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Stimulation of Non-oxidative Glucose Utilization by L-carnitine in Isolated Myocytes

Salah Abdel-aleem, Mohamed Sayed-Ahmed, Mohamed A. Nada[1], Steven C. Hendrickson, James St. Louis and James E. Lowe

Duke University Medical Center, Department of Surgery and [1]Pediatrics, Durham, North Carolina, 27710, USA

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S. Abdel-aleem, M. Sayed-Ahmed, M. A. Nada, S. C. Hendrickson, J. St. Louis and J. E. Lowe. Stimulation of Non-oxidative Glucose Utilization by L-carnitine in Isolated Myocytes. *Journal of Molecular and Cellular Cardiology* (1995) 27, 2465–2472. The effects of L-carnitine on $^{14}CO_2$ release from [1-^{14}C]pyruvate oxidation (an index of pyruvate dehydrogenase activity, PDH), [2-^{14}C]pyruvate, and [6-^{14}C]glucose oxidation (indices of the acetyl-CoA flux through citric acid cycle), and [U-^{14}C]glucose (an index of both PDH activity and the flux of acetyl-CoA through the citric acid cycle), were studied using isolated rat cardiac myocytes. L-carnitine increased the release of $^{14}CO_2$ from [1-^{14}C]pyruvate, and decreased that of [2-^{14}C]pyruvate in a time and concentration-dependent manner. At a concentration of 2.5 mM, L-carnitine produced a 50% increase of CO_2 release from [1-^{14}C]pyruvate and a 50% decrease from [2-^{14}C]pyruvate oxidation. L-carnitine also increased CO_2 release from [1-^{14}C[pyruvate oxidation by 35%, and decreased that of [2-^{14}C]pyruvate oxidation 30%, in isolated rat heart mitochondria. The fatty acid oxidation inhibitor, etomoxir, stimulated the release of CO_2 from both [1-^{14}]pyruvate and [2-^{14}C]pyruvate. These results were supported by the effects of L-carnitine on the CO_2 release from [6-^{14}C]- and [U-^{14}C]glucose oxidation. L-carnitine (5 mM) decreased the CO_2 release from [6-^{14}C]glucose by 37%, while etomoxir (50 μM) increased its release by 24%. L-carnitine had no effect on the oxidation of [U-^{14}C]glucose. L-carnitine increased palmitate oxidation in a time- and concentration-dependent manner in myocytes. Also, it increased the rate of efflux of acetylcarnitine generated from pyruvate in myocytes. These results suggest that L-carnitine stimulates pyruvate dehydrogenase complex activity and enhances non-oxidative glucose metabolism by increasing the mitochondrial acetylcarnitine efflux in the absence of exogenous fatty acids.

Key Words: L-carnitine; Glucose oxidation; Pyruvate dehydrogenase; Cardiac myocytes.

Introduction

Under normal conditions, L-carnitine functions as an essential cofactor in the oxidation of long-chain fatty acids (Fritz, 1959). The use of L-carnitine has been shown to protect the myocardium from ischemic injury, and improves cardiac function in cardiomyopathies resulting from carnitine deficiency (Liedtke and Nellis, 1979; Paulson and Shug, 1981; Hulsmann *et al*., 1985; Suzuki *et al*., 1981; Paulson *et al*., 1984; Rodrigues *et al*., 1989). The mechanism whereby L-carnitine exerts these effects is unknown. It has been suggested that protective effects of L-carnitine may be due to its effect on buffering toxic intermediates of fatty acid oxidation such as long chain acyl-CoA derivatives (Liedtke *et al*., 1984; Majos *et al*., 1991).

Recent studies have suggested that L-carnitine may have other roles in intermediary metabolism in addition to its role in fatty acid oxidation (Bremer, 1983; Vary *et al*., 1981). In rat heart mitochondria, carnitine reduces acetyl-CoA levels by increasing

Please address all correspondence to: Dr Salah Abdel-aleem, Duke University Medical Center, Box 3954, Durham NC 27710, USA.

