Effects of L-carnitine treatment on oxidant/antioxidant state and vascular reactivity of streptozotocin-diabetic rat aorta

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Abstract

In this study, the effects of L-carnitine treatment on lipids, lipid peroxidation of plasma, reactivity and antioxidant enzyme activity of aorta were evaluated in streptozotocin (STZ)-diabetic rats. Treatment with L-carnitine ($0.6\,g\,kg^{-1}$ daily, i.p.) was started 8 weeks after the induction of diabetes and continued for 2 weeks. Diabetes was induced by a single injection of streptozotocin ($45\,mg\,kg^{-1}$, i.p.). Plasma cholesterol, triglyceride and thiobarbituric acid reactive substance (TBARS) levels and blood glucose levels were significantly increased, although free carnitine levels were markedly decreased in diabetic rats. L-Carnitine treatment completely normalized plasma cholesterol, triglyceride, free carnitine and TBARS levels but partially restored blood glucose levels of diabetic rats. STZ-diabetes caused a significant reduction in the endothelium-dependent relaxation response to acetylcholine (ACh). In diabetic aorta, TBARS levels and catalase (CAT) activity were significantly increased but glutathione peroxidase (GSH-Px) activity was unchanged. Treatment of diabetic rats with L-carnitine resulted in partial restoration of the endothelium-dependent relaxation response to ACh and completely normalized the oxidant/antioxidant state. These results suggested that the beneficial effects of L-carnitine treatment partially improve vascular reactivity and antioxidant property beyond its reduction of plasma lipids and it may have an important therapeutic approach in the treatment of diabetic vascular complications.

Introduction

Vascular dysfunction may play a major role in the pathogenesis of diabetic cardiovascular diseases (Regan et al 1983; Keegan et al 1995; Taylor et al 1995). Mechanisms underlying the vascular dysfunction are multifactorial; undoubtedly hyperglycaemia plays a pivotal role and oxidative stress and dyslipidaemia could also be important factors (Cohen 1993). Hyperglycaemia induces generation of free radicals by involving oxidation of glucose, oxidative degeneration and protein glycation (Hunt et al 1988). Dyslipidaemia leads to increasing lipid peroxidation, damaging the structure and disrupting the functions of cells and tissues (Yu 1994). Cellular defence mechanisms (antioxidant enzymes, non-enzymic antioxidants) are also altered, impairing the balance between oxidant stress/antioxidant defence system to protect the cells and tissues against oxidative injury seen in diabetes (Oranje & Wolffenbuttel 1999). Treatment with either lipid-lowering drugs (cholestyramine, statins, fibrates) or antioxidants (vitamin E, *N*-acetylcysteine, probucol) has been shown to improve the vascular function of diabetic rats and it has been suggested that oxidative stress and dyslipidaemia may contribute to vascular dysfunction (Kamata et al 1996; Cinar et al 2001; Ozansoy et al 2001). L-Carnitine (β-hydroxy-trimethylaminobutyric acid) is a small water-soluble quaternary amine that plays an important role in lipid metabolism. It serves as an essential cofactor for the transport of long-chain fatty acids from the cytoplasmic compartment into inner mitochondrial membrane. Thus adequate carnitine levels are required for normal fatty-acid metabolism and regulation of carbohydrate metabolism (Tein et al 1996; Lopaschuck 2000). In diabetic man and rats, a significant reduction in plasma carnitine levels has been shown, which suggests that L-carnitine may play an important role in the pathophysiology of diabetic complications (Paulson et al 1984; Rodrigues et al 1988). It has reported that carnitine administration has

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a beneficial effect in the treatment of some diabetic complications, such as retinopathy, neuropathy and cardiomyopathy (Paulson et al 1984; Lowitt et al 1993; Cotter et al 1995).

The effects of endogenous carnitine depletion or carnitine deficiency in diabetic vascular dysfunction have not been investigated. Therefore, the first aim of this study was to test whether treatment with L-carnitine could prevent the alteration of function of diabetic aorta. The second aim was to investigate whether diabetes-induced changes in carbohydrate and lipid metabolism and oxidative/antioxidative markers might be important for the actions of L-carnitine.

