Iranian Journal of Pharmaceutical Sciences
Winter 2007: 3(1): 19-24
www.ijps.ir

Original Article

Effects of L-Carnitine on Cardiac Apoptosis in Ischemic-Reperfused Isolated Rat Heart

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Abstract

Carnitine is a vital biologic substance for transporting fatty acids into myocytes. It also facilitates fatty acids β-oxidation for energy production. In this study, effects of L-carnitine (L-Car) on apoptosis in the ischemic isolated rat heart were investigated. Male Sprague-Dawley rats were divided into four groups and anesthetized by sodium pentobarbital. The heart was removed and mounted on a Langendorff apparatus then perfused by a modified Krebs-Henseleit (K/H) solution under a constant pressure at 37 °C. In the control group, the hearts were perfused only by normal K/H solution at stabilization, 30 min. regional ischemia and 120 min. reperfusion, while in each of the test groups, the hearts were perfused during ischemia-reperfusion with 0.5, 2.5 and 5 mM of L-Car-enriched K/H solution, respectively. At the end of reperfusion, immunohistochemical detection of apoptotic cells was performed by using an *in situ* apoptosis detection kit. The number of TUNEL-positive cardiomyocytes was counted in five random high-power fields in each sample. In the control group, the number of apoptotic cells were 48±3 while addition of L-Car (0.5, 2.5 and 5 mM) to the solution reduced the number of apoptotic cells to 6±1, 4±1 and 3±1, respectively (p<0.001 for all concentrations). There was no significant difference between and within test groups using ANOVA one-way. Considering these results, we conclude that L-Car has a protective effect against cardiac ischemia-reperfusion induced injuries as a reduction of apoptotic cardiomyocytes.

Keywords: Apoptosis; Isolated heart; L-Carnitine; Rat.
Received: August 14, 2006; *Accepted*: October 26, 2006

1. Introduction

Carnitine is an essential cofactor under physiologic conditions in the intermediary metabolism and transport of long-chain fatty acids (LCFAs) from the cytoplasm of myocytes to mitochondrial matrix for ATP production [1]. Myocardial ischemia results in inhibition of fatty acid -oxidation followed by accumulation of their deleterious metabolites, which affect recovery of myocardial function at the reperfused heart [1, 2]. Apoptosis occurs during normal embryonic development of the

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cardiovascular system and in the adult heart and vasculature. Apoptosis in the mature cardiovascular system has been observed in the settings of ischemic heart disease, congestive heart failure, acute myocardial infarction and normal cardiac aging [3]. Ischemia reperfusion (I/R)-induced cardiomyocyte apoptosis has been shown both in animals and humans [4]. In isolated rabbit hearts, the first signs of apoptosis revealed 50 min. following ischemia [5]. In isolated rat hearts, Stewart *et al*. showed that signs of apoptosis first appeared after 10 min. of ischemia and reached maximum level after 30 min. of ischemia with little change after reperfusion [6]. Although some studies have shown that L-carnitine (L-Car) could improve the recovery of post-ischemic cardiac function and reduce myocardial injury after I/R, its effect on I/R-induced myocardial apoptosis is not clear. In this study, effects of L-Car on cardiomyocyte apoptosis were studied during 30 min. regional ischemia followed by 120 min. reperfusion in isolated rat heart.

2. Materials and methods

2.1. Chemicals and equipments

L-carnitine was purchased from Sigma Chemical Co. NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgSO_4$, $CaCl_2$, D-glucose, hematoxylin, eosin, xylene, methanol, and formalin were from Merck Company. Sodium pentobarbital was from Kela Company, Belgium, and heparin was purchased from Daru-Pakhsh Pharmaceutical Company, Iran. TUNEL *in situ* cell death detection kit, proteinase K, DAB+substrate, and POD-converter solution were from Roche Company.

The equipments used in this study were light microscope (Leitz HM-Lux 3, Germany), autotechnicum (Shandon, UK) and microtome (LEICA-RM2145, Germany).

2.2. Animals

Male Sprague-Dawley rats weighing 270-330 g were used in this study. One day before the experiment, they were transferred to the lab with free access to food and water.

2.3. Surgical procedure

Rats were pretreated with 300 IU heparin (i.p.) and then anaesthetized by sodium pentobarbital (50-60 mg/kg, i.p.) injection. As soon as deep anesthesia was achieved, thoraxes were opened [7, 8] and the hearts were excised rapidly and mounted on a non-recirculating Langendorff apparatus under 100 mmHg pressure at 37 °C and perfused throughout the experiments with modified Krebs-Henseleit (K/H) buffer solution that were equilibrated with 95% O_2-5% CO_2 in advance. The buffer contained (in mM): NaCl (118.5), $NaHCO_3$ (25.0), KCl (4.8), $MgSO_4$ (1.2), KH_2PO_4 (1.2), D-glucose (12.0) and $CaCl_2$ (1.7) [8, 9]. Hemodynamic factors and ECG were recorded during the experiments. Induction of regional ischemia was achieved by temporary occlusion of left anterior descending coronary artery.