mitochondrial efflux of acetylcarnitine, resulting in a 10–20-fold increase in the CoA-SH/acetyl-CoA ratio (Lysiak *et al.*, 1988). L-Carnitine stimulates [1-^{14}C]pyruvate oxidation with a comparable increase in pyruvate dehydrogenase complex (PDH) activity in human skeletal muscle mitochondria (Uziel *et al.*, 1988). Although the effects of L-carnitine on the PDH complex is well established in isolated mitochondria, these effects need to be evaluated with a system such as isolated tissue or cells where all regulatory processes are operative. In addition, L-carnitine has been shown to stimulate glucose oxidation and inhibit palmitate oxidation in an isolated working heart preparation (Broderick *et al.*, 1992). These studies are consistent with the hypothesis that L-carnitine activates PDH by reducing mitochondrial acetyl-CoA and increasing the free CoA-SH/acetyl-CoA ratio. However, it remains unclear how L-carnitine stimulates glucose oxidation if it increases the trapping of the mitochondrial acetyl-CoA produced from pyruvate. Theoretically, stimulation of the mitochondrial efflux of acetyl-CoA by L-carnitine should decrease its oxidation via the Krebs cycle. Studies using [U-^{14}C]glucose to calculate the effects of carnitine on glucose oxidation may be misleading because $^{14}CO_2$ is released at the level of PDH and the Krebs cycle, it is unknown whether the increased $^{14}CO_2$ production is due to activation of the PDH reaction or to stimulation of acetyl-CoA flux through the Krebs cycle (Broderick *et al.*, 1992). To avoid this confusion, the rate of oxidative glucose metabolism can be determined by measuring the $^{14}CO_2$ released from a radiolabeled substrate such as [6-^{14}C]glucose, which does not generate pyruvate labeled at carbon one.

The present study is designed to investigate the overall effects of L-carnitine on glucose oxidation in isolated cardiac myocytes. These effects include the effect on PDH complex, the efflux of acetylcarnitine, and oxidative glucose metabolism.

Materials and Methods

Animals

Male Sprague–Dawley rats, weighing 200–250 g, were obtained from Charles River Laboratory (Raleigh, NC, USA). Rats were allowed free access to standard diet and water *ad libitum*. The animal protocol was approved by Duke University's Animal Use Committee. Animals were killed by decapitation.

Materials

[U-^{14}C]-D-glucose, [2-^{14}C]pyruvate, [1-^{14}C]pyruvate, [1-^{14}C]palmitate, and [6-^{14}C]glucose, were purchased from New England Nuclear (Boston, MA, USA). Sigma was the source of bovine serum albumin (BSA, essentially fatty acid free). Etomoxir was a gift from Dr Stanley Sherratt (University of Newcastle, Newcastle upon Tyne, UK). L-carnitine was a generous gift from Sigma-Tau Pharmaceuticals, Inc. (Gaithersburg, MD, USA). Joklik essential medium was purchased from Gibco Laboratories (NJ, USA). Collagenase type II was purchased from Worthington (NJ, USA).

Isolation of myocytes

Adult rat heart myocytes were isolated using the published method of (Frangakis *et al.*, 1980). Myocytes were isolated with Joklik essential medium containinng 5.55 mM glucose, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The viability of myocytes isolated by this procedure was 80–90% as determined by trypan blue exclusion.

Metabolic studies using myocytes

Myocytes (2 mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 mM HEPES (pH 7.4), were placed in a 25-ml Erlenmeyer flask. Cells were pre-incubated with the desired concentrations of L-carnitine, or etomoxir for 10 min at 37°C. To this cell suspension was added 0.1 ml of a single labeled metabolic substrate yielding a final concentration of 0.2 mM [1-^{14}C]-palmitic acid (2.2×10^5 dpm), 2 mM [2-^{14}C]pyruvate (2×10^5 dpm), 2 mM [1-^{14}C]pyruvate (2.1×10^5 dpm), 5.55 mM [U-^{14}C]-D-glucose (2×10^5 dpm), or 5.55 mM [6-^{14}C]-D-glucose (1.8×10^5 dpm). The Erlenmeyer flask was then closed with a rubber septum containing a plastic center well. The incubation was continued during shaking at 37°C for 30 min. An injection of 0.4 ml of 1 M hyamine hydroxide was administered through the center well septum to absorb the released CO_2, and the reaction was terminated by injecting 0.4 ml of 7% perchloric acid through the center well into the incubation medium. The flasks were then shaken continuously for 2 h at 37°C, at which time the plastic center well was removed, placed into a scintillation vial containing 10 ml of Scinti Verse BD, and counted in a liquid scintillation

counter. Control experiments with $NaH^{14}CO_3$ added to the cell suspension showed that the release of $^{14}CO_2$ was complete 1 h after the addition of perchloric acid. All results were corrected for non-specific release of $^{14}CO_2$ by subtracting blank counts after the addition of radioactive substrate to the cell suspension.