Materials and Methods

Drugs

All chemicals used in experiments were purchased from Sigma Chemical (St Louis, MO) and dissolved in saline.

Experimental animals

The Animal Care Ethics Committee of Ankara University approved the study. Wistar albino male rats (180–230 g) were used in this experiment. During the experimental periods (10 weeks) the rats had free access to standard chow and water. At the beginning, rats were randomly divided into two groups: control and diabetic. Diabetes was induced by a single injection of streptozotocin (STZ; $45\,\text{mg kg}^{-1}$, i.p.). Seventy-two hours after the injection, hyperglycaemia was confirmed by measuring blood glucose levels in tail vein. Rats with blood glucose levels of $\geq 250\,\text{mg dL}^{-1}$ were considered to be diabetic. To investigate the reversal effect of L-carnitine treatment, we started the L-carnitine treatment 8 weeks after the STZ injection (the 8-week period was based on previous studies, since during that period vascular dysfunction occurred). The diabetic group was divided into untreated and treated with L-carnitine by daily intraperitoneal injection of ($0.6\,\text{g kg}^{-1}$) for 14 days (Rodrigues et al 1988).

Blood analysis

Blood samples were taken by cardiac puncture and plasma was obtained for determination of lipids, thiobarbituric acid reactive substance (TBARS) and free carnitine levels and stored at $-70\,°\text{C}$ until assayed. Blood glucose concentration was measured by an Accutrend Glucometer (Boehringer, Mannheim, Germany). Plasma triglyceride and cholesterol concentrations were determined with an automatic analyser (Allain et al 1974; Fossati & Prencipe 1982). Plasma TBARS levels were measured according to the method of Dazhong (Dazhong 1995). Plasma free L-carnitine levels were measured by the fluorimetric method described by Maehara et al (1988).

Vascular studies

The thoracic aorta was removed, rapidly placed in cold physiological solution (PSS) and carefully cleaned of surrounding connective tissue and cut into transverse rings of approximately 3–4 mm length each. The rings were mounted horizontally between stainless hooks in 10-mL organ baths, filled with PSS of the following composition (mM): NaCl 118.0; KCl 7.4; $CaCl_2$ $2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4$ $7H_2O$ 1.2; $NaHCO_3$ 25 and glucose 10.0. The PSS was gassed with 95% O_2–5% CO_2 and thermoregulated at $37\,°\text{C}$. Each ring was connected to a force displacement (Force displacement Transducer-Grass). Changes in isometric tension were recorded on a data acquisition system (Mikroelektrik Ltd, Ankara, Turkey). The suspended aortic rings were allowed to equilibrate for 60 min under a resting load of 2 g.

During this period, the bath solution was replaced every 10 min. At the end of the equilibration period, concentration–response curves to cumulative concentrations of phenylephrine were performed on each ring. After reaching a plateau, each ring was serially washed to baseline and equilibrated. Rings were contracted with a sub-maximal concentration of phenylephrine, which produced approximately 80% of the maximum response. This concentration was usually $1\,\mu\text{M}$ but was occasionally varied (10^{-6} to $3 \times 10^{-6}\text{M}$) to obtain equi-effective agonist activity. After reaching a plateau of contraction, cumulative concentration–response curves to ACh (10^{-8} to 10^{-5}M) and then sodium nitroprusside (10^{-10} to 10^{-7}M) were obtained to evaluate endothelium-dependent or -independent relaxation.

Measurement of lipid peroxidation and antioxidant enzyme activity

Remaining parts of the thoracic aorta were washed with saline, blotted with a piece of filter paper and homogenized. TBARS were measured according to the fluorimetric method of Yagi (1987). Catalase (CAT) was measured spectrophotometrically by the method of Aebi (1983). Glutathione peroxidase (GSH-Px) activity was determined according to the method of Lawrence & Burk (1976). Protein levels of aorta were determined by the method of Lowry et al (1951).

Statistical analysis

Results are expressed as the mean $\pm$ s.e.m. Relations to cumulative doses of agonists were expressed as a percentage of the maximum contractile response of phenylephrine. The sensitivity to the agonists was evaluated as the pD_2 (–log EC50 (concentration producing half maximal response)). Statistical analysis was carried out using one-way analysis of variance followed by Newman–Keul's test. Results were considered significantly different if $P < 0.05$.