2.4. Experimental protocol

A special protocol was designed for preparing the isolated rat heart samples. The hearts were randomly assigned to one of the control or test groups. After 15 min. stabilization, they were subjected to 30 min. of regional ischemia followed by 120 min. of reperfusion. In the control group (n=6), the hearts were perfused by drug free K/H solution for the whole period of I/R while in the test groups (3 groups, n=6 in each group), they were perfused during 30 min of ischemia and 120 min. of reperfusion with 0.5, 2.5 or 5 mM of L-Car-enriched K/H solution, respectively. At the end of reperfusion, the hearts were sliced transversely in a plane perpendicular to the apico-basal axis into 2 mm-thick sections. During 24 h, the sections were fixed in formalin (10%), dehydrated in graded alcohols and xylene in autotechnicum and then embedded in paraffin. Sections, 4

micron thick, were cut by microtome from the paraffin blocks and mounted on glass slides. Then, sections were deparaffinized (24 h in 80 °C) and rehydrated for apoptosis assay [10].

2.5. Detection of apoptosis by TUNEL technique

During apoptosis, activated endonucleases cleave the DNA into fragments of multiples of 180-200 base pairs. Cleavage of the DNA may yield double-stranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks (nicks) in high molecular weight DNA. The DNA fragments generate blunt ends and single-strand 3' overhangs. Presence of 39 hydroxyl termini at the DNA strand breaks can be detected by a labeling reaction with modified nucleotides like biotin, digoxigenin, or fluorescein-labeled dUTP (deoxyuridinetriphosphate). The TUNEL method uses deoxynucleotidyltransferase (TdT) enzyme to add biotinylated, BrdU or digoxygenin-labelled nucleotides to DNA strand breaks [11-13]. We applied TUNEL staining method by using a commercial *in situ* cell death detection kit to assay apoptosis in ischemic-reperfused isolated rat hearts according to the kit package construction. At the end of the TUNEL staining procedure, the slides in each group were stained by hematoxylin-eosin dyes for morphological studies. The number of TUNEL-positive nuclei was determined by counting all of the stained nuclei present in the five random microscopic fields for the control and treated groups.

The results were expressed as mean±sem. A one-way ANOVA with LSD post hoc test was carried out to test any difference between the mean numbers of TUNEL-positive cardiomyocytes in each group. The differences between groups were considered significant at a level of $p<0.05$.

3. Results

The effects of L-Car on the mean number of apoptotic cells are shown in Figure 1. The mean number of TUNEL-positive cardiomyocytes in five microscopic field in the control group were 48±3 while administration of L-Car (0.5, 2.5 or 5 mM) in the test groups markedly reduced the mean number of apoptotic cells to 6±1, 4±1 and 3±1, respectively ($p<0.001$ for all concentrations). The effect did not show significant differences between the test groups. Reduction in apoptotic cells number for all of the used concentrations was more than 87% with a linear correlation ($R^2=0.99$) with concentration of L-Car.

4. Discussion

In this study, we focused on the pharmacological effects of L-Car on myocardial apoptosis in isolated rat hearts. There are few

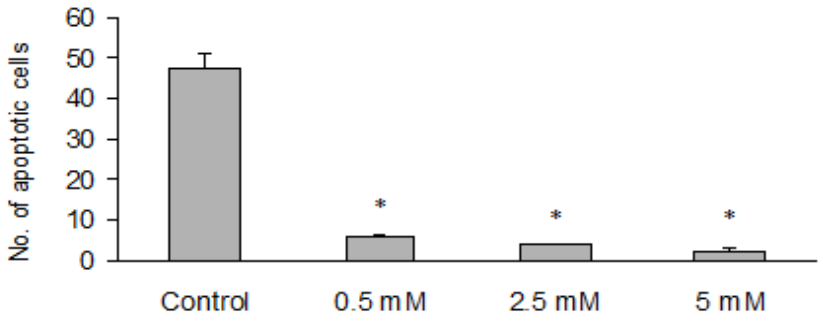

Figure 1. The number of apoptotic cells in the control and isolated rat hearts receiving 0.5-5 mM L-Car during 30 min. regional ischemia followed by 120 min. reperfusion. Data are expressed as mean ±sem.* $p< 0.001$ versus control. (n=6 in each group).