Isolation of rat heart mitochondria

Rat heart mitochondria were isolated by the procedure of (Chappel and Hansford, 1969). The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl (pH 7.4), and 1 mM EGTA. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Richmond, VA, USA).

Metabolic studies with rat heart mitochondria

Substrate oxidation in mitochondria was measured using published methods (Yang *et al*., 1987; Abdelaleem *et al*., 1992). In summary, mitochondria (0.5–1 mg protein) suspended in 0.9 ml of the reaction mixture, containing (in mM) Tris-HCl, 50 (pH 7.4); KCl, 120; ATP, 10; CoA-SH, 0.1; and EDTA-K_2, 0.5 (pH 7.4), were placed in a 25-ml Erlenmeyer flask. To this suspension was added 20 μl of L-carnitine to give the desired concentration. After incubating the mitochondria with carnitine for 10 min at 37°C under constant shaking, 0.1 ml of a single metabolic substrate was added to the mitochondria suspension to give a final concentration of 2 mM [2-^{14}C]pyruvate (2×10^5 dpm), or 2 mM [1-^{14}C]pyruvate (2.1×10^5 dpm). The rate of pyruvate oxidation was determined by measuring the $^{14}CO_2$ as previously described with myocytes.

Analysis of acetylcarnitine by tandem mass spectrometry

Myocytes (2 mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgCl_2$, and 0.5 mM $CaCl_2$ were pre-incubated with varied concentration of L-carnitine (0.2–5.0 mM) for 10 min at 37°C. Myocytes were then incubated for additional 30 min at 37°C after the addition of 0.1 ml of 2 mM pyruvate. At the end of this period, cell suspensions were centrifuged for 2 min at 4°C. To 0.1 ml aliquots of the supernatant, 20 pmol of [$^2H_3$]acetylcarnitine was added as an internal standard. The mixture was extracted with 0.8 ml methanol, centrifuged and clear supernatant was dried under nitrogen and immediately prepared for analysis of acetylcarnitine. The dried aliquots containing acetylcarnitine were incubated with 100 μl of 3 M HCl in n-methanol at 50°C for 15 min in a capped 1 ml glass vial. The esterifying agent was removed by evaporation under nitrogen and the derivatized sample was dissolved in 50 μl of methanol:glycerol (1:1;v/v) containing 1% octyl sodium sulfate (matrix). A QUATTRO tandem quadrupole mass spectrometer (Fisons-VG Instruments, Danvers, MA, USA) equipped with a liquid secondary ionization source and a cesium ion gun was used for the analysis of acetylcarnitine. This method of analysis is based on the detection of a common fragment ion of acetylcarnitine methyl esters produced by collision-induced dissociation as previously described by Millington *et al.* (Millington *et al*., 1991). Approximately 2 μl of sample matrix was analysed and the data recorded and processed as previously described. The final spectra displayed the relative intensities of ions corresponding to the molecular weights of the individual acylcarnitine methyl esters. The concentrations of acetylcarnitine corresponding to m/z 218 were determined based on their intensities relative to the internal standard, labeled acetylcarnitine, which corresponds to m/z 221.

Statistical analysis

Statistical analysis were performed by determining the paired *t*-test and comparing groups to control. A $P<0.05$ was considered significant.

Results

The effects of L-carnitine and etomoxir on $^{14}CO_2$ release from [2-^{14}C]pyruvate and [1-^{14}C]pyruvate were studied using isolated cardiac myocytes. The production of $^{14}CO_2$ from [1-^{14}C]pyruvate is an index of PDH activity, while its release from [2-^{14}C]pyruvate is representative of flux through the Krebs cycle. As shown in Figure 1a, L-carnitine resulted in stimulation of the release of $^{14}CO_2$ from [1-^{14}C]pyruvate, and inhibition of that from [2-^{14}C]pyruvate in a concentration-dependent manner. L-carnitine at a concentration of 2.5 mM produced 50% increase of $^{14}CO_2$ production from [1-^{14}C]pyruvate, and 50% decrease of its production from [2-^{14}C[pyruvate. The carnitine palmitoyltransferase I inhibitor, etomoxir, stimulated the release of $^{14}CO_2$