Results

Metabolic parameters

Blood glucose, plasma triglyceride and cholesterol levels were significantly increased in diabetic rats. Plasma triglyceride and cholesterol levels of diabetic rats were

completely normalized by treatment with L-carnitine. Blood glucose levels of L-carnitine-treated diabetic rats were partially reduced but the rats were still hyperglycaemic (Table 1).

Induction of diabetes caused carnitine deficiency; plasma-free carnitine concentrations were significantly decreased in diabetic rats. L-Carnitine treatment markedly increased free carnitine levels towards control levels. Plasma TBARS levels were higher in diabetic rats than in control rats and indicated that an increase in plasma lipid peroxidation occurred in diabetic rats. L-Carnitine treatment of diabetic rats normalized TBARS levels (Table 1).

Lipid peroxidation levels and antioxidant enzyme activity of aortas

CAT activity and the level of TBARS were increased, whereas GSH-Px activity was unchanged, in diabetic rats (Table 2). L-Carnitine treatment restored CAT activity and TBARS level of diabetic aorta. No difference was found in GSH-Px activity in all groups.

Vascular studies

Addition of ACh and sodium nitroprusside resulted in concentration-dependent relaxation in all aorta rings pre-contracted with phenylephrine. The ACh-induced relaxation was significantly reduced in diabetic rats relative to control rats. L-Carnitine treatment partially reversed the defective endothelium-dependent relaxation of diabetic aorta strips. The relaxation response to sodium nitroprusside, the endothelium-independent vasodilatation, has been found not to be significantly different in

experimental groups. Concentration–response curves for both ACh and sodium nitroprusside are represented in Figure 1. On the other hand, the sensitivity of aorta from all experimental groups to ACh and sodium nitroprusside was unchanged (Table 3).

Discussion

The results of this study verify those of previous researchers, which showed the biochemical alterations such as hyperglycaemia, increased plasma concentration of blood lipids and decrease in endothelium-dependent relaxation in diabetic aorta (Kakkar et al 1995; Özçelikay et al 2000; Stefek et al 2000; Ozansoy et al 2001). In addition, this study demonstrated that the imbalance between oxidative stress and antioxidant status is characterized by increased plasma concentration of TBARS and activity of CAT in aortas of diabetic rats.

This study also showed that short-term (2 weeks) treatment with L-carnitine restored the increased plasma concentrations of TBARS and blood lipids and normalized augmented aorta CAT activity; also, it normalized the decreased plasma free-carnitine concentrations of diabetic rats. Moreover, L-carnitine treatment also partially normalized the endothelium-dependent response to ACh in diabetic aortas.

In our studies and previous studies, it has been shown that induction of diabetes increases blood lipids and lipid peroxidation, which has a direct link with the augmentation of TBARS levels (Yu 1994; Kubow et al 1996; Oranje & Wolffenbuttel 1999; De La Cruz et al 2000). As expected, this leads to the alteration of some endogenous

Table 1 General characteristics of control, diabetic and L-carnitine-treated diabetic rats.

Characteristics	Control rats (n=7)	Diabetic rats (n=7)	L-Carnitine-treated diabetic rats (n=7)
Blood glucose (mg dL^{-1})	90.85 ± 6.40#	449.20 ± 13.56*	273.00 ± 48.34*#
Plasma triglyceride (mg dL^{-1})	83.2 ± 6.3#	274.7 ± 28.0*	90.2 ± 5.0#
Plasma cholesterol (mg dL^{-1})	69.1 ± 5.7#	101.3 ± 4.6*	55.1 ± 5.0#
Plasma TBARS (μM)	1.50 ± 0.09#	2.39 ± 0.07*	1.63 ± 0.29#
Plasma free L-carnitine (μM)	125 ± 16.2#	73.8 ± 7.7*	128.2 ± 7.6#

Data are mean ± s.e.m.; n, number of rats. *$P < 0.001$ vs controls; #$P < 0.001$ vs diabetic rats. TBARS, thiobarbituric acid reactive substance.

