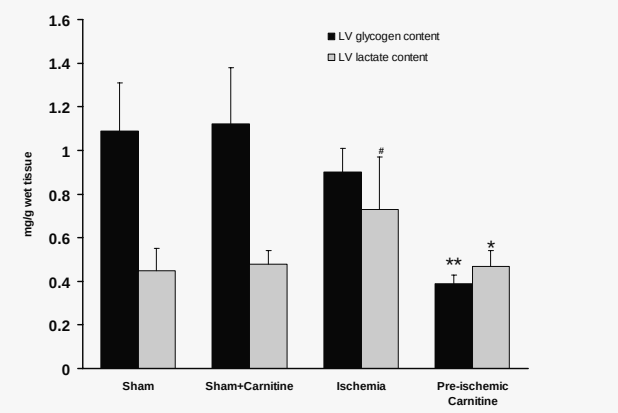

Fig. 4: Left ventricular (LV) glycogen (black columns) and lactate (gray columns) content in the hearts perfused with krebs in aerobic condition in absence (sham) or presence of 2.5 mM L-Car (sham+L-Car); in hearts subjected to 30 min regional ischemia without any treatment (ischemia) and in hearts that received L-Car only for 15 min prior to ischemia (pre-ischemia L-Car). *$p<0.05$; **$p<0.01$ *vs* ischemia group; $^{\#}p<0.01$ *vs* sham. Data are expressed as mean ±SD.

DISCUSSION

Several studies have shown that L-carntine reduces myocardial ischemia and reperfusion injury by preventing the accumulation of free fatty acids and by stimulating the activity of pyruvate dehydrogenase and thus improving glucose metabolism (Ferrari et al., 2004). We tried three different concentrations of L-Car (0.5, 2.5 and 5 mM). Pre-ischemic administration of all three concentrations of L-Car could precondition the heart as evidenced by its ability to lower the infarct size markedly ($p<0.001$) and improved postischemic ventricular functional recovery. At the reperfusion phase, pre-ischemic use of L-Car reduced LVEDP elevation. Heart rate (HR) and coronary flow rate (CFR) did not show significant changes in treated groups compared to the control. Rate pressure product (RPP) was increased during the reperfusion (maybe related to LVDP increase) but it had significant value only at 15th min reperfusion time by 2.5 and 5 mM of L-Car (Table 1). Improvement of LVDP and RPP at reperfusion time and decreasing of LVEDP during reperfusion times indicate cardiac functions improvement. Among the three different concentratins, 2.5 mM L-Car was found to be optimal for preconditioning purpose. It was also found in this study that pre-ischemic application of L-Car in ischemic/reperfused hearts preconditions the hearts by reduction of left ventricular glycogen and lactate content.

Glucose catabolism in the heart consists of two distinct pathways, glycolysis and mitochondrial glucose oxidation. In aerobic condition, the majority of ATP produced from glucose catabolism in the heart is normally derived from glucose oxidation (Schönekess *et al.,* 1997). During ischemia, anaerobic glycolysis from either glucose or glycogen results in the production of ATP and as well as the intracellular accumulation of lactate and protons (H^+) as metabolic end products (Heather *et al.,* 1996). Glycolytic ATP production can be beneficial during myocardial ischemia. In contrast, elevated lactate and H^+ during ischemia may exacerbate myocardial injury (Heather *et al.,* 1996**).** The effect of high glycogen during ischemia may therefore depend on whether the advantage of increased glycolytic ATP production outweighs the detrimental effect of accumulation of lactate and H^+. In fact, there are discrepancies on carnitine effect on ischemic/reperfusion injury. Diaz *et al.* (2008) claimed that in an anaerobic condition, carnitine administration worsens both injury and recovery of contractile functions in ischemic/reperfusion rat hearts. However, the protective effects of L-carnitine against ischemic/ reperfusion injury has been reported in many different studies and models (Hosgorler *et al.*, 2010; Guan *et al.*, 2009; Xie *et al.*, 2006; Onem *et al.*, 2006).