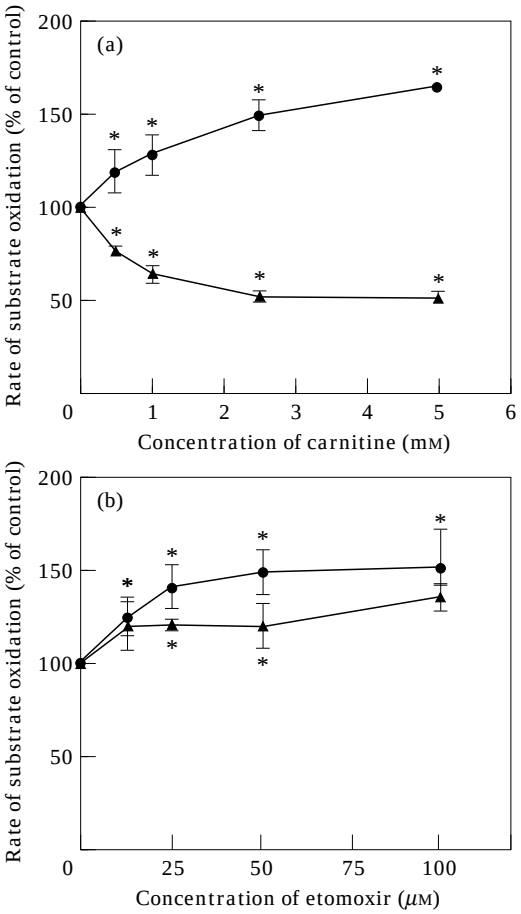

Figure 1 The effect of L-carnitine and etomoxir on pyruvate utilization in isolated cardiac myocytes. The effect of (a) carnitine, and (b) etomoxir on the oxidation of [1-^{14}C]pyruvate (●), and [2-^{14}C]pyruvate (▲) were studied using isolated myocytes. The control values for the oxidation of [1-^{14}C]pyruvate and [2-^{14}C]pyruvate were 146 ± 9.42, and 43.24 ± 1.5 (nmol/mg protein/30 min), respectively. Values are presented as the mean $\pm$ S.D. of at least three separate experiments. Asterisks indicate a $P<0.05$ of the effect of carnitine or etomoxir *v* control.

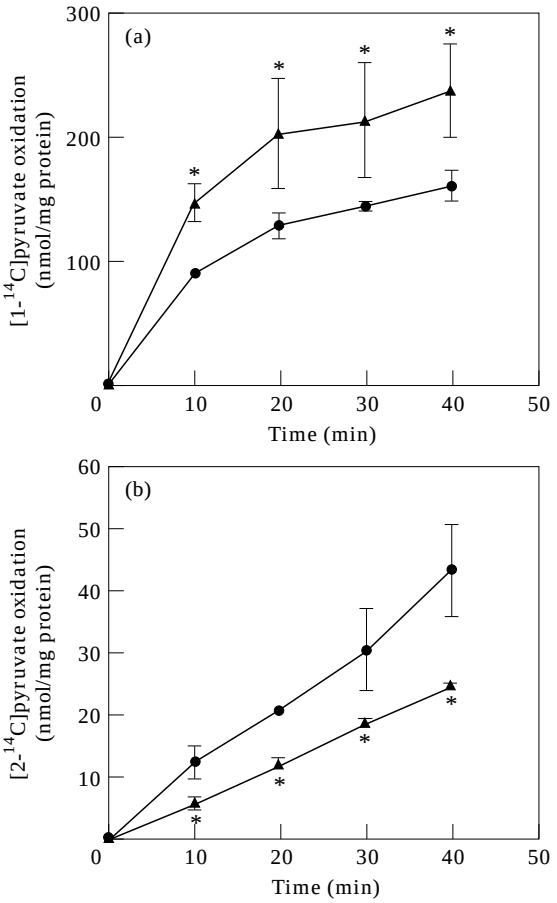

Figure 2 Time course of the effect of L-carnitine on pyruvate utilization in isolated myocytes. Myocytes were pre-incubated with 5 mM carnitine (▲) or without carnitine (●), then the oxidation of (a) [1-^{14}C]pyruvate, and (b) [2-^{14}C]pyruvate were determined at indicated time periods. Values are presented as the mean $\pm$ S.D. of at least three separate experiments. Asterisks indicate a $P<0.05$ of the effect of carnitine or etomoxir *v* control.

from both [1-^{14}C]pyruvate and [2-^{14}C]pyruvate (Fig. 1b).