Table 2 Values of oxidant/antioxidant markers in aorta of control, diabetic and L-carnitine-treated diabetic rats.

Oxidant/antioxidant markers	Control rats (n=7)	Diabetic rats (n=7)	L-Carnitine-treated diabetic rats (n=7)
TBARS (nmol (mg protein)$^{-1}$ MDA)	0.040 ± 0.002#	0.056 ± 0.001*	0.043 ± 0.001#
CAT (ks (mg protein)$^{-1}$)	0.016 ± 0.004#	0.034 ± 0.008*	0.019 ± 0.004#
GSH-Px (nmol min^{-1}(mg protein)$^{-1}$)	0.075 ± 0.006	0.095 ± 0.006	0.086 ± 0.005

Data are mean ± s.e.m.; n, number of rats. *$P < 0.001$ vs controls; #$P < 0.001$ vs diabetic rats. TBARS, thiobarbituric acid reactive substance; GSH-Px, glutathione peroxidase; CAT, catalase.
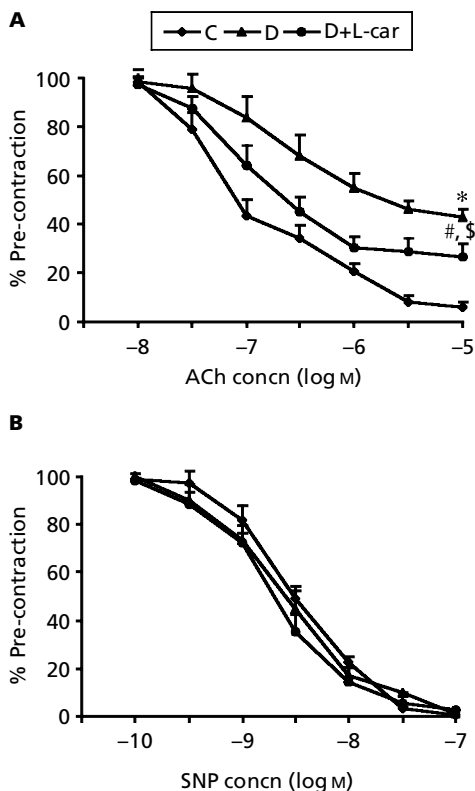
Figure 1 Cumulative concentration–response curves to acetylcholine (ACh) (A) and sodium nitroprusside (SNP) (B) in aorta rings pre-contracted with phenylephrine from control (C, n = 7), diabetic (D, n = 7) and L-carnitine-treated (D + L, n = 7) diabetic rats. #P < 0.01, *P < 0.001, vs control group; $P < 0.01 vs diabetic group.

antioxidant systems in diabetes. The results of our measurements show that CAT activity was increased, whereas GSH-Px activity was unchanged in diabetic aorta.

These results agree with previous research on vascular and non-vascular tissues (Wohaieb et al 1987; Pieper et al 1995; Ozansoy et al 2001). Both GSH-Px and CAT are antioxidant enzymes that protect cells against oxidative injury by breakdown of H_2O_2, which was shown to be elevated in diabetes. CAT was localized mainly in the peroxisomal compartment in mitochondria, as was fatty acyl-CoA oxidase, and it was effective at excess concentration of H_2O_2, whereas GSH-Px was found in cytosol and detoxified H_2O_2 at lower concentrations.

The increased CAT activity without a significant change in GSH-Px may be due to excess H_2O_2 formation in the peroxisomal compartment (Doroshow et al 1980). However, CAT activity was increased but TBARS were still found to be higher in diabetic aorta, suggesting that oxidative stress exceeded the capacity of the CAT, which is responsible for scavenging oxygen free radicals in mitochondria of diabetic aorta (Kakkar et al 1995).

Consistent with previous studies, we found that ACh-induced endothelium-dependent relaxation response was significantly decreased but that there was no alteration in its sensitivity in diabetic rat aorta. Endothelium-dependent relaxation by ACh, muscarinic receptor operated effect and the decrease in response to ACh indicate that post-receptor events rather than receptor mechanisms on endothelium cells were involved in the impaired response. The possible mechanism of decreased endothelium-dependent relaxation to ACh is that it decreased the bioavability of NO by increasing the rate of production of free radicals in diabetic aorta. It has been reported that free radicals, particularly superoxide and hydroxyl radicals, react with NO and this results in production of the less potent vasodilators, such as peroxynitrite, NO_2 and NO_3. These products may be responsible for the impairment of endothelium-dependent relaxation (Gryglewski et al 1986; Mügge et al 1991; Beckman et al 1994).