In the present study, left ventricular glycogen store was decreased (56%) by pre-ischemic perfusion of L-Car in ischemic hearts, and this was associated with significantly reduction in lactate content during the subsequent ischemic episode. The cardioprotective action of pre-ischemic used L-Car against ischemic reperfusion injury, probably by reduction of glycogen content and therefore, of lactate content is consistent with the glycogen hypothesis. The glycogen hypothesis states that glycogen depletion occurs during the preconditioning episodes and that the resultant lower glycogen levels during prolonged ischemia contribute to ischemic preconditioning (Bradamante *et al.*, 2000; Schaefer, 1995) by reduction of lactate and protons production. However, the cardio-protective effects of L-Car against ischemia/reperfusion injury can be related to different mechanisms such as stimulating fatty acid oxidation during ischemia, restoring the balance between fatty acid and glucose oxidation, reducing of toxic effects of long chain free fatty acids metabolites, mitigating the noxious effects of oxygen free radicals in the reperfused hearts, increasing in coronary blood flow and anti-arrhythmic effect (Lango *et al.*, 2001). Furthermore, some researches suggest that L-Car is also crucial in the regulation of carbohydrate metabolism in addition to its role in the oxidation of fatty acids (Lopaschuk, 2000). Increase in fatty acid oxidation, which can be detrimental to cardiac recovery during

Table 1: Effects of pre-ischemic administration of L-Car (0.5-5 mM) on Heart Rate (HR; beats/min), Rate Pressure Product (RPP; mmHg.beats.min^{-1}) and Coronary Flow Rate (CFR; ml/min) in control and treated isolated rat hearts immediately before and during reperfusion period. Data are expressed as mean ±SEM.

Groups		Pre-reperfusion	Reperfusion					
			5 min	15 min	30 min	60 min	90 min	120 min
Control	H R RPP CFR	224±15 22319±1904 4.9±0.6	240±23 27697±2870 7.4±0.5*	224±16 25224±1816 6.6±0.5	219±17 23384±1836 5.6±0.5	220±17 21250±1910 4.6±0.5	227 ± 16 20619±2032 4.2 ± 0.5	190±13 15054±1133* 3.9±0.7
L-Car (0.5 mM)	H R RPP CFR	177±16 19880±1916 2.1±0.4	184±42 23027±5334 4.4±0.9	201±22 24260±2729 3.9±0.5	185±23 21619±2656 3.3±0.8	179±15 19873±1641 2.8±0.8	140±20 15472±2028 2.5 ± 0.8	114±15** 16511±2185** 3.5±0.3
L-Car (2.5 mM)	H R RPP CFR	198±16 23328±2591 3.3±0.3	227±17 29856±2570* 6.6±0.4*	212±10 29490±1339* 5.8±0.3*	205±10 27578±1071 5.1±0.5	221±13 27997±2221 4.6±0.5	189±21 22410±2539 4.0±0.5	178±13 20362±1804 3.3±0.4
L-Car (5 mM)	H R RPP CFR	245±20 30160±2736 3.2±0.3	289±5 41801±1367** 6.9±0.5**	273±28 39496±5102** 5.9±0.4*	256±13 35960±2463 5.2±0.5*	249±8 33348±2493 4.2±0.6	231±17 29709±2713 3.7±0.5	197±25 24601±2996 3.5±0.5

******* p< 0.001, ****** p< 0.01 ,***** p< 0.05 *vs* pre-reperfusion value by using repeated ANOVA with LSD post test. n=6 in each group.

reperfusion in ischemic tissue (Lopaschuk, 2000), may not ascribe solely the protective efficacy of L-Car. Recovery after ischemia has been shown to be improved by reducing the availability of fatty acid during reperfusion (Lopaschuk *et al.,* 1990). It appears that L-Car may be beneficial in myocardial ischemia and reperfusion by increasing pyruvate dehydrogenase activity and shifting cellular energy production from oxidation of fatty acids to glycolysis from glycogen and therefore by reducing lactate accumulation.

CONCLUSION

The finding of this study indicates that L-Car may act as a pharmacological preconditioning agent. Pre-ischemic administration of L-Car could precondition the heart as evidenced by its ability to lower the infarct size profoundly (p<0.001) and to improve postischemic functional recovery. The present study also shows that pre-ischemic perfusion of isolated rat hearts by 2.5 mM L-Car reduces both the myocardial content of glycogen and lactate. It can be concluded that L-Car preconditions the heart in part by recovery of postischemic ventricular hemodynamic functions, depletion of glycogen and therefore reduction of lactate accumulation.

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