Figure 2 shows the time course of the effect of L-carnitine on the release of $^{14}CO_2$ from [1-^{14}C]pyruvate, and [2-^{14}C[pyruvate oxidation in isolated myocytes. The stimulation of $^{14}CO_2$ production from [1-^{14}C]pyruvate (Fig. 2a), and the inhibition of its release from [2-^{14}C]pyruvate (Fig. 2b) by L-carnitine was time-dependent over a period of 10–40 min.

Table 1 shows the effect of L-carnitine on $^{14}CO_2$ release from [2-^{14}C]pyruvate, and [1-^{14}C]pyruvate oxidation in isolated rat heart mitochondria. This compound significantly increased $^{14}CO_2$ release from [1-^{14}C]pyruvate oxidation by 35%, and decreased that from [2-^{14}C]pyruvate by 30%.

The effect of L-carnitine and etomoxir on the release of $^{14}CO_2$ from [6-^{14}C]- and [U-^{14}C]glucose in isolated myocytes is shown in Table 2. [6-^{14}C]glucose is used because, during its metabolism, the $^{14}CO_2$ is only produced from the Krebs cycle. [6-^{14}C]glucose is converted into [3-^{14}C]pyruvate via glycolysis, then [2-^{14}C]acetyl-CoA is generated by the PDH complex, and finally $^{14}CO_2$ is produced as the latter intermediate passes through the Krebs cycle. L-carnitine (5 mM) inhibited its production by 37%, whereas, etomoxir (50 μM) increased its release by 24%. L-carnitine had no effect on $^{14}CO_2$ production from [U-^{14}C]glucose, while, etomoxir (50 μM) increased its release by approximately two-fold.

Table 1 The effect of L-carnitine on the utilization of pyruvate in rat heart mitochondria

Addition	Oxidation rate (nmol/mg/protein/30 min)	
	[1-^{14}C]Pyruvate	[2-^{14}C]Pyruvate
Control	476.9 ± 10.6	101.9 ± 0.32
L-Carnitine	644.0 ± 19.6*	72.0 ± 0.20*

Values are presented as the mean ± S.D. of at least three separate experiments.
* $P<0.05$ of effect of carnitine *v* control.

Table 2 The effect of L-carnitine and etomoxir on the oxidation of [6-^{14}C] and [U-^{14}C]glucose in myocytes

Addition	Rate of glucose oxidation (nmol/mg protein/30 min)	
	[6-^{14}C]glucose	[U-^{14}C]glucose
None, Control	1.71 ± 0.07	4.78 ± 0.54
Carnitine (5 mM)	1.46 ± 0.06*	5.16 ± 0.62
Etomoxir	1.88 ± 0.08*	9.40 ± 0.75*

Myocytes were pre-incubated with 5 mM carnitine, or 50 μM etomoxir for 30 min at 37°C.
Values are presented as the mean ± S.D. of at least three separate experiments.
* $P<0.05$ of the effect of carnitine or etomoxir *v* control.

The fate of the mitochondrial acetylcarnitine generated from pyruvate was investigated using isolated myocytes. As shown in Figure 3, the efflux rate of acetylcarnitine generated from pyruvate utilization increased by increasing the concentration of L-carnitine in the medium. In absence of L-carnitine there is minimal accumulation of acetylcarnitine in the medium (4.13 ± 0.38 nmol/mg protein/30 min). At lower carnitine concentrations (0.2–1.0 mM), the efflux rate was linear, then it becomes saturable at higher concentrations (2.5–5.0 mM).