Responses to sodium nitroprusside, which induced endothelium-dependent relaxation by directly activating vascular smooth muscle soluble guanylate cylase, were not altered. This indicate that the response of smooth muscle to NO was not impaired in diabetic aorta and the effect should be in the endothelium cell (Özçelikay et al 2000).

In the study we found that short-term L-carnintine treatment had some beneficial effects in 8-week diabetic rats: the hyperglycaemia was partially restored; the plasma concentration of free carnitine was increased towards control levels; the vascular dysfunction was partially restored; increased blood lipids and lipid peroxidation were normalised.

In the treatment of various cardiovascular diseases, such as angina pectoris, arrhythmias, essential hypertension and diabetes, the vital role of L-carnintine is understood better than in the past but its mechanisms are not fully explained (Digiesi et al 1989; Bartels et al 1994; Mondillo et al 1995; Tatlican et al 1998). L-Carnintine is an essential cofactor for transport and oxidation of long-chain acids,

Table 3   pD$_2$ values of agonists in aorta of control, diabetic and L-carnitine-treated diabetic rats.

pD$_2$ values	Acetylcholine	Sodium nitroprusside
Control rats (n = 7)	6.86 ± 0.23	8.37 ± 0.10
Diabetic rats (n = 7)	6.81 ± 0.13	8.37 ± 0.11
L-Carnitine-treated diabetic rats (n = 7)	6.83 ± 0.43	8.53 ± 0.56

Data are means ± s.e.m.; n, number of rats.

which are the preferred substrate for energy production in various tissues, such as myocardium vessels and skeletal muscle (Neely & Morgan 1974). Any alteration in L-carnintine levels might result in an inhibition of the β-oxidation of fatty acids, and energy production causes the accumulation of long-chain fatty acids and fatty acid esters and might disturb membrane lipid composition and dysfunctions in these tissues (Breker 1983).

It has shown that cardiac contractile dysfunction is associated with the reduction of carnintine levels in plasma and myocardium in experimentally induced diabetes and in diabetic and aged patients (Abu-Erreish et al 1977; Paulson et al 1984; Arsenian 1997). In diabetic rats, there are several mechanisms that could be postulated to explain the decrease of plasma carnitine levels. These include the augmentation of L-carnintine degradation and alteration in cellular mechanisms for transport and release, a defect in biosynthesis–secretion (Bieber 1988). In addition, defiency of carnitine can arise from increased excretion of L-carnintine (Randle et al 1964; Neely 1982; Vary & Neely 1982; Ido et al 1994; Cameron & Cotter 1997). During chronic diabetes L-carnitine administration resulted in a restoration of the carnitine defiency in diabetic cardiopathy, neuropathy and retinopathy (Cedarbaun et al 1984; Whitmer 1987; Hotta et al 1996). It may mediate repletion of L-carnitine levels that would facilate the transfer of acyl groups from acyl-CoA to L-carnintine, resulting in an increase in mitochondrial free CoA. As shown in this study, the effects of L-carnitine on blood glucose in diabetic rats are consistent with previous studies (Paulson et al 1984; Rodrigues et al 1988). The results from different investigators about the effect of L-carnitine treatment on blood glucose in diabetic rats are conflicting. Plasma glucose concentrations in diabetic rats have been observed to be decreased and unchanged with L-carnitine treatment (Paulson et al 1984; Rodrigues et al 1989, 1990). Rodrigeus et al (1989) have shown that treatment of 6-week diabetic rats with intraperitoneal high-dose L-carnitine for 2 weeks partially reversed some of the biochemical parameters, such as plasma glucose, blood lipids and functional alterations. In other experiments, they demonstrated that low-dose intraperitoneal L-carnitine had the preventive effect on 6-week diabetic rats (Rodrigues et al 1989). However, Rodrigeus has shown that oral L-carnitine treatment did not restore plasma glucose levels and heart function (Rodrigues et al 1990). In this study, we show the partial reduction of blood glucose levels with L-carnitine treatment in 8-week diabetic rats. These results indicate that the blood-glucose-lowering effect of L-carnitine treatment is related to the route of administration and treatment duration. The central role of carnitine on lipid metabolism is well known, but the role of carnitine in carbohydrate metabolism is less understood. It has been reported that L-carnitine stimulates the activity of the pyruvate dehyrogenase complex by decreasing the intramitochondrial acetyl-CoA–CoA ratio through the trapping of acetyl groups. The simultaneous reduction of acetyl-CoA levels in the cytosol further contributes to the activation of the glycolytic patway (Lysiak et al 1988). Also, L-carnitine caused decreased plasma lipid