reports in this area and fewer on I/R-induced apoptosis in isolated rat hearts. However, our results clearly showed that administration of L-Car (0.5-5 mM) during 30 min. of regional ischemia followed by 120 min. of reperfusion, reduced the mean number of apoptotic cells in the treated groups ($p<0.001$) versus control. The effect had a linear correlation ($R^2 = 0.99$) with concentration of L-Car. Our results are consistent with the results of Cui *et al*. [14]. They perfused isolated rat hearts with L-Car (0.5 or 5 mM) for 10 min. before 30 min. of global ischemia followed by 120 min. of reperfusion. They reported that L-Car (5 mM) reduced percentage of apoptotic cardiomyocytes from their control values of 23.5±3.3% to 16.5±2.5% ($p< 0.05$). However, lower concentrations (<5 mM) of L-Car failed to reduce apoptotic cell death in their study [14]. In contrast to the above study, perfusion of 0.5 mM of L-Car in our experiments markedly reduced the mean number of apoptotic cells similar to 5 mM (Figure 1). It seems that the differences in the effects of 0.5 mM between the two studies mainly relate to the type of ischemia, duration and time of L-Car administration. Thus, administration of 0.5 mM L-Car for 150 min. (30 min. ischemia plus 120 min. reperfusion) resulted in a greater resistance against I/R-induced apoptosis compared to short perfusion time.

During myocardial ischemia, depressed oxygen supply results in the inhibition of fatty acid β-oxidation leading fatty acid metabolic intermediates accumulation such as acyl carnitine, β-hydroxy fatty acid intermediates and acyl-CoA. Accumulation of fatty acids and their intermediates during myocardial ischemia has been shown to be deleterious to the recovery of myocardial function of the reperfused heart. It has been suggested that at least some post-ischemic damage mediated by the accumulation of fatty acids is through the activation of apoptosis. Several biochemical mechanisms are possible that could lead to apoptosis in the post-ischemic heart in the presence of fatty acids. One mechanism involves the accumulation of palmitoyl CoA, which is the precursor, along with serine, for the synthesis of sphingosine and ceramide (a pro-apoptotic lipid). Another mechanism through which alterations in fatty acid metabolism in the ischemic heart may elicit myocardial apoptosis is through the accumulation of palmitoylcarnitine. Palmitoylcarnitine has been shown to activate the pro-apoptotic caspases [2]. It has been shown that palmitoyl-carnitine reversed the inhibition of caspase activity by L-Car. Accumulation of long-chain acylcarnitines in ischemic myocardium and the balance between carnitine and its acyl derivatives could contribute to the development of apoptosis [14]. Additionally, in the presence of LCFAs, L-Car removes toxic LCFAs (such as palmitoyl CoA and palmitoylcarnitine) from cardiomyocytes. L-Car also has an ability to reduce production of oxygen free radicals [2]. It is suggested that beside L-Car effects on fatty acid β-oxidation, augmentation of glucose metabolism and glycolytic activity probably might participate in the protection of cardiomyocytes against I/R-induced apoptosis by supplying required ATP and improvement of cardiac function during I/R [15]. It has been suggested that L-Car reduces apoptosis through inhibition of caspase activation [2]. Other *in vitro* investigations strongly supported that L-Car is able to inhibit the death planned, most likely by preventing sphingomyelin breakdown and consequent ceramide synthesis [16]. Furuno *et al*. suggested that the protective effect of L-Car on cellular apoptosis could be explained by inhibition of mitochondrial permeability transition and its ability to remove toxic LCFAs through the formation of dissociable complexes [17]. Study on the cultured neurons from the cerebral cortex of 18-day-old rat embryos revealed that L-Car (100 μM) decreased apoptosis and promoted neuronal survival

and mitochondrial activity in a concentration dependent manner [18]. Andrieu-Abadie *et al.* described that a 1 h treatment of isolated adult rat cardiac myocytes with doxorubicin (0.5 μM) induced DNA fragmentation associated with the classic morphological features of apoptosis observed after 7 days of culture [19]. Pre-treatment of cardiac myocytes with L-Car (200 μg/ml), was found to concentration-dependently inhibit the doxorubicin-induced sphingomyelin hydrolysis and ceramide generation as well as subsequent cell death [19]. Western analysis using caspase 2 as a marker for apoptosis showed that L-Car inhibited caspase 2 processing, suggesting that L-Car is acting upstream of caspase 2 cleavage. L-Car at 5 mM inhibited the activity of caspase 8. In addition, 5 mM L-Car inhibited the processing of caspase 3 and caspase 9 by caspase 8. L-Car was also able to inhibit proteolytic activation of caspase 9 mediated by cytochrome c and ATP [20]. Vescovo *et al.* speculated the below mechanisms for anti-apoptotic effects of L-Car: Blocking of tumor necrosis factor-alpha and sphingolipids activation cascade, inhibiting the cleavage of caspases substrates at mitochondrial level, making it a general caspase inhibitor. They also showed that the mitochondrial pathway was certainly involved, in that activated caspase 3 and 9 are inhibited and Bcl-2 is increased [21].

In conclusion, it seems that a conclusive interpretation about L-Car effects on apoptosis is not easy yet. Our data and the results of some previous studies show only a partial improvement in preventing apoptosis in I/R-induced myocardial apoptosis. Further studies are needed for better understanding of L-Car effects on apoptosis at the set of I/R. Irrespective of the mechanism(s) of cell death, the present study documents that L-Car could reduce apoptotic cell death, which may contribute to the post-ischemic ventricular recovery.

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