The effect of L-carnitine on the oxidation of palmitate is shown in Figure 4. L-carnitine at 1 mM,

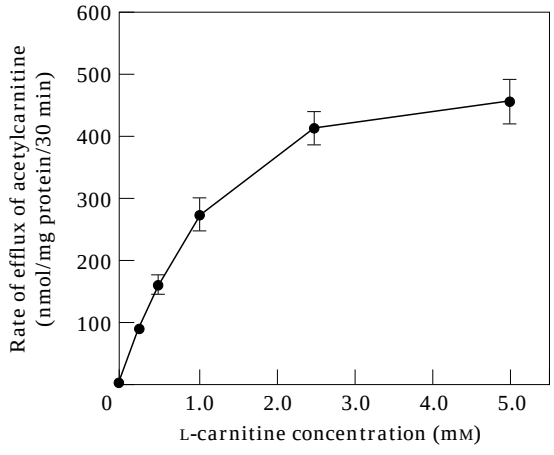

Figure 3 The effect of L-carnitine on the efflux of acetylcarnitine in myocytes. Cells were pre-incubated with varied concentrations of L-carnitine for 10 min. For more experimental details see section under Materials and Methods. Values are presented as the mean ± S.D. of three separate experiments.

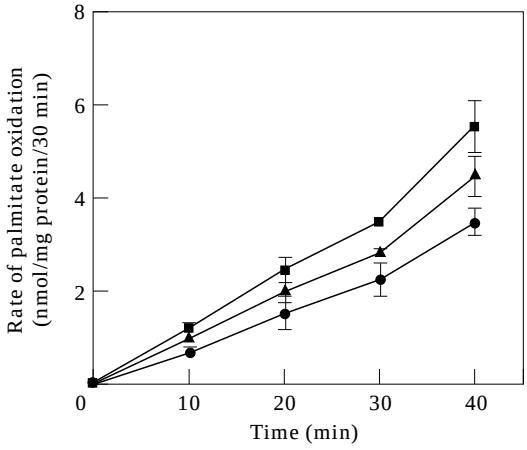

Figure 4 The effect of L-carnitine on palmitate oxidation in myocytes. Cells were preincubated without (control, ●), with 1 mM L-carnitine (▲), or 5 mM L-carnitine (■) for 10 min. Values are presented as the mean ± S.D. of three separate experiments.

and 5 mM significantly increased palmitate oxidation as a function of incubation time. The rate of palmitate oxidation was increased by 28% and 59% when myocytes preincubated for 40 min with 1 mM, and 5 mM of L-carnitine, respectively.

Discussion

The increase of $^{14}CO_2$ release from [1-^{14}C]pyruvate along with its decrease from [2-^{14}C]pyruvate by L-carnitine suggests that this compound stimulates PDH activity and enhances the mitochondrial efflux of acetyl-CoA, produced from pyruvate metabolism in isolated myocytes. Acetylcarnitine transferase in the mitochondrial matrix catalyses the transformation of acetyl-CoA into acetylcarnitine, which is then, transported across the mitochondrial membrane by the carnitine acetylcarnitine translocase (Murthy and Pande, 1985). It is unlikely that L-carnitine decreases CO_2 release from [2-^{14}C]pyruvate by inhibiting a specific site in the citric acid cycle because palmitate oxidation, which ultimately utilizes this pathway, is not inhibited by this compound. These findings suggest that L-carnitine stimulates PDH by decreasing mitochondrial acetyl-CoA levels leading to an increase in the CoA-SH/acetyl-CoA ratio. Regulation of PDH by the CoA-SH/acetyl-CoA ratio has been reported previously (Wieland, 1983). These results are consistent with prior studies which demonstrated stimulation of PDH activity by L-carnitine in isolated human skeletal muscle mitochondria (Uziel *et al*., 1988). The results of the present study are also supported by the study of Lysiak *et al.* (1986) where L-carnitine decreased the CO_2 release from [2-^{14}C]pyruvate oxidation in isolated rat heart mitochondria.

The flux of pyruvate through the PDH is threefold higher than the acetyl-CoA flux through the Krebs cycle in absence of L-carnitine. This difference in fluxes may be due to the oxidation of acetyl-CoA from endogenous fatty acids. However, the relatively small stimulation of [2-^{14}C]pyruvate oxidation by etomoxir in absence of exogenous fatty acids suggest that the influence of basal endogenous fatty acid oxidation is minimal in these preparations. The other possibility which may explain the difference in these fluxes is the limitation of the acetyl-CoA flux through the Krebs cycle by citrate synthetase activity or the rate of supply of oxaloacetate under these conditions.