levels, a result which is similar to the lipid-reducing effect of L-carnitine in hyperlipidaemic and diabetic rats (Paulson et al 1984; Maccari et al 1987; Rodrigues et al 1988; Rauchova et al 1998). The hypolipidaemic effects of L-carnitine may be due to increased extramitochondrial fatty-acid synthesis or to decreased basal lipolysis in adipose tissue (Paulson et al 1984).

It has been reported that many pathological conditions that cause elevation of lipid peroxidation were prevented by L-carnitine. A similar improving effect of L-carnitine on the oxidant/antioxidant imbalance of brains of old rats and stress-induced lipid peroxidation in rat gastric mucosa has been shown (Izgut-Uysal et al 2001; Rami & Pannerselvam 2002). Recently, Sayed-Ahmed et al (2001) have shown that L-carnitine prevented the progression of atherosclerotic lesions by normalizing aorta ultrastructure and restored the increased oxidative stress by reversing the decrease in glutathione (non-enzymic antioxidant) levels in hypercholesterolaemic rabbits. In our study, we found that L-carnitine normalized the changed enzymatic activity of CAT to normal levels in diabetic rat aorta and improved the increased level of lipid peroxides in aorta and plasma. The L-carnitine has two effects on TBARS: a scavenger effect and a preventive effect on the formation of oxygen reactive species (Ronca et al 1992; Di Giacomo et al 1993). Another possible mechanism is normalized lipid peroxidation due to lower blood lipids.

Furthermore, this study has provided the first evidence that L-carnitine partially restores vascular dysfunction of diabetic aorta. The main mechanisms of this improvement in vascular dysfunction in diabetic rats are unknown, but there are a number of possibilities. One of the effects of L-carnitine treatment is arranging altered cellular membrane and function of cellular membrane, such as protein kinase C and Na/K ATP ase activity (Breker 1983). Another effect of L-carnitine might be caused by its ability to reduce blood lipids. The decrease in oxidative stress after L-carnitine treatment may lead to a reduction of NO inactivation by free radicals with enhanced NO bioavailability and improved endothelium-dependent vasodilatation. It has also been shown that the endothelium-dependent vasodilatory effect of L-carnitine seems to be mostly mediated by NO (Cameron & Cotter 1997; Herrera et al 2002), since free radicals and dyslipidaemia are considered to be among the contributing factors that play a pivotal role in vascular complications of diabetes mellitus (Yu 1994; Oranje & Wolffenbuttel 1999).

However, L-carnitine treatment partially restores the endothelium-dependent relaxation of aorta that is damaged by STZ-diabetes, indicating that hyperglycaemia is the major factor rather than oxidative stress and dyslipidaemia for diabetic vascular dysfunction. Also, it has been found that in 8-week diabetic rats, L-carnitine treatment partially decreased the severity of hyperglycaemia and reduction of hyperglycaemia has a direct relation with partial restoration of endothelium-dependent relaxation in diabetic aorta. It would be reasonable to assume, however, that the partial restoration of reduced aortic response to ACh produced by L-carnitine might be, at least partly, due to its effect on oxidative stress via its hypolipidaemic and antioxidant effects.

Conclusion

This study has shown L-carnitine to have some beneficial effects against cardiovascular and metabolic complications that are caused by diabetes, via its hypolipidaemic and antioxidant properties.

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