The recent study by Broderick *et al.* (1992) suggested that L-carnitine increases glucose oxidation and decreases palmitate utilization in the perfused working heart preparation. However, it remains unclear how this compound increases the oxidative metabolism of glucose if it enhances the removal of acetyl-CoA, produced from pyruvate by PDH complex. In contrast, the trapping of mitochondrial acetyl-CoA by L-carnitine should lead to a decrease of its flux through the Krebs cycle. [U-^{14}C]Glucose was used as the metabolic substrate in Broderick's (1992) study, and rates of glucose oxidation were determined by measuring CO_2 production. These measurements include CO_2 produced from the PDH reaction, as well as the Krebs cycle. To avoid this interference, the rate of glucose oxidation in the present study was determined by measuring the CO_2 release from [6-^{14}C]glucose. This substrate is converted to [3-^{14}C]pyruvate, and then into [2-^{14}C]acetyl-CoA ($^{14}CH_3$-CO-SCoA). Thus, the CO_2 in this case is released only through the Krebs cycle. L-Carnitine decreased oxidation of [6-^{14}C]glucose and had no effect on the oxidation of [U-^{14}C]glucose. This effect confirms our hypothesis that the overall effect of L-carnitine is to decrease oxidative metabolism of glucose. The absence of an effect of L-carnitine on the oxidation of [U-^{14}C]glucose is due to release of CO_2 at the level of the PDH reaction. Previous studies from this laboratory have shown that etomoxir, an inhibitor of carnitine palmitoyltransferase 1 (Eistetter and Wolf, 1989; Wolf, 1992), stimulates CO_2 release from [U-^{14}C]glucose in isolated cardiac cells (Abdel-aleem *et al*., 1994). In the present study, etomoxir stimulated CO_2 release from [1-^{14}C]pyruvate, [2-^{14}C]pyruvate, and [6-^{14}C]glucose which suggests that it stimulates both the PDH reaction and flux of acetyl-CoA through the Krebs cycle. It should be also pointed out that the present study was performed by determining the effect of L-carnitine on the oxidation of glucose in the absence of exogenous fatty acids which may not necessarily behave this way under all physiological conditions. It is possible that the effect of L-carnitine on [6-^{14}C]glucose oxidation is more apparent in the presence of exogenous fatty acids.

Reduction of the flux of acetyl-CoA generated from pyruvate through the Krebs cycle is supported by measurements of acetylcarnitine which leaves myocytes and accumulates in the medium. These results demonstrated the stimulation of the rate of efflux of acetylcarnitine as a function of carnitine concentration. However, it remains unclear why L-carnitine decreases the flux of acetyl-CoA generated from pyruvate through the Krebs cycle and does not affect the flux of acetate produced from fatty acid β-oxidation. In the present study, the flux acetate produced from palmitate through Krebs cycle was enhanced by L-carnitine. These results suggest that the acetyl-CoA produced from car-

bohydrate oxidation may be more accessible to the mitochondrial carnitine system as proposed by Lysiak *et al.* (1988). It is assumed that the increase in the rate of export of acetylcarnitine under these experimental conditions is accompanied by alterations in intramitochondrial acetyl-CoA. The study by Wang *et al.* (1991) has demonstrated the decrease of acetyl-CoA and increase of free CoA-SH by L-carnitine during state 4 respiration in isolated rat heart mitochondria.

The contribution of the anaplerotic pathways such as the formation of oxaloacetate by pyruvate carboxylase and malic enzyme has been reported in cardiac tissues (Russel and Taegtmeyer, 1991; Lancha *et al.*, 1994). Although pyruvate flux through these pathways was not determined in this study, their involvement will not change the proposed mechanism of L-carnitine. Pyruvate flux was determined by measuring the CO_2 release from [2-^{14}C]pyruvate through the Krebs cycle, therefore, this measurement includes the CO_2 released from acetyl-CoA or oxaloacetate.

Increasing glycolysis during cardiac ischemia has been shown to protect the myocardium from ischemic injury (Owen *et al.*, 1990; Jeremy *et al.*, 1993). Therefore, the stimulation of non-oxidative glucose oxidation by L-carnitine may be the key mechanism by which this compound improves cardiac function during ischemia.

In conclusion, these data show that L-carnitine stimulates pyruvate dehydrogenase activity, and decreases the flux of acetyl-CoA generated from pyruvate through the Krebs cycle. These effects may be due to enhancing the mitochondrial efflux of acetyl-CoA by L-carnitine.

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